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Feasibility of Assessing the Community Composition of Prasinophytes at the Helgoland Roads Sampling Site with a DNA Microarray▽

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The microalgal class Prasinophyceae (Chlorophyta) contains several picoeukaryotic species, which are known to be common in temperate and cold waters and have been observed to constitute major fractions of marine picoplankton. However, reliable detection and classification of prasinophytes are mainly hampered by their small size and few morphological markers. Consequently, very little is known about the abundance and ecology of the members of this class. In order to facilitate the assessment of the abundance of the Prasinophyceae, we have designed and evaluated an 18S rRNA gene-targeted oligonucleotide microarray consisting of 21 probes targeting different taxonomic levels of prasinophytes. The microarray contains both previously published probes from other hybridization methods and new probes, which were designed for novel prasinophyte groups. The evaluation of the probe set was done under stringent conditions with 18S PCR fragments from 20 unialgal reference cultures used as positive targets. This microarray has been applied to assess the community composition of prasinophytes at Helgoland, an island in the North Sea where time series data are collected and analyzed daily but only for the nano- and microplankton-size fractions. There is no identification of prasinophytes other than to record them numerically in the flagellate fraction. The samples were collected every 2 weeks between February 2004 and December 2006. The study here demonstrates the potential of DNA microarrays to be applied as a tool for quick general monitoring of this important picoplanktonic algal group.

Phytoplanktonic cells between 0.2 and 2 μm are termed picoplankton, and in spite of their small size, they can contribute greatly to the global carbon cycle, biomass, and productivity in the sea (6, 8). The prokaryotic picophytoplankton consists of two cyanobacterial genera, *Synechococcus* (23, 48) and *Prochlorococcus* (4); in contrast, the eukaryotic part of the picophytoplanktonic community is less well known. Recently, several new classes have been described (2, 16, 37). As one of the key taxa in the marine eukaryotic picoplankton, the Prasinophyceae are an exceptionally interesting group (36). To date, 20 genera with 180 species are known within the Prasinophyceae; a few are recently described (46, 49). They are distributed worldwide and attain high abundances in several environments (40, 47, 51). However, their differentiation and detection are mainly hampered by the absence of reliable methods to identify and monitor these small cells with few morphological features (45, 50). With classical methods, such as light, epifluorescence, and electron microscopy, it is not possible to identify picoplanktonic groups down to the species level, and morphologically indistinguishable species with hidden genetic diversity make a correct classification impossible (20, 51). High-performance liquid chromatography can be used to identify the major classes of the phytoplankton community (18), but taxonomic resolution below the class level is limited. Several groups do not possess specific diagnostic pigments or may share overlapping ones (28).

In the last decade, molecular methods have facilitated the investigation of the physiology, ecology, and distribution of this important part of the marine food web (5). In this respect, especially molecular probes have been shown to be very useful in terms of the detection and monitoring of microbial diversity, especially in the pico-sized fraction. Target genes for molecular probes are frequently the small and large subunit rRNA genes. They feature both highly conserved regions and variable regions that allow the development of probes at different taxonomic levels. The probes can be used in combination with a wide variety of hybridization-based methods (13, 14). Furthermore, the probes can be tailored as phylogenetic markers to identify phytoplankton at different taxonomic levels in a hierarchical fashion from classes down to species or strains using whole-cell and cell-free formats (21, 33, 34). The fluorescence in situ hybridization (FISH) of Prasinophyceae has shown great potential (3, 36), but the processing and quantitative analysis of FISH samples with fluorescent microscopy can be tedious, slow, and time demanding, as only one probe can be processed at a time because of the limited choice in fluorochromes. The application of molecular probes and FISH techniques in combination with flow cytometry have greatly increased the speed, detection, and accuracy of the monitoring of picoplanktonic communities (3, 34), but the limitation of fluorochrome choice still applies. However, the application of new methods, e.g., microarrays, for the detection of picoplanktonic eukaryotes with potential for high-throughput analysis of samples

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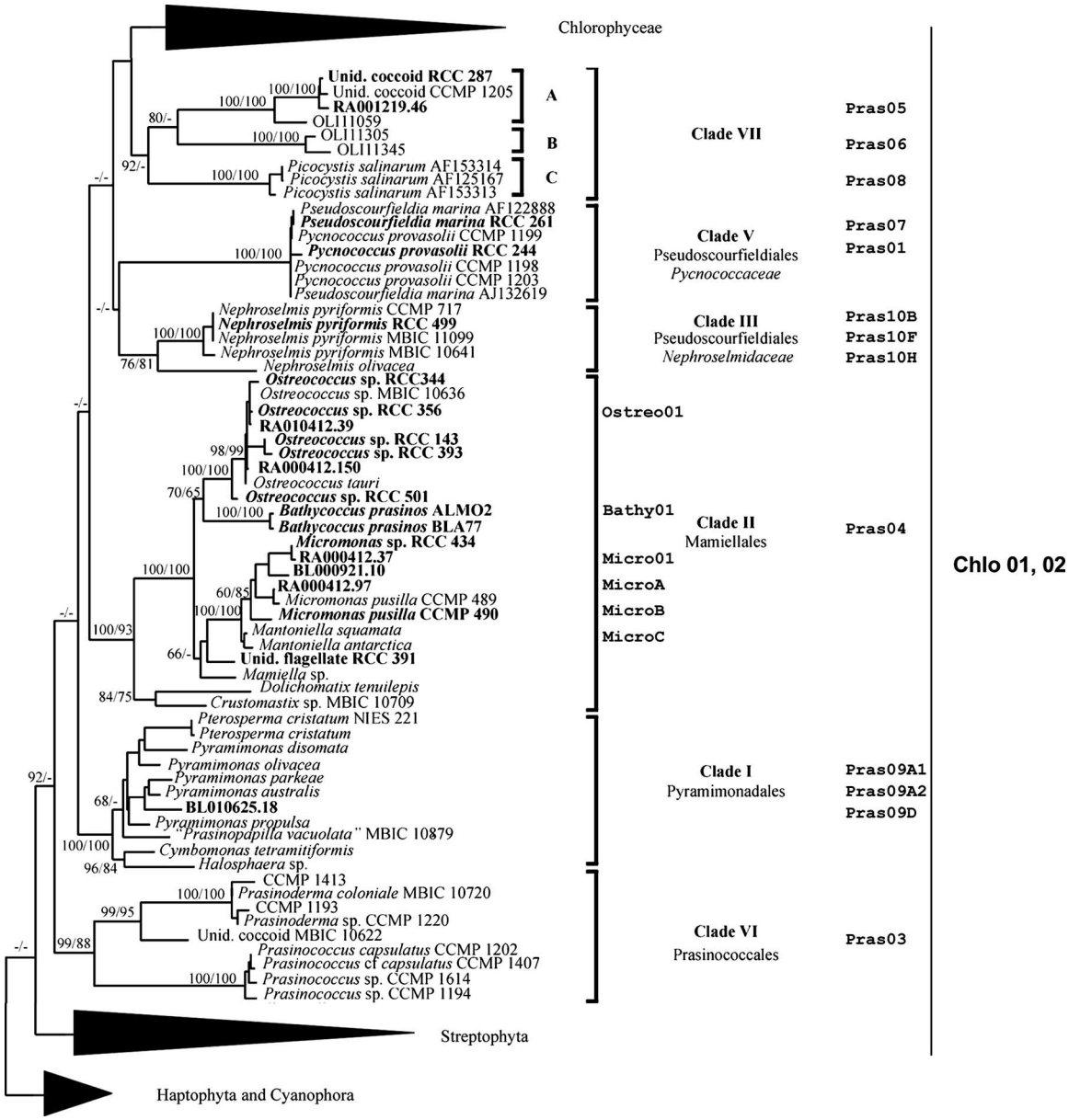

