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Application of Sequence-Specific Labeled 16S rRNA Gene Oligonucleotide Probes for Genetic Profiling of Cyanobacterial Abundance and Diversity by Array Hybridization

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DNA sequence information for the small-subunit rRNA gene (16S rDNA) obtained from cyanobacterial cultures was used to investigate the presence of cyanobacteria and their abundance in natural habitats. Eight planktonic communities developing in lakes characterized by relatively low algal biomass (mesotrophic) and in lakes with correspondingly high biomass (eutrophic) were selected for the study. The organismal compositions of the water samples were analyzed genetically, using multiplex sequence-specific labeling of oligonucleotide probes targeted to 16S rDNA and subsequent hybridization of the labeled probes to their respective complements spotted onto a solid support (DNA array). Ten probes were established to determine the relative abundances of the discernible cyanobacteria encountered in the selected lakes. The probes were generally specific for their targets, as determined through analyses of clone cultures. Reproducible abundance profiles were established for the lakes investigated in the subsequent analyses of natural cyanobacterial communities. The results from the genetic analyses were then compared with information obtained from standard hydrobiological and hydrochemical analyses. Qualitatively, there were relatively good correlations among the groups of organisms (*Nostoc*, *Microcystis*, and *Planktothrix* species) found in the different lakes. The levels of correlation were lower for the quantitative data. This may, however, be due to differences in sample processing technique. The conclusions from these comparisons are that the genetic abundance profiles may provide a foundation for separating and quantifying genetically distinct groups of cyanobacteria in their natural habitats.

The cyanobacteria are a widely distributed and diverse group of unicellular and multicellular photosynthetic prokaryotes that possess chlorophyll *a* and conduct oxygenic photosynthesis. These organisms are important in the biosphere, being among the main groups of primary producers (5, 29). Several species also produce cyanotoxins (20). For classifying cyanobacteria, a phylogenetic system based on the 16S rRNA gene (rDNA) sequence information retrieved from organisms in pure cultures has been developed (12, 45, 46). The primary genetic analysis of these organisms in their natural habitats, however, has been technically challenging (10, 11, 34, 37).

The most widely applied strategy for accessing cyanobacterial biodiversity in nature has been through 16S rDNA cloning, sequencing, and phylogenetic reconstruction (10, 11, 37). This strategy, however, is not suited for large-scale screenings due to the complexity of this approach. Another approach used to extract information about biodiversity is denaturing gradient gel electrophoresis (DGGE) (24a). DNA array hybridization has also been applied to 16S rDNA and 16S rRNA through nonspecific labeling of the nucleic acids (12a). Recently, in situ-based hybridization methods have been developed for cyanobacteria (38). The limitation of in situ hybridization is that only one organism, or at most a few, can be analyzed simultaneously.

There is an apparent need for new approaches to the genetic analysis of complex cyanobacterial communities. These approaches include both sample preparation and DNA detection methods. We have recently developed a simple method for concentration of cyanobacterial cells and subsequent DNA purification, using the same paramagnetic beads for both purposes (31). A complete diagnostic assay was produced by the combination of the sample preparation method with a method for quantitative detection of several polymorphisms in a single reaction (33). Here, we demonstrate that this assay can be successfully applied for the analysis of natural cyanobacterial populations. Using this assay, the species compositions and abundances of cyanobacteria in eight lake communities were determined. The analyzed samples originated from selected lakes along a trophic gradient, ranging from localities moderately influenced by plant nutrients (mesotrophic) to lakes heavily affected by plant nutrients (eutrophic).

Ten 16S rDNA probes to identify the cyanobacterial genera *Microcystis*, *Planktothrix*, *Anabaena*, and *Aphanizomenon*, in addition to a probe which corresponds to the *Nostoc* group (which includes *Nostoc*, *Anabaena*, and *Aphanizomenon* spp.) and a universal probe for eubacteria (including chloroplasts), were created. This collection of 10 probes was used in a multiplex assay of the relevant prokaryotes both in laboratory culture and in these organisms' natural habitats. The results from the 16S rDNA probe assay were compared to data obtained from an analysis by light microscopy and to the corresponding hydrochemical data (i.e., pH, total organic carbon content, conductivity, chloride concentration, total phosphorus content, and total nitrogen content) from the lakes. The work presented here is a step toward defining the genetic diversity of natural cyanobacterial populations and toward the generation of methods to assess this diversity.

MATERIALS AND METHODS

A schematic representation of the approach used to generate the genetic abundance profiles is shown in Fig. 1.

