(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
2 December 2004 (02.12.2004)

PCT

(10) International Publication Number
WO 2004/104211 A2

(51) International Patent Classification[7]: C12Q

(21) International Application Number: PCT/FI2004/000310

(22) International Filing Date: 21 May 2004 (21.05.2004)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
20030771 21 May 2003 (21.05.2003) FI

(71) Applicants (*for all designated States except US*): **HELSINGIN YLIOPISTO** [FI/FI]; Yliopistonkatu 4, PL 33, FI-00014 Helsingin Yliopisto (FI). **UNIVERSITY OF LIEGE** [BE/BE]; Quai van Beneden, 25, B-4020 Liege (BE). **CONSIGLIO NAZIONALE DELLE RICERCHE** [IT/IT]; Instituto di Technologie Biomediche, Via Fratelli Cervi, 93, I-20090 Segrate Milano (IT). **CONSIGLIO NAZIONALE DELLE RICERCHE** [IT/IT]; Instituto per lo Studio degli Ecosistemi, Largo Vittorio Tonolli, 50/52, I-28922 Pallanza Verbania (IT).

(72) Inventors; and
(75) Inventors/Applicants (*for US only*): **SIVONEN, Kaarina** [FI/FI]; Versokuja 6 B, FI-00790 Helsinki (FI). **RANTALA, Anne** [FI/FI]; Vuorimiehenkatu 5 B 30, FI-00140 Helsinki (FI). **ROUHIAINEN, Leo** [FI/FI]; Versokuja 6 B, FI-00790 Helsinki (FI). **FEWER, David** [FI/FI]; Mustankivenaukio 3 B 32, FI-00980 Helsinki (FI). **RAJANIEMI, Pirjo** [FI/FI]; Mäkelänkatu 34 A 6, FI-00510 Helsinki (FI). **WILMOTTE, Annick** [BE/BE]; Rue de la charrette, 83, B-4130 Tilff (BE). **BOUTTE, Christophe** [FR/FR]; 4 rue Louis Odin, F-42500 Le Chambon Feugerolles (FR). **GRUBISIC, Stana** [BE/BE]; 6, rue Saint Julien, B-4020 Liege (BE). **BALTHASART, Pierre** [BE/BE]; rue de Serbie, 85, B-4000 Liege (BE). **DE BELLIS, Gianluca** [IT/IT]; via Fratelli Cervi, 93, I-20090 Segrate Milano (IT). **RIZZI, Ermanno** [IT/IT]; via Fratelli Cervi, 93, I-20090 Segrate Milano (IT). **FROSINI, Andrea** [IT/IT]; via Fratelli Cervi, 93, I-20090 Segrate Milano (IT). **CASTIGLIONI, Bianca** [IT/IT]; via Fratelli Cervi, 93, I-20090 Segrate Milano (IT). **VENTURA, Stefano** [IT/IT]; via Franco Bolognese 2, I-40129 Bologna Bo (IT). **MUGNAI, Maria Angela** [IT/IT]; via A. Cadamosto 14, I-59100 Prato Po (IT).

(74) Agent: **SEPPO LAINE OY**; Itämerenkatu 3 B, FI-00180 Helsinki (FI).

(81) Designated States (*unless otherwise indicated, for every kind of national protection available*): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NA, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) Designated States (*unless otherwise indicated, for every kind of regional protection available*): ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *without international search report and to be republished upon receipt of that report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 2004/104211 A2

(54) **Title:** METHOD FOR DETECTING TOXIC AND NON-TOXIC CYANOBACTERIA

(57) **Abstract:** This invention is related to a method for detecting toxic and non-toxic cyanobacteria. The method comprises that nucleic acid from a biological sample is brought into contact with an oligonucleotide designed to be specific for the mcy gene, in particular mcyE and/or mcyD, and with an oligonucleotide designed to be specific for 16SrDNA, and the presence or absence of toxic cyanobacteria is detected by a suitable molecular biology method. The invention is related also to oligonucleotides used in the method.

METHOD FOR DETECTING TOXIC AND NON-TOXIC CYANOBACTERIA

This invention relates to a method for detecting toxic and non-toxic cyanobacteria. This invention relates also to oligonucleotides, which can be used in the detection method.

5 **BACKGROUND OF THE INVENTION**

Cyanobacteria produce a wide variety of bioactive compounds. Many of these are potent toxins, which cause health problems for animals and humans when producer organisms occur in masses in lakes and water reservoirs (Sivonen and Jones, 1999). Most well known of the
10 cyanobacterial toxins are the hepatotoxic heptapeptides, microcystins. The general structure of microcystins is *cyclo*(-D-Ala-X-D-MeAsp-Z-Adda-D-Glu-Mdha-), where X and Z are variable L-amino acids, D-MeAsp is D-*erythro*-β-methylaspartic acid, Mdha is N-methyldehydroalanine and Adda is 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid. More than 65 structurally different microcystins are known (Sivonen and Jones,
15 1999). Most common variants have L-leucine and L-arginine in the positions of X and Z, respectively, and demethylated forms are also frequently found. Toxicity of microcystins is caused by the inhibition of protein phosphatases 1 and 2A (MacKintosh et al., 1990). The level of inhibition varies depending on the structure, but the Adda and D-Glu moieties, which are almost invariable in microcystins, are essential for the inhibition (Goldberg et al., 1995)
20 and hence for the toxicity.