FIG. 1. Tree of prasinophyte diversity, showing which probes correspond to which clades. (Modified from reference 17 with permission from Elsevier.)

taken at close temporal and spatial scales can greatly facilitate the assessment of picoplanktonic species abundances, ecology, and physiology. DNA microarrays with species-specific probes have the unparalleled opportunity to detect thousands of targets in one experiment because the target is labeled, not the probes, and many probes can be used simultaneously. Although originally developed for gene expression applications, this innovative technique is being applied to species identification and has offered a promising experimental platform for microbial ecology. The most demanding challenge for the applicability of microarrays for microbial species identification is the high number of unknown environmental species and their corresponding DNA sequences that may result in unspecific signals or an oversight of species without a probe on the chip (11). However, the accuracy of the microarray can be enhanced by the application of hierarchical probes at different taxonomic levels because the detection of species is assessed by more than one positive probe signal (34). The development of a functional chip is an elaborate task because the set of probes on the microarray has to be developed such that all constituents work specifically under the same hybridization conditions (10, 34). The applicability of microarrays for the identification of phytoplankton has been demonstrated in various publications, e.g., for the detection and monitoring of harmful algae (12, 25) and marine microalgae at the class level (30, 33).

In this study, a phylochip for the detection and monitoring of the picoplanktonic prasinophytes was developed. Some of the probes were initially designed for FISH and showed specific hybridization signals, whereas other probes were designed for

TABLE 1. Origin of algal cultures used in this study and their corresponding phylogenetic clades according to Guillou et al. (17)

No.	Species[a]	Medium	Temperature (°C)	Origin	Specific target for clade
1	Unidentified Prasinophyceae sp. strain CCMP1413	K	20	North Atlantic	VI
2	*Prasinococcus capsulatus* CCMP1193	f/2-Si	20	North Atlantic, Gulf Stream	VI
3	*Prasinoderma coloniale* CCMP1220	K	20	North Atlantic, Gulf Stream	VI
4	*Prasinococcus* cf. *capsulatus* CCMP1194[b]	K	20	North Atlantic, Gulf Stream	VI
5	*Prasinococcus* cf. *capsulatus* CCMP1202	K	20	North Atlantic, Caribbean Sea	VI
6	*Bathycoccus prasinos* RCC496	K	15	Mediterranean Sea, Spanish coast	II
7	*Bathycoccus prasinos* CCMP1898	K	15	Mediterranean Sea, Gulf of Naples	II
8	*Ostreococcus tauri* RCC116	K	20	Mediterranean Sea, Thau lagoon	II
9	*Ostreococcus* sp. strain RCC344	K	20	Atlantic Ocean, Moroccan upwelling	II
10	*Micromonas pusilla* CCMP490	K	20	North Atlantic, Nantucket Sound	II
11	*Micromonas pusilla* CCMP1195	f/2-Si	15	North Atlantic, Gulf of Maine	II
12	*Mantoniella squamata* CCMP480	K	20	Norfolk, United Kingdom	II
13	Unidentified coccoid RCC287	K	20	Pacific Ocean, Equatorial Pacific	VIIA
14	Unidentified Chlorophyceae sp. strain CCMP1205	f/2-Si	20	Collection site unknown, Trident cruise	VIIA
15	*Pycnococcus provasolii* CCMP1203	K	20	North Atlantic	V
16	*Pycnococcus provasolii* CCMP1199	K	20	North Atlantic, Gulf of Mexico	V
17	*Picocystis salinarum* CCMP1897	f/2-Si	20	North Pacific, San Francisco Bay	VIIC
18	*Pterosperma cristatum* NIES221	K	20	Harima-Nada, Seto Inland Sea, Japan	Part of I
19	*Pyramimonas parkeae* CCMP724	f/2-Si	15	North Pacific, Santa Catalina Island	Part of I
20	*Nephroselmis pyriformis* CCMP717	K	15	North Atlantic, Galveston Channel	Part of III

[a] CCMP, Provasoli-Guillard Center for Culture of Marine Phytoplankton, United States; RCC, Roscoff Culture Collection, France; NIES, National Institute for Environmental Studies, Japan.
[b] *Prasinococcus* cf. *capsulatus* is a *Prasinococcus* sp. that looks like *Prasinococcus capsulatus* but is not taxonomically defined.

new groups of prasinophytes. The microarray contains 21 probes at different hierarchical levels. The specificity and discriminative potential of the probes were tested extensively under stringent conditions. Furthermore, the potential of the microarray was used to assess the occurrence of Prasinophyceae and the variation of its population composition over three annual cycles at the Helgoland Roads time series station.

MATERIALS AND METHODS

Specificity. (i) Probe development. One group of the probes that were evaluated in this study has already been published (36, 41, 42), and these probes were used for the Chlorophyta Prasinophyceae clades II, V, VI, VIIA, and VIIB (see reference 17 for phylogenetic details) and the species *Bathycoccus prasinos*, *Micromonas pusilla*, and *Ostreococcus tauri* (Fig. 1 and Table 1). They were tested in dot blot and FISH analyses (Table 2). Seven new probes for clades I, III, and VIIC (17) were designed with the probe design and probe match tool of ARB software (27) to cover the remaining prasinophyte diversity described by Guillou et al. (17) as shown in Fig. 1. The group is paraphyletic (35, 44), and because of this, probe design was difficult. It was impossible to design a probe for the entire group, and even at a clade level, multiple probes were sometimes necessary to cover all members of a clade. Probe specificity was tested in silico with the BLAST tool (1). All probe sequences are listed in Table 2.

(ii) Culture conditions. All algal strains for the specificity tests were cultured under sterile conditions in seawater-based f/2 and K media (15, 24) at 150 μEinstein to 200 μEinstein with a light:dark cycle of 14:10 h and at 15 or 20°C (Table 1).

(iii) DNA extraction. The template DNA was extracted from pure cultures with the DNeasy plant mini kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol.