Organisms and culture procedure. The following clonal isolates from the Norwegian Institute for Water Research (NIVA) Culture Collection were used:

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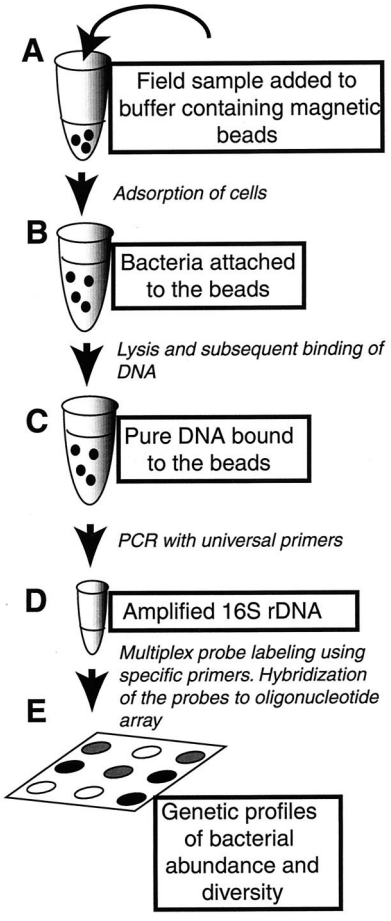

FIG. 1. Schematic representation of the approach used for determining the genetic profiles of the water samples. The field samples were added to a buffer containing magnetic beads (A), with subsequent adsorption of cyanobacteria onto the beads (B). The bacteria and the beads were then collected by a magnet and lysed, and the released DNA was purified (C). For each sample, the 16S rDNA gene was subsequently amplified (D). Finally, probes were labeled sequence specifically and hybridized to their respective complements (E).

Aphanizomenon gracile NIVA-CYA 103, *Anabaena lemmermannii* NIVA-CYA 266/1, *Nostoc* sp. strain NIVA-CYA 124, *Phormidium* sp. strain NIVA-CYA 203, *Planktothrix prolifica* NIVA-CYA 320, *Microcystis aeruginosa* NIVA-CYA 143, *Microcystis flos-aquae* NIVA-CYA 144, and *Pseudanabaena limnetica* NIVA-CYA 276/6. The cultivations were performed under standard laboratory conditions, using Z8 medium (24). Illumination was provided by fluorescent lamps exposing the cultures to 30 microeinsteins m^{-2} s^{-1}.

Limnological, hydrobiological, and hydrochemical methods. The lake localities were selected on the basis of their geographical characteristics (Table 1). The quantitative and qualitative samples for the chemical and microbiological analyses were collected in the free water bodies, 10 cm below the surfaces of the lakes. The fieldwork was carried out in April 1996 and September 1998 and followed limnological on-site procedures and sample storage methods (41).

The chemical compositions of the water samples were analyzed at the laboratory of the Norwegian Institute for Water Research, Oslo, Norway. The determinations comprised pH, conductivity, total phosphorous, total nitrogen, total organic carbon, and chloride analyses. The chemical procedures applied were methods approved by the Norwegian Standards Association (23).

Sample preparation and DNA purification. All of the strains used in this work were isolated from single cells or filaments (clone cultures). Aliquots of dense clone cultures (1 ml each, containing approximately 10^7 cells/ml) were pelleted in a microcentrifuge (model 2231M; Hermle GmbH, Goshe, Germany) at 5,000 rpm for 10 min and immediately frozen at −80°C. The DNA in the frozen pellets was purified by the use of a magnetic-bead-based DNA DIRECT DNA isolation kit (Dynal A/S, Oslo, Norway), with the manufacturer's protocol being modified for the purification for cyanobacteria (30).

The samples collected in the field were preserved at the site in 50% (vol/vol) isopropanol, as described for the cell concentration step below. Three samples were collected from each sampling site. The samples were then transported to the laboratory and processed further. The DNA was purified by a newly developed cell concentration-DNA purification protocol (31). In this solid-phase protocol, cells from 0.8 ml of aqueous solution were adsorbed for 20 min onto paramagnetic beads (final volume, 2 ml) in a buffer containing 50% (vol/vol) isopropanol, 0.75 M ammonium acetate, and 1 U (the beads in 200 μl of lysis buffer) of DNA DIRECT Dynabeads (Dynal A/S). The magnetic beads and the adsorbed microorganisms were attracted to the side of a 2-ml microcentrifuge tube by a MPC-Q magnet (Dynal A/S). Then 20 μl of 4 M guanidine thiocyanate–1% (wt/vol) Sarkosyl was added, and the incubation was continued at 65°C for 10 min. The DNA was precipitated onto the beads by addition of 40 μl of 96% ethanol, with subsequent incubation at room temperature for 5 min. Finally, the DNA-bead complexes were washed twice with 500 μl of 70% (vol/vol) ethanol, with a magnet being used between washings. The complex was dried at 65°C for 5 min to remove residual ethanol. The beads with the bound DNA was then used directly (no elution of DNA) in the amplification reactions.

Probe construction. Partial 16S rDNA sequences covering variable regions V6 to V8 (positions 346 to 845 relative to the published *Escherichia coli* 16S rDNA sequence [3]) from a representative collection of 59 cyanobacterial sequences in the EMBL nucleotide sequence database (release 55, August 1999; Cambridge, England) were aligned both manually and by using the computer algorithm PILEUP from the GCG package (Genetic Computer Group, Madison, Wis.) (13). A phylogenetic tree was constructed by the neighbor-joining method (35), using the Treecon software package (47). The Kimura two-parameter model (16), with a transversion:transition weight of 2:1, was used to compute the distance matrix for the neighbor-joining analysis. The identification of the different signature sequences was done manually in a multiple-sequence alignment. The probes constructed from these regions are shown in Table 2.

PCR amplification. Ribosomal DNA was amplified by using the primer set CC-CD, which is targeted to universally conserved regions (34). The amplification reactions were performed with a GeneAmp 2400 PCR thermocycler (Perkin-Elmer, Norwalk, Conn.). The reaction mixtures included 10 pmol of primers, 200 μM each deoxynucleoside triphosphate, 10 mM Tris-HCl (pH 8.8), 1.5 mM $MgCl_2$, 50 mM KCl, 0.1% (wt/vol) Triton X-100, 1 U of DynaZyme DNA polymerase (Finnzymes Oy, Espoo, Finland), and purified DNA or DNA-bead complexes, in a final volume of 50 μl. Prior to amplification the DNA was denatured for 4 min at 94°C, and after amplification an extension step (7 min at 72°C) was performed. The amplification required 35 cycles of 96°C for 15 s, 70°C for 30 s, and 72°C for 1 min.

Multiplex cyclic labeling of probes. Twenty-microliter volumes of the PCR products from the amplification reactions were used in the cyclic labeling reactions. The deoxynucleoside triphosphates were dephosphorylated by addition of 100 nmol of Tris-HCl (pH 8.0), 50 nmol of $MgCl_2$, and 1 U of shrimp alkaline phosphatase (U.S. Biochemical Corp., Cleveland, Ohio), with subsequent incubation at 37°C for 1 h. Finally, the phosphatase was inactivated by heating the solution to 96°C for 10 min.

The cyclic labeling reactions were carried out in 80-μl volumes containing 3 pmol of each of the primers shown in Table 2; 10 pmol of ddATP, 10 pmol of ddGTP, and 10 pmol of ddTTP (all from Boehringer GmbH, Mannheim, Germany); 7 pmol of fluorescein-12-ddCTP (NEN, Boston, Mass.); 1.25 μl of Thermo Sequenase reaction buffer; 1.1 μl of enzyme dilution buffer; 0.15 μl of 32-U/μl Thermo Sequenase (Amersham Pharmacia plc, Little Chalfont, Buckinghamshire, England); and 25 μl of phosphatase-treated PCR product. The labeling was done for 10 cycles of 95°C for 30 s and 50°C for 4 min.

Probe hybridization and chromogenic detection. Primers (0.5-μl volumes of 100-pmol/μl solutions) complementary to those used in the labeling reaction were spotted onto Hybond membrane strips (4 by 5 cm; Amersham Pharmacia International plc) and then UV cross-linked (5,000 joule/cm^2). The strips were prehybridized for 2 h at 37°C in a prehybridization solution containing 0.7× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 1× SPEP, 5× Denhardt's solution, and 100 μg of heterologous DNA/ml (33). The products from the cyclic labeling reactions were added to 0.5-ml volumes of hybridization solution (0.7× SSC, 1× SPEP, 1× Denhardt's solution, 10% dextran sulfate, and 100 μg of heterologous DNA/ml) in 2-ml microcentrifuge tubes and denatured at 95°C for 5 min. The strips were added, and the incubation was continued with gentle rotation for 2 h at 37°C. The membrane strips were washed in 50 ml of a solution containing 1× SSC and 1% (wt/vol) sodium dodecyl sulfate, then in 50 ml of a solution comprising 0.1× SSC and 0.1% (wt/vol) sodium dodecyl sulfate, and finally twice in 50 ml of 0.10 M Tris-HCl (pH 7.5) containing 0.15 M NaCl. Each washing was carried out with brief vortexing at room temperature.

The membrane strips were blocked with 20 ml of 0.10 M Tris-HCl (pH 7.5) containing 0.15 M NaCl and 0.5% (wt/vol) skimmed milk for 1 h and incubated in 20 ml of the same buffer containing 1/1,000 volume of antifluorescein-horseradish peroxidase conjugate (NEN) for an additional hour. The membrane strips were washed three times, with brief vortexing, in 50 ml of 0.10 M Tris-HCl (pH 7.5) containing 0.15 M NaCl. For chromogenic detection of horseradish peroxidase, a RENAISSANCE 4CN *Plus* kit was used for 5 min, according to the manufacturer's recommendations (NEN).

The relative signal strengths were determined by scanning the membranes with an Agfa Snapscanner 600 (Agfa Gevaert N.V., Montsel, Belgium) and analyzed by using the Gel-Pro ANALYZER software (Media Cybernetics, Silver Spring, Md.).

TABLE 1. Geographical and limnological characteristics of the localities

Water body sampled	Geographic position			Altitude above sea level (m)	Analytical variable					
	County	Latitude	Longitude		pH	Conductivity (mS/m)	Phosphorus concn (μg/liter)	Nitrogen concn (μg/liter)	Total dissolved carbon (mg/liter)	Chlorine concn (mg/liter)
Lake Steinfjorden[b]	Buskerud	60°04′N	10°20′E	63	7.6	10.3	7	250	5.1	5.1
Lake Gjersjøen[a]	Akershus	59°40′N	10°53′E	40	7.6	19.7	6	1,500	6.5	18.5
Lake Stensrudtjernet[a]	Oslo	59°48′N	10°55′E	133	7.2	16.0	12	435	9.5	26.4
Lake Langen[a]	Akershus	59°45′N	10°58′E	126	6.9	5.86	17	420	8.7	6.5
Lake Østensjøvannet[a]	Oslo	59°54′N	10°49′E	107	7.3	14.0	124	1,180	8.9	9.4
Lake Holstadvatnet[a]	Oslo	59°40′N	10°53′E	91	7.9	22.9	135	1,200	10.2	19.0
Lake Årungen[a]	Akershus	59°41′N	10°45′E	34	7.6	26.8	69	3,940	11.3	25.6
Lake Fløtjønna[c]	Møre & Romsdal	63°20′N	8°04′E	1	—[d]	—	—	—	—	—

[a] Date of sampling: 3 September 1998 (surface water).
[b] Date of sampling: 21 September 1998 (surface water).
[c] Lake influenced by marine water. Date of sampling: 29 May 1996 (surface water).
[d] —, not determined.