(iv) PCR amplification of the 18S rRNA gene. For the probe specificity tests, the entire 18S gene was amplified from the target DNA (approximately 100 ng template DNA/100 μl PCR) with the universal PCR primers 1F (5′-AAC CTG GTT GAT CCT GCC AGT-3′) and 1528R (5′-TGA TCC TTC TGC AGG TTC ACC TAC-3′) without the polylinkers (29). The PCR protocol was 5 min at 94°C, 2 min at 94°C, 4 min at 54°C, and 2 min at 72°C for 29 cycles and an extension for 7 min at 72°C. All PCR experiments were carried out in a Mastercycler (Eppendorf, Hamburg, Germany). For the positive control in the microarray hybridization experiments, the yeast *Saccharomyces cerevisiae* was used. A 250-bp fragment of the TATA-box binding protein gene (TBP) of *S. cerevisiae* was amplified with the primers TBP-F (5′-ATG GCC GAT GAG GAA CGT TTA A-3′) and TBP-R (5′-TTT TCA GAT CTA ACC TGC ACC C-3′). The TBP PCR amplification protocol was 5 min at 94°C, 1 min at 94°C, 1 min at 52°C, and 1 min at 72°C for 35 cycles and an extension for 10 min at 72°C. All PCR fragments were purified with the QIAquick PCR purification kit (Qiagen, Hilden, Germany) with modifications of the protocol to enhance the yield of PCR fragments. Step 8 (elution with elution buffer EB) was repeated with the same eluate. The DNA concentration was measured with a Nanodrop spectrophotometer (Peqlab, Erlangen, Germany).

(v) Biotin labeling of the purified PCR fragments. In the hybridization experiments, the Biotin DecaLabel DNA labeling kit (Fermentas, St. Leon-Rot, Germany) was utilized. One labeling reaction contained 200 ng of PCR fragments and was incubated at 37°C overnight (17 to 20 h) to achieve the best biotin incorporation. The labeled PCR fragments were purified with the MinElute PCR purification kit (Qiagen, Hilden, Germany) with modifications of the manufacturer's protocol as above to enhance the yield. The DNA concentration was measured as above.

Field samples. (i) Sampling site and filtration. The analyzed field samples were taken in the North Sea on the island of Helgoland as part of the Helgoland Roads time series (14, 39). Samples were taken every 2 weeks between February 2004 and December 2006. The Helgoland time series site is an anchorage area between the two islands of Helgoland, termed the Roads (54°11.3′N, 07°54.0′E). Picoplankton samples were obtained either by filtration of 1 to 1.5 liters of unfractionated seawater onto a 0.2-μm Isopore GTTP membrane filter (Millipore, Schwalbach, Germany) so that the entire phytoplankton community was collected on the filter or by filtration of ~1.5 liters of fractionated seawater that was prefiltered through 10-μm and 3-μm Isopore TCTP membrane filters and finally collected onto a 0.2-μm Sterivex-GP filter (Millipore, Schwalbach, Germany). For DNA extraction of the latter set of samples, the 10-μm and the 0.2-μm filters were used; thus, the fraction between 10 μm and 3 μm was missing. The picoplankton are operationally defined as the <2-μm fraction, and thus, the prasinophytes were present in both sets of samples. All filters were immediately stored at −20°C.

(ii) DNA extraction. DNA from the field samples was extracted as described previously for the extraction of genomic DNA from laboratory cultures.

(iii) PCR amplification of the 18S rRNA gene. For sample analysis, a fragment of the 18S gene was amplified with the universal specific PCR primers 82F

TABLE 2. Probe sequences for the microarray

Probe	Probe sequence (5′ to 3′)	Target	Reference or source
Chlo01	GTG GTG GTC CGC ACC TCG	Chlorophyta	42
Chlo02	CTT CGA GCC CCC AAC TTT	Chlorophyta	41
Pras01	ACG GTC CCG AAG GGT TGG	*Pseudoscourfieldia marina, Pycnococcus provasolii* (clade V)	36
Pras03	GCC ACC AGT GCA CAC CGG	*Prasinococcales* (clade VI)	36
Pras04	CGT AAG CCC GCT TTG AAC	Mamiellales (clade II), except genus *Dolichomastix*	36
Pras05	GCC AGA ACC ACG TCC TCG	Clade VIIA (CCMP1205 plus RCC287)	36
Pras06	AAT CAA GAC GGA GCG CGT	Clade VIIB (environmental sequences, OLI1059, 11305, 11345)	36
Pras07	CCG ACA GAA AGA CGC AGA	*Pseudoscourfieldia marina, Pycnococcus provasolii* (clade V)	36
Pras08	ATT GTG TGG GTC TTC GGG	*Picocystis salinarum* (clade VIIC)	C. Gescher, this study
Pras09A1	GGT TGC GTT AGT CTT GCT	*Pterosperma cristatum* (clade I)	C. Gescher, this study
Pras09A2	GCC GCC TTC GGG CGT TTT	*Pyramimonas* sp., *Prasinopapilla* sp., *Cymbomonas* sp. (clade I)	C. Gescher, this study
Pras09D	AAC TGG CTC GGT ACG CGG	*Halosphaera* sp. (clade I)	C. Gescher, this study
Pras10B	TAA AAG ACC GAC CGC TTC	*Nephroselmis pyriformis, Pseudoscourfieldia marina* (clade III)	C. Gescher, this study
Pras10F	CGT TTC AAC TCG ACC AGT	*Nephroselmis pyriformis* (different from 10B) (clade III)	C. Gescher, this study
Pras10H	CAC TGG CGC GCC CCA TCT	*Nephroselmis olivacea* (clade III)	C. Gescher, this study
Bathy01	ACT CCA TGT CTC AGC GTT	*Bathycoccus prasinos*	36
Micro01	AAT GGA ACA CCG CCG GCG	*Micromonas pusilla*	36
Ostreo01	CCT CCT CAC CAG GAA GCT	*Ostreococcus tauri*	36
MicroA	CCG TCA AGA GGC CGC GGT	*Micromonas pusilla* (clade A, according to Guillou et al. [17])	N. Simon, unpublished data
MicroB	CAC GAC CAA CAG ACG GTT	*Micromonas pusilla* (clade B, according to Guillou et al. [17])	N. Simon, unpublished data
MicroC	ACG GCG GCG AAC CGC AAT	*Micromonas pusilla* (clade C, according to Guillou et al. [17])	N. Simon, unpublished data
Positive control	ATG GCC GAT GAG GAA CGT	*S. cerevisiae*, TBP	33
Negative control	TCC CCC GGG TAT GGC CGC		33

(5′-GAA ACT GCG AAT GAA TGG CTC-3′) and 1528R (5′-TGA TCC TTC TGC AGG TTC ACC TAC-3′) from the target DNA (approximately 100 ng template DNA/100 μl PCR) in a Mastercycler (Eppendorf, Hamburg, Germany). The PCR protocol of the 18S amplification and the TBP of *Saccharomyces cerevisiae* (positive control) and the purification and determination of the DNA concentration were identical to those for the specificity tests.

(iv) Biotin labeling of the purified PCR fragments. Labeling of the PCR fragments was conducted as described above for the specificity test.

Chip design and hybridization. (i) Probe synthesis. The molecular probes, including the positive and negative controls, were synthesized by Thermo Electron Corporation (Ulm, Germany) with a C6/MMT aminolink at the 5′ end.