RESULTS

The specificities of the 16S rDNA probes were first tested on unialgal clone cultures. The probes were then used to determine the relative distributions and abundances of cyanobacteria in samples from the eight lakes. Finally, the results from the molecular analyses were compared with data obtained by hydrobiological and hydrochemical methods.

Construction and validation of the probes. The probes were constructed from informative nucleotide positions in the alignment of the 59 representative cyanobacterial sequences, in addition to that of *E. coli*. These informative sites were determined by phylogenetic reconstruction (Fig. 2A). The probes (whose names are in parentheses) were targeted to the positions defining the branches leading to the genera *Aphanizomenon* (APHA), *Anabaena* (ANAB), *Phormidium* (PHORM), *Planktothrix* (PLAN#1 and PLAN#2), and *Microcystis* (MICR#1 and MICR#3); to *M. aeruginosa* NIVA-CYA 143, 57, 228/1, 43, 123/1, 31, and 166 as well as *M.* cf. *ichthyoblabe* NIVA-CYA 279, *M.* cf. *wesenbergii* NIVA-CYA 172/5, and *M. viridis* NIVA-CYA 122/2 (MICR#2); and to the genera *Nostoc*, *Aphanizomenon*, and *Anabaena* (NOSTOC); and finally the branch leading to all the eubacteria investigated here (UNIVER).

The region in the 16S rDNA with the most informative sequence is the variable region V7. The probe sequences (MICR#2 and MICR#3, NOSTOC, and ANAB) are located in this region (Fig. 2B). The probe PHORM sequence is located in an insertion that is unique to *Phormidium* sp. strain NIVA-CYA 203. The probe PLAN#1 is the only probe that is constructed solely for discrimination by hybridization and not for the combination of hybridization and probe labeling. That is, the position used for labeling is conserved (i.e., shared with other groups) while the variable positions are in the hybridizing region. Finally, the probe UNIVER was constructed from a conserved region between V6 and V7 (Fig. 2B). This probe is conserved among all the investigated eubacteria with respect to both probe labeling and hybridization.

The specificities of the constructed probes were tested on eight different cyanobacterial strains, representing the major lineages. The outgroup *Chlorobium* sp. was included as an additional control for probe specificity. Approximately 5% of the DNA purified from the unialgal cultures was used in 50-μl PCR amplifications with the primer pair CC-CD (34). The products were visualized on an ethidium bromide-stained 1.5% agarose gel in order to confirm the success of the amplification reaction. All samples were uniformly amplified, yielding a strong band at approximately 600 bp and no additional visible bands (results not shown).

The universal probe for all eubacteria (UNIVER) was labeled relatively uniformly for the strains tested (Fig. 3A). The different cyanobacterium-specific probes (except for PLAN#1) gave the signals expected for the respective signature sequences (compare Fig. 2 and 3). The low specificity of PLAN#1 may have been caused by the hybridization conditions, which may not have been stringent enough for this probe. On the other hand, high specificity for the *Planktothrix* group was observed for the probe PLAN#2, which was constructed for discrimination by a combination of labeling and

TABLE 2. Oligonucleotide probes used in this study

Probe	Probe sequence[a]	Positions[b]
PHORM	5′CCTCTGGTACCGTCAGGTTGCTTTCACAA3′	491–472
MICR#1	5′CCCTGAGTGTCAGATACAGCCCAGTAG3′	761–735
MICR#2	5′GCAGGTGGTCAGCCAAGTCTGC3′	580–601
MICR#3	5′TCTGCCAGTTTCCACCGCCTTTAGGT3′	649–620
PLAN#2	5′TACAGGCCACACCTAGTTTCCATCGTTTAC3′	840–811
ANAB	5′CTGCTGTTAAAGAGTCTGGCTCAACCAGAT3′	594–625
APHA	5′CCCCTAGCTTTCGTCCCTCAGTGTCAGT3′	775–748
NOSTOC	5′GCTCAACCARATMARAGCAGTGGAAACTA3′	616–644
PLAN#1	5′CAATCATTCCGGATAACGCTTGCATCC3′	565–539
UNIVER	5′CCGTMTTACCGCGGCTGCTGGCA3′	537–515

[a] Primers complementary to these probe sequences were spotted on the membranes.
[b] Positions are relative to the published *E. coli* 16S rDNA sequence (3).

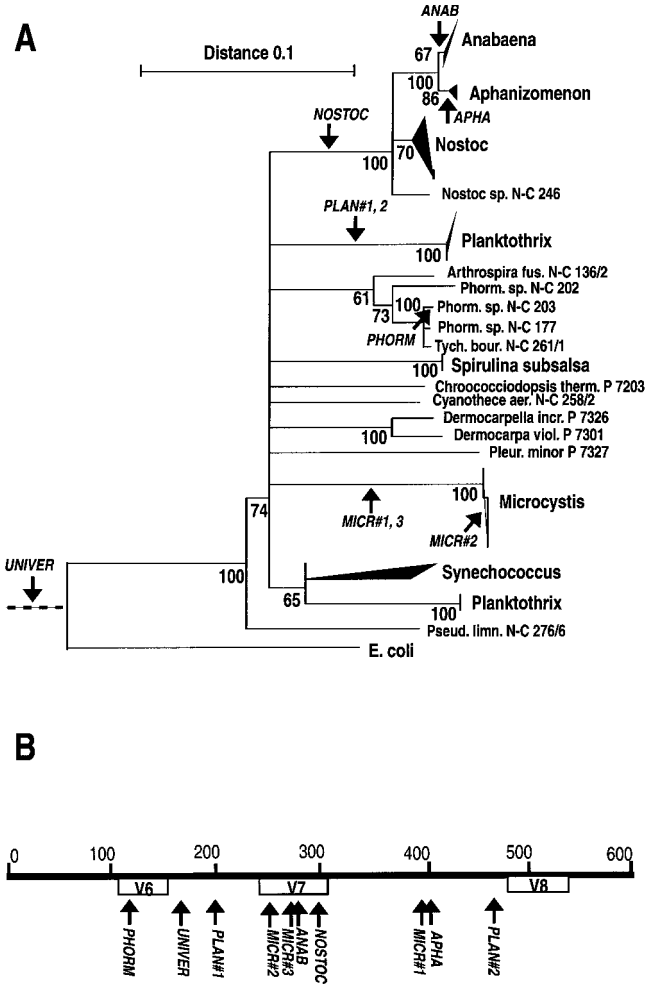

FIG. 2. Phylogenetic positions of the different strains and probes (A) and their locations in the 16S rDNA gene (B). (A) The distance tree was built with the neighbor-joining algorithm (35), using distance matrixes from the Kimura two-parameter model (16) and bootstrap analysis (Treecon software package) (47). The distance between two organisms, expressed in substitutions per nucleotide, is obtained by adding the horizontal branches connecting them. The rectangles define groups of organisms. Numbers at the nodes indicate the percentage of 500 bootstrap trees in which the cluster descending from the node was found. The bars indicate the phylogenetic positions of the constructed probes. (B) Probe positions shown relative to nucleotides 346 to 845 of the published *E. coli* 16S rDNA sequence (3). Sequences with the following EMBL accession numbers were used in the phylogenetic reconstruction: z82808, z82784, z82785, z82786, z82783, z82775, z82780, z82779, z82796, z82799, z82795, z82798, z82994, z82790, z82793, z82791, z82778, z82787, z82788, z82802, z82797, z82801, z82800, z82806, z82809, z82803, z82776, z82805, z82804, z82789, z82807, z82810, z82777, z82782, z82781, y12604, y12605, y12606, y12607, y12608, y12609, y12610, y12611, y12612, y12613, y12614, y12676, y12677, y12678, y12679, y12680, y12681, y12682, y12683, y12684, y12685, y12686, y12687, and y12688. Abbreviations: fus., *fusiformis*; Phorm., *Phormidium*; Tych. bour., *Tychonema* bour *bourrellyi*; therm., *thermalis*; aer., *aeruginosa*; incr., *incrassata*; viol., *violacea*; Pleur., *Pleurocapsa*; Pseud. limn., *Pseudanabaena limnetica*.