(ii) Microarray production. Probes were spotted onto epoxy-coated Nexterion Slide E slides (Peqlab Biotechnologie GMBH, Erlangen, Germany) at a final concentration of 1 μM in 3× saline sodium citrate buffer (SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate]). We utilized the pin printer VersArray ChipWriter Pro (Bio-Rad Laboratories GmbH, München, Germany) and split pins (Point Technologies, Inc., CO). Subsequently, the slides were incubated at 60°C for 30 min in a Shake 'n' Stack hybridization oven (Thermo Hybaid, Ulm, Germany). The microarrays were stored at −20°C. The chip contained four replicates of each probe in four independent blocks.

(iii) Standard hybridization protocol. The hybridization solution was prepared with 1× hybridization buffer (1 M NaCl–10 mM Tris [pH 8]–0.005% Triton X-100–1 mg/ml bovine serum albumin–0.1 μg/μl herring sperm DNA) and the biotin-labeled PCR fragment at a final concentration of 11.25 ng/μl. The TBP fragment from *S. cerevisiae* was added as the positive control at a final concentration of 4.7 ng/μl. Blocking of the background noise was conducted by the prehybridization of the slides at 58°C for 1 h in a slide box with 50 ml 1× STT buffer (1 M NaCl–10 mM Tris [pH 8]–0.005% Triton X-100–1 mg/ml bovine serum albumin). Next, the slides were centrifuged, and the hybridization solution was incubated at 94°C for 5 min. A special coverslip, the Lifter Slip (Implen, München, Germany), was used for the hybridization. A volume of 30 μl hybridization solution was pipetted under the coverslip, and capillary action ensured even dispersal of the hybridization solution between the chip and the coverslip. The slide was placed in a humid chamber, which was constructed from a 50-ml Sarstedt tube filled with tissues moistened with hybridization solution. The hybridization was conducted at 58°C for 1 h; afterward, the slide was washed with 2× SSC-10 mM EDTA-0.05% sodium dodecyl sulfate and then 1× SSC-10 mM EDTA for 15 min and dried by centrifugation.

(ii) Staining. The hybridized PCR fragments on the chip were stained with streptavidin-CY5 (Amersham Biosciences, Freiburg, Germany) in 1× hybridization buffer at a final concentration of 100 ng/ml. The chip was placed at room temperature for 30 min in a humid chamber and was washed afterward, twice with 2× SSC buffer for 5 min and once with 1× SSC buffer for 5 min, to remove excess staining moieties.

(iii) Scanning and quantification of microarrays. The chip was scanned with a GenePix 4000B scanner (Molecular Devices, Sunnyvale, CA), and analysis of the obtained fluorescence signal intensities was done with GenePix 6.0 software (Molecular Devices, Sunnyvale, CA). A grid of circles was superimposed onto the scanned image to calculate the fluorescent signals and the surrounding background intensity.

(iv) Data preparation and analysis. The hybridization data obtained from the array scanner were processed with the program PhylochipAnalyzer (32). This program implements the computation of signal-to-noise ratios (S/Ns) across the hierarchical probe set, while eliminating false positives. As we are analyzing time-dependent S/Ns for 21 probes, we must apply techniques to reduce the complexity for a structural analysis. This is done here by methods similarly applied in the analysis of differential gene expression profiles (9) as follows. Before that, all S/N values below 2, i.e., intensities below twice the background standard deviation (derived from the negative control signals), are set to zero. The samples with only zero values are removed from the data set. The resulting data are shown in Fig. 5A in color-coded form. Values above 26 are displayed in white, whereas zero values are in brown. These data are used for hierarchical clusterings (22) as displayed in Fig. 5B. Dissimilarities are computed from Euclidian distances of rows of data (for probe clustering) or columns of data (for time clustering). A complete-linkage clustering is computed from the dissimilarity matrices by standard procedures within the R package (http://www.r-project.org). In both figures, seasons are depicted in color-coded form. In Fig. 5B, this season data row is permuted, according to the order induced by time clustering (attached at the bottom).

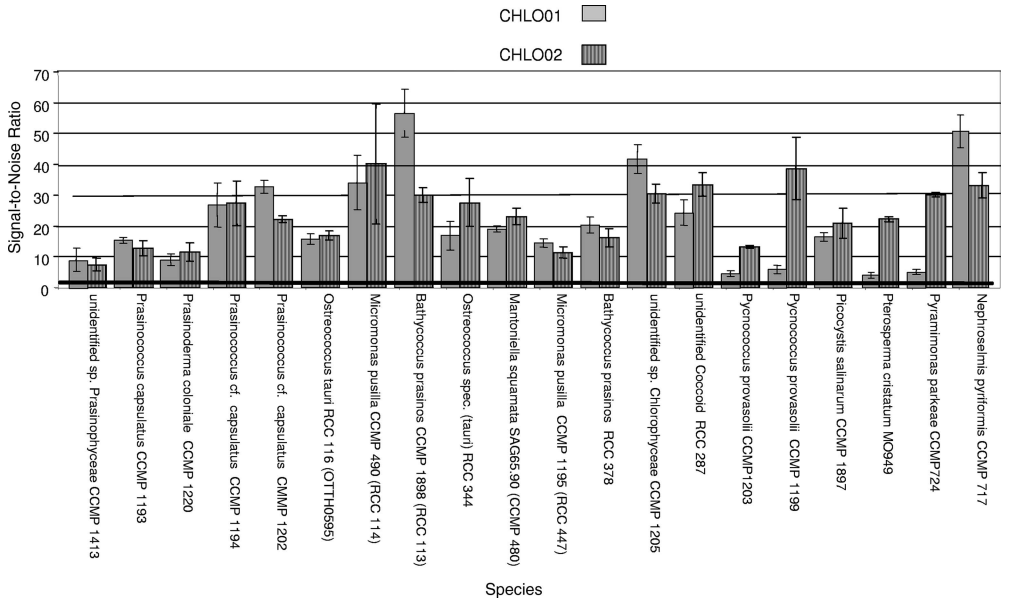

FIG. 2. Comparison of the signal intensities of probes Chlo01 and Chlo02 across all strains tested.

RESULTS

Specificity tests. The phylogenetic analysis of the 18S rRNA gene of the Prasinophyceae by Guillou et al. (17) identified six clades, of which five are recognized at the order level. Here, we present a DNA microarray that contains a probe set that is suited to identify Prasinophyceae according to this classification at the clade or order level (Fig. 1). Additionally, the microarray contains probes for the identification of certain prasinophyte genera. Probe specificity on the microarray was tested under stringent conditions (the same hybridization temperature and washing conditions for all probes and chips) with laboratory cultures. Overall, the majority of the probes on the chip showed specific hybridization results. Some probes displayed weak cross-hybridization to a significant number of nontarget species, but these could be distinguished from much stronger target signals. The S/Ns of perfectly matched targets hybridized to their specific probes showed different signal strengths, demonstrating that the intensities of individual probes vary strongly in their sensitivity. For example, a comparison of the S/Ns for two probes for the Chlorophyta was conducted (Fig. 2). All strains showed signals for the Chlo01 and 02 probes, but they varied over a broad range. The signals for probe Chlo02 were higher than those for Chlo01.

The data of the hybridization signals are shown in three-dimensional plots with probes with algal strains that were used as positive targets (Fig. 3A to C).

Hybridization results of the 20 reference cultures to the Prasinophyta probes at the clade level are shown in Fig. 3A to C. The specificity tests are discussed, referring to clades in the tree in Fig. 1, beginning with the clade at the top of the tree and moving to the bottom of the tree.

(i) Clade VII—unnamed order containing the genus *Picocystis*. Three probes were designed for this clade, each recognizing one of the three subclades in this undescribed order of prasinophytes. Given the genetic diversity among the three subclades, the groups should be described at least at the genus level. Probe Pras05 (clade VIIA) showed no cross-hybridization with nontarget strains and gave very strong specific signals for one target strain, unidentified Chlorophyceae sp. strain CCMP1205 (Fig. 3A). The other target strain identified from Fig. 1, unidentified coccoid RCC287, did not give a signal. Probe Pras06 for clade VIIB was tested only with nontarget species because no target strains were available from this clade (Fig. 3A). Here we detected only one weak cross-hybridization with *Micromonas pusilla* CCMP490. Probe Pras08 (clade VIIC) showed a signal for its target species *Picocystis salinarum* CCMP1897 (Fig. 3B). Several strains in other clades also cross-hybridized with this probe, but most signals were below the threshold of 2 (Fig. 4).

(ii) Clade V, order Pseudoscourfieldiales, family Pycnococcaceae. Probes Pras07 and Pras01 recognize this family of the Pseudoscourfieldiales, which contains two described genera, *Pseudoscourfieldia* and *Pycnococcus*. Pras07 represents an improvement over probe Pras01 and detects more species in this clade, as displayed in Fig. 1. As shown in Fig. 3, they both showed specific signals for the target strains and Pras07. Two weak cross-hybridizations occurred for probe Pras07, in contrast to none for probe Pras01, but signals for the targets were much stronger for Pras07 than for Pras01.

(iii) Clade III, order Pseudoscourfieldiales, family Nephroselmidaceae. The three probes designed for this clade are also family- and genus-level probes because only the genus *Nephroselmis* belongs to this clade. Pras10H showed one weak cross-hybridization to a nontarget strain, whereas Pras10F showed no unspecific binding with any closely related species (Fig. 3B). Pras10B showed specific results in hybridization with its target strain *Nephroselmis pyriformis* CCMP717 and minor cross-hybridizations with some other strains (Fig. 3B).

(iv) Clade II, Mamiellales. Pras04 recognizes all members of this order (Fig. 3C). It showed clear signals with all seven

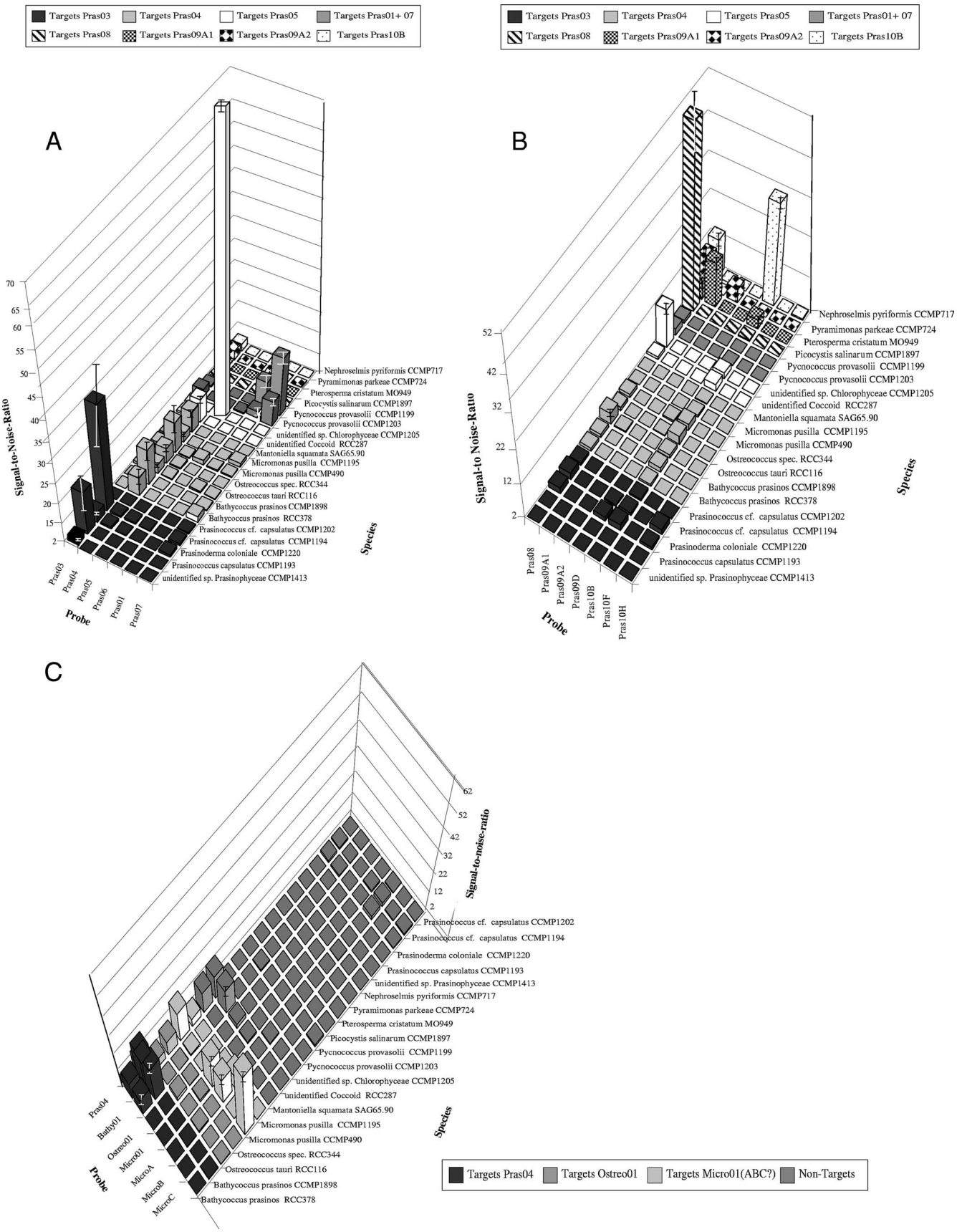

FIG. 3. Three-dimensional plot of the signal intensities for all probes and all strains. (A) S/Ns for probes Pras03, 04, 05, 06, 01, and 07; (B) S/Ns for probes Pras08, 09, and 10; (C) S/Ns for Pras04 and genus and species probes in this clade.