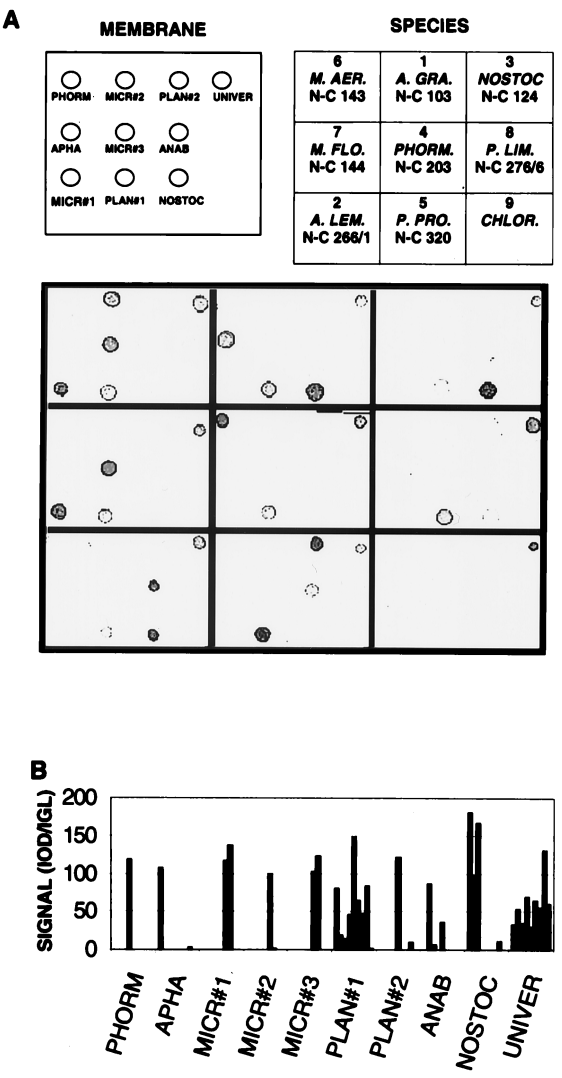

FIG. 3. Multiplex sequence-specific labeling for clonal isolates of organisms in culture (A), with corresponding signal intensities (B). (A) The probe locations for the nine membranes (bottom) are shown above them (MEMBRANE). The species order is shown in the upper right panel (SPECIES). Abbreviations: *M. AER*. N-C 143, *M. aeruginosa* NIVA-CYA 143; *M. FLO*. N-C 144, *M. flos-aquae* NIVA-CYA 144; *A. LEM*. N-C 266/1, *Anabaena lemmermannii* NIVA-CYA 266/1; *A. GRA*. N-C 103, *Aphanizomenon gracile* NIVA-CYA 103; *PHORM*. N-C 203, *Phormidium* sp. strain NIVA-CYA 203; *P. PRO* N-C 320, *Planktothrix prolifica* NIVA-CYA 320; *NOSTOC* N-C 124, *Nostoc* sp. strain NIVA-CYA 124; *P. LIM* N-C 276/6, *Pseudanabaena limnetica* NIVA-CYA 276/6; CHLOR., *Chlorobium* sp. (B) Signal intensities, determined by measuring the pixel density in an 8-bit grayscale image (IOD/IGL), are shown. Positions (left to right) correspond to the numbering in the SPECIES panel above.

hybridization. High specificities were also obtained for the probes that were constructed for discrimination through labeling alone. For instance, there is only a single base pair difference in the amplified region between *M. aeruginosa* strains NIVA-CYA 143 and 144. Using the probe MICR#2, this single-base difference was sufficient to separate NIVA-CYA 143 and 144 with a signal-to-noise ratio of 80.

The incorporation efficiency of fluorescently labeled dideoxynucleotides is sequence dependent (27, 28). The base composition and the melting point, in addition to the sequences flanking the probe region, could affect the probe hybridization and, subsequently, the labeling efficiencies (42). The relevant labeling efficiencies ranged from 1.0 to 5, relative to the labeling of the universal probe UNIVER. For the individual probes, the efficiencies were as follows: PHORM, 1.7; MICR#1, 2.2; MICR#2, 1.4; MICR#3, 1.9; PLAN#2, 4.0; ANAB, 1.6; APHA, 3.3; NOSTOC, 4.3; and PLAN#1, 4.9.

Presence of cyanobacteria in the lakes. The developed assay was tested on water samples from eight different localities with water conditions ranging from mesotrophic to eutrophic. The water quality of the samples was evaluated by hydrochemical

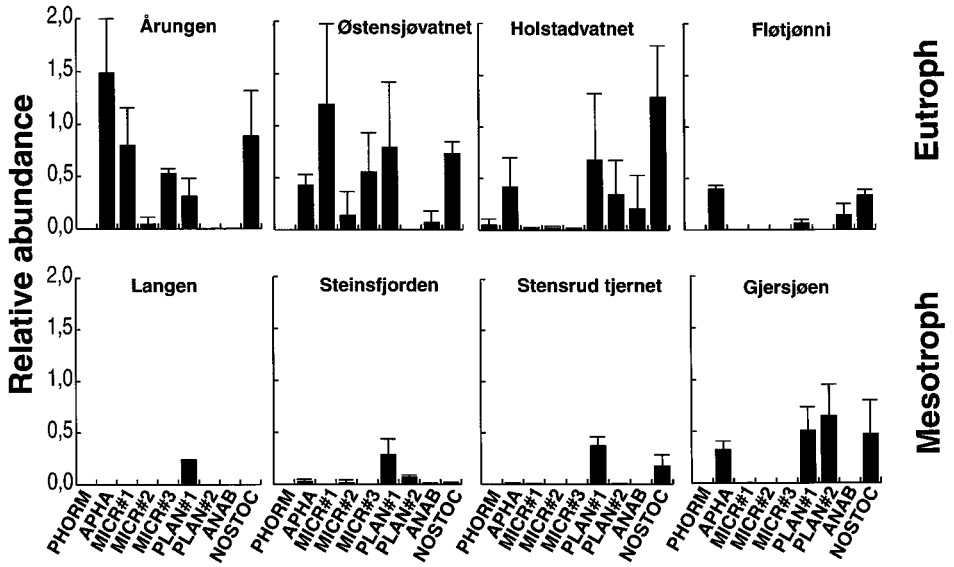

FIG. 4. Water profile analysis for eight Norwegian lakes. The lakes have been classified as mesotrophs (relatively low contents of biomass) or eutrophics (those with high biomass contents). The signal intensities relative to the universal probe UNIVER were multiplied by a factor obtained from clonal isolates to correct for differences in probe labeling efficiencies to obtain the relative abundances of the different genotypes in the samples (see Results). The error bars represent standard deviations.

analyses (Table 1). The pHs of the water samples ranged from 7 to 8, while the conductivities ranged from 6 to 27 mS/m. The total amounts of organic carbon (composed mainly of natural organic substances) in the samples were in the concentration range 6.5 to 11.3 mg/liter.