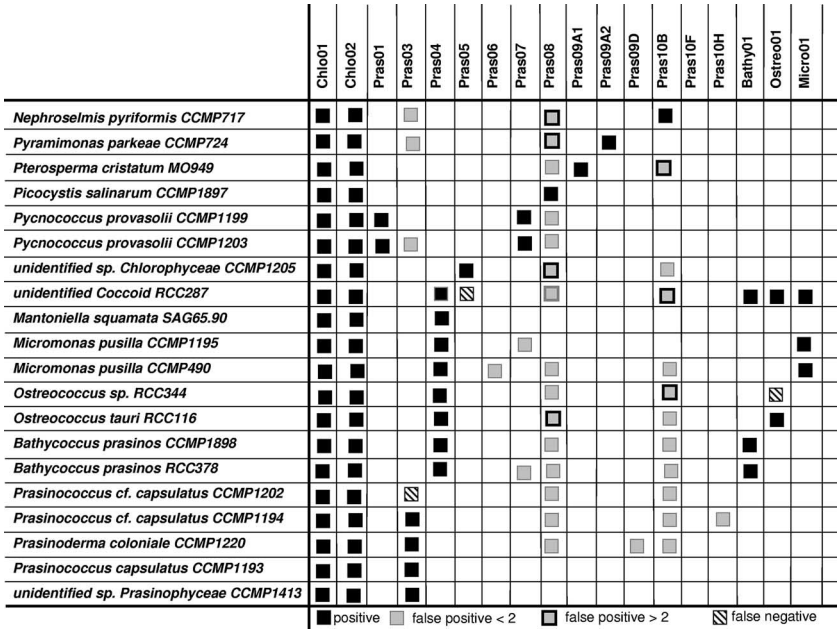

FIG. 4. Summary of specificity testing for clade and genus probes.

strains tested. An unspecific signal to coccoid strain RCC287, a nontarget species, was observed. To resolve this, we sequenced this culture (data not shown) and found that it was a mixture of *Bathycoccus prasinos* and *Micromonas pusilla* (data not shown), and both contained the target sequence of probe Pras04. Thus, the culture used in this experiment is a mixture, and the signal can be regarded as correct. There are three described genera in this clade: *Bathycoccus*, *Ostreococcus*, and *Micromonas*. We tested genus-level probes for each of these genera. The probe for *Bathycoccus*, Bathy01, showed a signal for the specific cultures *B. prasinos* RCC378 and CCMP1898 and for unidentified coccoid RCC287 (Fig. 3C). The two *Ostreococcus* cultures, *O. tauri* RCC116 and RCC344, showed signal intensities for probe Ostreo01, but they were just above or at the threshold level, respectively (Fig. 3C). The probe Micro01 is specific for the genus *Micromonas*. Two cultures of *M. pusilla*, CCMP490 and CCMP1195, are target species for probe Micro01 and showed a signal for this probe (Fig. 3C). The signal for *M. pusilla* CCMP1195 was very low but above the threshold (Fig. 3C). The probes MicroA, B, and C are specific for different subclades within the *Micromonas pusilla* species complex (17). At least five morphologically indistinguishable different groups can be detected in this complex (43). The culture *M. pusilla* CCMP490, which belongs to the *Micromonas* clade A (17), gave a signal for MicroA and also for MicroC (Fig. 3C). One possible explanation for the mixed signals for *M. pusilla* CCMP490 could be that the culture is not clonal. Representatives of the different *M. pusilla* clades occur worldwide (43), and members of two clades could easily be present in one culture. The subclade affiliation of culture *M. pusilla* CCMP1195 is unknown, because it was not in the study of Guillou et al. (17). This culture showed only signals for probe MicroA (Fig. 3C). No signals were observed for MicroB.

(v) Clade I, Pyramimonadales. The known diversity in clade I was covered by three specific probes: Pras09A1, Pras09A2, and Pras09D (Fig. 3B). These three probes recognize the described genera *Pterosperma*, *Halosphaera*, *Cymbomonas*, *Prasinopapilla*, and *Pyramimonas*, the last of which is paraphyletic. Specific hybridization results were observed for Pras09A1 and Pras09A2 when hybridized with their target species, and they showed no nontarget hybridization. For the third probe of this clade, probe Pras09D, no target strains were available in culture, but one weak cross-hybridization occurred with *Prasinoderma coloniale* CCMP1220; thus, it can be assumed to be specific.

(vi) Clade VI, order Prasinococcales. This clade consists of two described genera, *Prasinococcus* and *Prasinoderma*, and one unidentified strain that shows sufficient genetic divergence to be another genus. Probe Pras03 for clade VI showed strong specific signals to three out of five hybridized target strains, one target strain showed a weaker signal, and another showed no signal at all (Fig. 3A). Weak cross-hybridizations with three strains from other clades (I, II, III, and V) were detected.

Field samples. The DNA microarray was used to characterize the community composition of the Prasinophyceae at the Helgoland time series site in the North Sea site over a 3-year cycle between February 2004 and December 2006. In total, 61 samples were taken every 2 weeks within this time period and have been analyzed in this study. Overall, comparable S/Ns could be observed for all samples analyzed. However, the observation of variable signals for different targets of the same probe and inconsistencies within the hierarchies (e.g., for Chlo02 and Pras04) of the probes for some samples forced us to cut back our analysis to a rather general view. Hence, the overall signals per probe were counted as a first step in the analysis. The group with the highest number of positive signals consisted of the probes Chlo02, Pras04, Bathy01, Pras08, and Micro01. They were displayed in ~40 to 70% of the samples with a positive signal (Fig. 5A). Furthermore, probes Chlo01, Pras04, and Bathy01 each showed one sample with a rather