The cell concentrations and DNA purifications were done as described in Materials and Methods. PCR amplification products were obtained for the samples from Lakes Østensjøvatnet, Gjersjøen, and Årungen when 90% of the purified DNA was used in the 50-μl PCR mixture. Using 9% of the material, all of the samples were amplified except for those from Lake Langen. With an input of 1% of the material, amplification reactions were achieved for all samples.

The water sample from Lake Langen seemed to contain PCR-inhibitory compounds. The water from this lake contained slimy substances that clogged the 25-μm-pore-size plankton net. These biopolymers have been attributed to a population of the flagellate *Gonyostomum semen* (9), which produces a sticky slime. The nature and possible inhibitory effects of the substances involved may explain the apparent inhibition of PCR for samples from Lake Langen.

The average signal strengths and standard deviations for the probe labeling studies were calculated based on data for the three parallel samples from each investigated locality (Fig. 4). The bars in Fig. 4 show the approximate relative genotypic composition of each sample. The abundance of genotypes is given relative to the total abundance of procaryotes in the samples (including chloroplasts and heterotrophic bacteria), which was determined by amplification with the PCR primer pair CC-CD that is universal for all eubacteria.

The dominating species of cyanobacteria for each locality were also identified based on morphological and/or cytological characteristics (40) (Table 3). Upon comparison, there were some discrepancies between the results obtained by the genetic and by the morphological-cytological analyses (compare Table 3 and Fig. 4). It was not possible to distinguish the phycologically defined genera *Anabaena* and *Aphanizomenon* by using the probe labeling assay for the natural isolates. On the contrary, these genera were readily discriminated with the probes ANAB and APHA, respectively, for the laboratory strains tested. Generally, however, the probes constructed in this work could discriminate between the genera while the morphologically and/or cytologically defined species could not be separated.

Qualitatively, there were relatively good correlations among the groups of organisms (*Nostoc*, *Microcystis*, and *Planktothrix* spp.) found in the different lakes. However, the *Planktothrix*-specific probe PLAN#2 gave a relatively strong signal in the probe labeling assay with samples from Lake Gjersjøen, while organisms belonging to this genus were not observed during the microscopic examinations. In addition, the correlations were lower for the quantitative data. The differences in the quantitative data may, however, be partially due to the use of different sampling methods. For the genetic analyses the water samples were analyzed directly, while for the morphological and cytological analyses the samples were filtered through a plankton net with a 25-μm-diameter pore size. This filtering may select for filaments and cyanobacterial colonies, with single cells being excluded.

DISCUSSION

Genetic detection of microorganisms in their natural habitats. Nucleic acid sequences provide objective and statistically testable criteria for systematic characterization and classification (25, 34). It is generally possible to construct probes for phylogenetic groups of various evolutionary levels. These probes subsequently may be used in a hierarchical classification system for practical purposes (43).

Until now, a limitation in the genetic analysis of microorganisms in naturally occurring ecosystems has been the problem of dealing with complex communities (14). The tools currently available, such as in situ techniques (1, 38, 44), dot blot hybridization (43), and selective PCR amplification (22), are generally designed for simultaneous detection of only one or, at most, a minimal selection of organisms. Recently, there have been efforts to employ multiplex analysis through the use of array hybridization assays (12a, 19, 36) and DGGE analyses

TABLE 3. Cyanobacterial presence in the lake communities in September 1998[a]-microscopic examination of plankton net samples (25 μm)

Cyanobacterium	Presence or absence of cyanobacteria in Lake[b]:							
	Steinsfjorden	Gjersøen	Fløtjønni	Langen	Stensrudtjernet	Holstadvatnet	Østensjøvannet	Årungen
Anabaena planktonica	−	+++++	−	−	−	−	−	+++++
Anabaena crassa	−	−	−	−	−	+	−	−
Anabaena spiroides	−	−	−	−	−	−	++++	−
Anabaena lemmermannii	+							
Anabaena cf. *curva*	+	++	−	−	−	−	−	−
Anabaena planktonica		−	−	−	−	+	−	−
Anabaena sp. strain Bory	−	++	+++++	−	−	−	−	−
Aphanizomenon cf. *klebahnii*	−	−	−	−	−	−	−	+++
Aphanizomenon cf. *gracile*	−	−	−	−	−	+	−	−
Aphanothece sp.	+++			−	−	−	−	−
Microcystis aeruginosa	−	+	−	−	−	−	++	+
M. cf. *flos-aquae*	−	−	−	−	−	+	+	−
M. botrys	−	+	−	−	−	−	+++	−
M. ichthyoblabe	−	−	−	−	−	−	+++	−
M. wesenbergii	−	−	−	−	−	−	+++	−
Planktothrix agardhii	−	−	−	−	−	+++++	−	+
P. prolifica	+++	−	−	−	−	−	−	−
P. rubescens	++++	−	−	−	−	−	−	−
Woronichinia naegeliana	+	−	−	−	−		−	−

[a] Determined by microscopic examination of plankton net samples (25 μm).
[b] −, not observed; +, sporadically seen; ++, few seen; +++, common; ++++, abundant; +++++, dominant.

(24a). Array hybridization, however, gives a relatively low signal-to-noise ratio (49), while for DGGE analyses the distinct nucleic acid populations must have different migration patterns in the gel.

We have combined the specificity and sensitivity obtained through enzymatic labeling of DNA probes with the multiplex detection obtained with array hybridization. A single base substitution could be detected with a signal-to-noise ratio of 80. This assay may make it possible to accurately analyze whole microbial communities and to directly relate sequence information obtained from organisms in type culture collections to the biodiversity that exists in nature.

Comparison of the labeling assay and the morphological-cytological analyses. Analyses of pure cultures indicated that the labeling assay in itself is specific and yields quantitative information (33). However, for the analyses of natural samples, each of the steps, from sample preparation to detection, must be quantitative for the complete assay to be quantitative.

As described in Results, for the two methods used, there were relatively good qualitative correlations for the groups of organisms (*Nostoc*, *Microcystis*, and *Planktothrix* spp.), in the samples, while the quantitative correlations were lower. With both methods there can be introduced errors, in the form of exclusion of single cells by the filtering protocol for the microscopic analyses and in the form of different amplification efficiencies for the different 16S rDNA populations in the genetic analyses. However, another major reason for the quantitative differences is that cells are counted in the microscopic analyses while gene copy numbers are determined for the genetic analyses. Furthermore, 16S rDNA copy numbers in different organisms may differ. In addition, the genome itself may be present in various numbers of copies per cell, depending on the growth conditions.

The use of genetic and morphological-cytological criteria in taxonomy. Morphological characteristics may actually be corresponding although the organisms concerned are evolutionarily divergent. This is the case, for example, for organisms in the genera *Tychonema* and *Planktothrix*. They can only be distinguished based on a few particular morphological and cytological characteristics (39). However, based on genetic criteria, the organisms belonging to these genera are relatively divergent (34). Furthermore, on the basis of morphological similarities, several *Synechococcus* species have been misclassified as being in the genus *Microcystis* (21). On the other hand, species that have so far been genetically indistinguishable, such as those constituting the genus *Planktothrix*, may be separated by their morphological and cytological differences, e.g., through differences in pigmentation and trichome diameter (32, 39).

Morphological and cytological studies concentrate on phenotypic characteristics, which are subject to direct selection by the environment, presumably leading to nonlinear evolutionary patterns (8). Genetic alterations, on the other hand, are predominantly caused by neutral mutations, which are assumed to have linear evolutionary patterns (15). The nonlinear evolutionary patterns involve apparent limitations connected with selective characteristics (e.g., morphological and cytological) used in classification. Horizontal gene transfer, on the other hand, will influence the usefulness of some genetic characteristics for taxonomic purposes (18). It is interesting that the cyanobacterial evolutionary tree consists of several genetically clustered groups of organisms with presumably relatively high frequencies of genetic exchange, while between these groups the gene exchange frequencies are low (26, 32). The *Aphanizomenon* and *Anabaena* probes constructed in this work were not specific for the species found in the investigated lakes, although they were specific for the organisms in culture. A possible explanation for this difference is gene transfer (32). Our study suggests that genetic characteristics can be used to separate an apparently genetically distinct group of organisms, while morphological-cytological characteristics are suited for decisions about systematic units at a basic level inside the genetic groups (26).

Polyphasic approaches in systematics, classification based on a synthesis of a multitude of different criteria (genotypic and phenotypic), are emerging in microbial taxonomy (4, 34). Such strategies will also advance the methodologies for analyses of complex natural communities (24a). However, as discussed

above, it is necessary to consider and interpret the evolutionary nature of the different systematic criteria applied.

Genetic profiles as tracers of environmental conditions. The use of living organisms to monitor environmental status and changes has been an important aspect of biometry throughout history (17). The presence or absence of indicator organisms or communities may foretell the long-term environmental effects. Cyanobacteria and other microalgae can be used as indicators of eutrophication (2). For instance, the role of phosphorus as a limiting nutrient for photosynthetic algae is well documented (48). Not surprisingly, a correlation between phosphorus and photosynthetic algae was also found in our data. However, in addition, we found that cyanobacterial biodiversity increased with increasing eutrophication (Fig. 4).

Bioremediators may also be used to monitor changes in undefined environmental conditions. Most likely, changes in the environment will lead to alterations of microbial community compositions (20). Thus, changes in unknown factors can be detected at an early stage through the monitoring of complex populations. Furthermore, genetic information about aquatic communities can be obtained by nucleic acid analysis of organisms in water samples without the organisms being characterized in culture (10, 38).

The use of high-density oligonucleotide arrays has transformed the field of genome analysis and expression studies (6, 7). The accuracy of array hybridization can be enhanced considerably by the combination of high-density DNA arrays with the sequence-specific labeling of oligonucleotide probes. The goal is a genetic assay that is suitable for analyses of composite microbial communities.

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