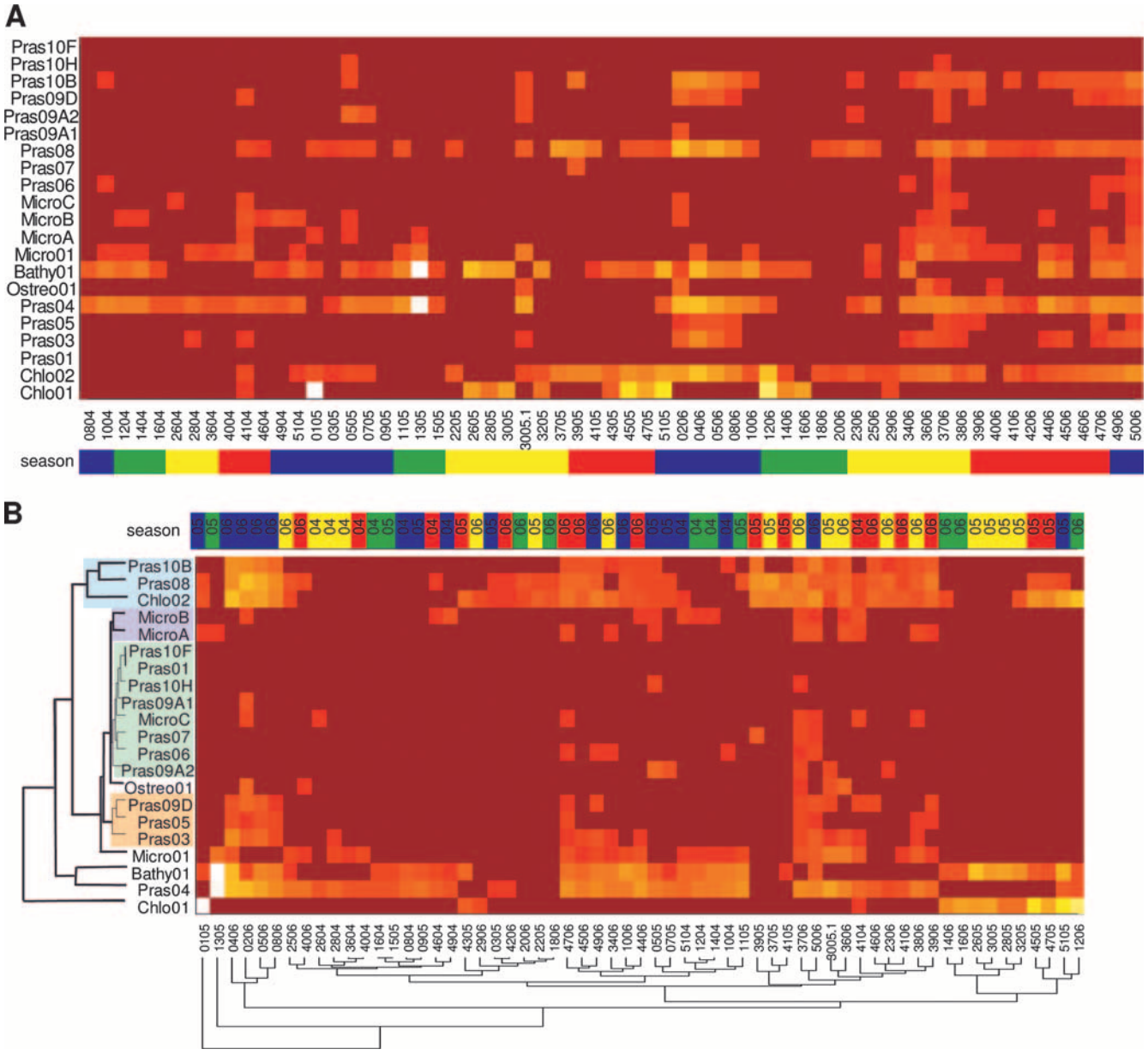

FIG. 5. (A) Color-coded representation of processed hybridization data. Top, S/Ns with the lower cutoff of 2 are in brown, the maximum value in white, and the medium values in red to yellow. Bottom, the color-coded seasons along the time axis are as follows: blue, winter; green, spring; yellow, summer; and red, autumn. The time-axis ticks are a combination of week and year (WWYY). (B) Clustered data. The data are horizontally (time) ordered by a hierarchical clustering (see text) shown at the bottom. The season color codes are ordered in the same way as described for panel A. The vertical (probe) order is given by hierarchical clustering displayed at the left.

strong signal (above 26; Fig. 5A). The remaining probes showed a positive signal in only less than ~25% of the samples. Some of the probes, such as Pras01 and Pras10F, did not show a positive signal at all. These data indicated that organisms belonging to the order Mamiellales, which are target species of the first group of probes, were the major contributors to the prasinophyte community at the Helgoland Roads sampling site. All other clades of the prasinophytes appeared to be minor components. The overall trend seems to be that within the Mamiellales, *Bathycoccus* made a larger contribution to the community composition than *Micromonas*. Similar results were detected with FISH (data not shown). Our data also indicated a seasonal variation in the community composition, because in the summers of the years 2004 and 2006 (approximately weeks 30 to 40), positive signals could be observed for *Micromonas* but not for *Bathycoccus*.

In order to assess the diversity within the prasinophytes, the complexity of the samples in terms of positive signals was addressed. It could be observed that the complexity (diversity) of the signals (columns) was strongly time dependent. Phases of higher complexity (diversity) for winter 2005/2006, late summer 2006, and winter 2006/2007 were observed.

Seasonal variation and succession were further assessed by grouping similar samples together. The data were reordered in time (horizontally) by the hierarchical clustering depicted as the attached tree below the matrix plot in Fig. 5B. The hierarchical approach sorted the data into blocks of similar signals. For example, at the right, a period between 1406 and 1206 showed similarity in the Bathy01 and Chlo01 signals. This phase is subdivided in two clades between 3205 and 4505, i.e., in the right clade, signals of probes Chlo02 and Pras04 appear. There were two samples of comparable high complexity (many signals), namely 3706 and 5006. Samples 0406, 0206, 0506, and 0806 are grouped at the left. They are persistently dominated by signals present in three groups: group I (Pras10B, Pras08, and Chlo02), group II (Pras09D, Pras05, and Pras03), and group III (Bathy01 and Pras04). These groups appeared as blocks in the matrix plot because of the vertical reordering, which is induced by the hierarchical clustering attached as a tree at the right in Fig. 5B. In contrast, the probes grouped in green showed much lower and fewer signals.

Despite these described similarities, overall, the clustering did not result in a clear picture that displayed a regular seasonal variation of species composition within the prasinophytes.

DISCUSSION

The eukaryotic picoplankton is of major importance in terms of its contribution to biomass and productivity of oceanic oligotrophic waters as well as coastal waters. However, the assessment of picoplankton ecology is hampered by their small size. A number of publications have taken advantage of molecular probes, e.g., in combination with FISH, to solve this problem. The major drawback of FISH is the limited throughput, only allowing the identification of one or a few organisms at a time with a restricted number of fluorochromes (7, 38). A comprehensive view of microbial communities is challenging, time consuming, and difficult to achieve with this method. Furthermore, Not and coworkers (36) reported heterogeneous signals for probes Pras01, 03, and 05 in FISH. One possible explanation may be that thick cell walls inhibit the penetration of probes (19). These problems do not occur in a microarray application because only nucleic acids are used and probes need not enter intact cells. The probes Pras04, Micro01, Ostreo01, and Bathy01 are specific for their target strains and delivered a bright fluorescence in FISH (36) and also in a microarray hybridization (30).

Specificity tests. In the first part of this study, 21 phytoplankton probes at different hierarchical levels were tested and evaluated with 20 algal cultures in a microarray hybridization format. Part of the probe set was previously published in the context of other hybridization techniques (30, 33), and others were newly designed. The previously published probes had to be reevaluated on the microarray, because probes that work specifically in the context of other hybridization methods perform quite differently, or even not at all, in a microarray format (31). However, for the current probe set, we observed that the majority of the probes resulted in hybridization signals on the microarray. Furthermore, most of the specific probes were significantly stronger than the signals for nontarget species. The probes Pras08 and Pras10B, for clades VII and III, respectively, showed cross-hybridization to a significant number of nontarget species, e.g., all tested species belonging to the Mamiellales. Therefore, these probes may be considered unspecific. An attempt to design new, more specific probes for these clades was hampered by the diversity of the clade. Nevertheless, if the strengths of the true-positive signal and the false-positive signal are taken into consideration for the interpretation of hybridization signals, the probes could be used for the characterization of prasinophyte communities. The true-positive signal of the probes is around 5× higher than the false-positive signal. In addition, the signal of the cross-hybridization to species that belong to the Mamiellales is in the same range as the signal for the respective probes targeting this group (e.g., Pras04). Thus, if a chip displays positive signals for probes Pras04, Pras08, and Pras10B and the signal of Pras08 and Pras10B is in the same range as the signal for Pras04, it can be concluded that the signal is originating from Mamiellales. This indicates that an identification of different clades within the Prasinophyceae with the presented probe set is feasible with a standardized hybridization protocol.

In the course of the specificity testing, we observed large differences in the signal intensities of probes hybridized to different perfectly matching targets, e.g., for the Chlo01 and Chlo02 probes, positive hybridization signals could be observed for all target species. However, the signal intensity varied over a broad range. This has been observed frequently for probes that cover broad taxonomic groups (36). Five probes (Pras01, 04, 05, and 09A1 and 09A2) were specific for their target clades (V, II, VIIA, and I, respectively) and did not cross-hybridize with closely related species. The same results were observed for nearly all species and subclade probes of clade II (Bathy01, Micro01, A, B, and C). However, Ostreo01 displayed relatively low signal intensities. For a group of four probes (Pras06, 09D, and 10F and 10H for clades VIIB, II, and III, respectively), no target strains were available. They showed no significant cross-hybridization with all other prasinophyte cultures. These results indicate a high specificity for these probes. Thus, interpretation of the field sample analysis can be complicated because it is not clear if the probes result in signals at all if a respective target species is present. Specific hybridization signals for the target and cross-hybridization with closely related species were observed for probes Pras03, 07, 08, and 10B (clades VI, V, III, and VIIC, respectively).

Three cultures resulted in inconsistent signals because they did not hybridize to the corresponding perfectly matching probe. Two of them did not result in a signal at all, whereas the third one did bind to another probe (Pras04 instead of Pras05). Sequencing of the 18S rRNA gene of the targets revealed that this result was correct and that the target species did contain the probe sequence, but this does not explain why this target species was placed in another clade in the phylogenetic analysis, unless there were originally multiple isolates in this culture which have been sequentially replaced over time. This displays the potential and accuracy of the method. DNA microarrays could be suited to serve as a valuable tool to ensure the quality of a culture collection. The other two strains, which did not result in a hybridization signal, were correctly identified. In these cases, hybridization must have been hampered by other factors. The observation that most probes displayed relatively strong variation if they were hybridized to different target organisms underpins the widely accepted observation

that in situ hybridization results for probes are not always consistent with their in silico predictions. Future research has to focus on the assessment of these discrepancies. Another weakness of the DNA microarrays are cross-hybridizations. They are almost impossible to avoid in a microarray hybridization format under stringent conditions (26). In this study, we evaluated probes that have been designed and optimized in respect to the hybridization temperature independently. The aim was to evaluate their performance under a single given hybridization protocol. For example, the unspecific signals for probe Pras08 could not be prevented under our hybridization conditions. It is likely that the probe may work specifically under more stringent hybridization conditions. However, it is not clear if all other probes on the microarray would keep their specificity and sensitivity under more stringent conditions. Therefore, we have to find ways to deal with cross hybridization, e.g., by the application of multiple probes for one target or hierarchical sets or by hybridization of the same sample at two different temperatures. Both approaches can prevent the misinterpretation of false-positive signals and will further improve the reliability of the microarray. Hierarchical probe sets consist of probes that target species at different taxonomic levels. In such a probe set, a signal at the species level should only be considered truly positive if all probes in the taxonomic hierarchy also show positive signals. The PhylochipAnalyzer software offers the possibility to examine all probes in a defined hierarchy and therefore represents a major advance in the data processing and interpretation of microarray experiments (32).

Field samples. In the second part of this study, the specificity-tested DNA microarray was used to assess the community composition of the picoeukaryotic class Prasinophyceae. In total, 61 samples were analyzed that were taken every 2 weeks between February 2004 and December 2006. Overall, the results of the different samples displayed comparable hybridization signals. However, a few inconsistencies among the probes that targeted different taxonomic levels within the prasinophytes were observed. The probes from the beginning of 2004 did not show positive signals for Chlo02, but the probes at lower hierarchical levels, such as Pras04, did. It was stressed before that hierarchical probe sets should contribute to the accuracy of the results from a DNA microarray. Technically, all signals that were observed in the absence of Chlo02 should be considered negative. However, it is very unlikely that this is the case because prasinophytes have been identified to be major contributors to the picoplankton. It is also unlikely that there are no prasinophytes in these earlier samples. We assume that we have a technical problem here that is related either to the production of the microarrays or to the normalization of the data. The quality of the chip production was assessed by staining one chip from each batch with Sybr green, a fluorescent dye that is suited to stain DNA on the chips. However, because the DNA microarrays have been spotted with a split pin system, it cannot be excluded that the pin was blocked at some point during the spotting process of the respective probes. The normalization of the data poses another problem. As an internal marker that could be used for the normalization of the data in respect to variations related to the hybridization process is missing, an external control was added to the hybridization mixture. The data were normalized to this external control. Nevertheless, this approach is very susceptible to variations in the concentration of the control, e.g., because of handling.

A general analysis of the data generated with the microarrays indicates that the major constituents of the prasinophytes at the Helgoland Reed sampling site belong to the order Mamiellales. This is in accordance with a previous publication that reported the order Mamiellales to be dominant among the Chlorophyta in the English Channel (36). However, in this publication, it was described that among the Mamiellales, *Micromonas pusilla* was the dominant species. Our data for the Helgoland sampling site indicate that the genus *Bathycoccus* could be the major contributor to prasinophyte composition. The probe targeting this genus has more positive signals compared to *Micromonas* and *Ostreococcus*, which play only a minor role in the community composition of the prasinophytes at Helgoland. Furthermore, our data indicate a seasonality of the occurrence of *Bathycoccus* and *Micromonas*, because the probe for *Bathycoccus* did not display a signal in the summers of the years 2004 and 2006, whereas *Micromonas* was detected during these periods. Nevertheless, these findings should be confirmed by other methods, e.g., quantitative PCR, but they were comparable to counts observed by FISH methods (data not shown).

Clustering. By the hierarchical clustering of the probes, similar time-dependent hybridization patterns can be identified. In contrast, the hierarchical clustering of the samples does not reveal a clear grouping when looking at the corresponding permutation of seasons, i.e., the time structure of the samples does not obviously correlate with seasons. This finding may be taken as an indication that there is no repetitive seasonal pattern of time course detectable within the hybridization patterns which, in contrast, one would expect for a community with strong interactions.

Conclusion. A DNA microarray has been developed and assessed for the characterization of the community composition of Prasinophyceae. It is shown that the probes evaluated and tested in this study offer the potential to generate quickly a general impression of the community structure of the Prasinophyceae. However, some limitations of the technology had to be acknowledged, and the demand for improvement has to be pointed out. Our study represents one of the first applications of a low-density phylochip for the simultaneous detection and identification of different picoplanktonic species. Our analysis identified the genus *Bathycoccus* to be the major constituent of the prasinophyte community in the North Sea at the island of Helgoland time series site.

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