Microcystins have been found predominantly in cyanobacteria of three planktonic, bloom-forming genera, *Anabaena, Microcystis* and *Planktothrix* (Sivonen and Jones, 1999). Not all members of these genera make microcystins and both toxic and non-toxic strains occur in the
25 same species. Toxic and non-toxic strains of *Anabaena*, *Microcystis* or *Planktothrix* cannot be separated based on the classical morphological taxonomy or ribosomal gene sequencing (Lyra et al., 2001). On the other hand, one strain may produce different microcystins and also other peptides simultaneously (Sivonen et al., 1992; Fujii et al., 1996; Fastner et al., 2001.

30 Peptide synthetase genes were shown to be required for the synthesis of microcystins (Dittmann et al., 1997). Recently, the gene clusters encoding microcystin synthetase were sequenced and characterized from the unicellular *Microcystis aeruginosa* (Nishizawa et al.,

2000; Tillet et al., 2000) and from the filamentous *Planktothrix agardhii* (Christiansen et al., 2003). It was demonstrated that the microcystins biosynthesis is a combination of peptide and polyketide synthesis (Nishizawa et al., 2000; Tillet et al., 2000).

5 The microcystin synthetase gene region spans about 55 kb, and includes genes for peptide synthetases (*mcy*A, -B, -C), polyketide synthases (*mcy*D), mixed peptide synthetase and polyketide synthases (*mcy*E, -G), and tailoring enzymes Tillett. *et al.* (2000), Nishizawa *et al.* (2000).

10 Microcystin producers among the filamentous, nitrogen-fixing genus, *Anabaena*, are found in North America, in France and in Northern Europe, where they frequently develop massive growth in lakes and reservoirs (Sivonen and Jones, 1999). The bioactive peptides produced by *Anabaena* 90 have been characterized: three microcystins (MCYST-LR, MCYST-RR and D-Asp-MCYST-LR; Sivonen et al., 1992), two seven-residue depsipeptides (anabaenopeptilide
15 90A and 90B), and three six-residue peptides having an ureido linkage (anabaenopeptins A, B and C; Fujii et al., 1996). However, the microcystin synthetase gene region from *Anabaena* has not been sequenced.

Based on the sequence data available, various DNA probes and primers have been designed
20 and used to discriminate between toxic microcystin-producing and non-toxic non-microcystin producing genotypes by hybridization and PCR. However, the existing primers deduced from *Microcystis mcy* genes, reliably identify potential microcystin-producers only in *Microcystis* and fail to amplify *mcy* sequences from part of microcystin containing strains of other genera. There is therefore a great need for oligonucleotides, which could be used as probes and primers
25 in detecting toxic cyanobacteria also in genera other than *Microcystis*. Such oligonucleotides should discriminate between toxic microcystin-producing and non-toxic non-microcystin producing genotypes in various molecular biology methods, such oligonucleotides should be specific to the studied cyanobacteria genera and the oligonucleotides should be able to discriminate the most important or dominating microcystin producing cyanobacteria genera
30 from one another.

It would be also of advantage if non-toxic cyanobacteria could be identified.

SUMMARY

It is the aim of the present invention to eliminate the problems associated with the prior art.

One object of this invention is to provide a method for the detection of toxic cyanobacteria.

In this invention it has been surprisingly found that by designing oligonucleotides to be specific for *mcyE* gene of the microcystin synthetase gene region, it is possible to detect cyanobacteria from all of the most potent toxin producing cyanobacteria genera. In addition it is possible to identify which cyanobacterial genus produces the toxin.

In particular, the oligonucleotides are designed to be specific for a region of *mcyE* gene responsible for adding Adda and D-glutamate to the immature synthesis product.

More specifically, the oligonucleotides are designed to be specific for a region of *mcyE* gene region catalyzing a peptide synthesis between Adda-D-glutamate and dehydroalanine and to the adenylating region. It is assumed that the step of adding Adda -D-glutamate-dipeptide is decisive for toxicity of the product. However, it is surprising that oligonucleotides designed to be specific for this region are genus specific and at the same time capable of identifying cyanobacteria from all other toxin-producing genera. Oligonucleotides of this invention can identify toxin producers at least among *Anabaena*, *Microcystis*, *Planktothrix*, *Nostoc* and *Nodularia* genera.

In this invention the whole microcystin synthetase gene region from *Anabaena* was sequenced. Before this invention it had not been possible to compare the sequences of microcystin synthetase gene region from the main microcystin-producing cyanobacteria genera.

The oligonucleotides of this invention can be used in detecting toxin-producing cyanobacteria by using various molecular biology methods. Such methods are for example hybridization, PCR, reverse transcriptase PCR, QRT-PCR, LCR, LDR and minisequencing.

These methods can be combined with a microarray method. In a preferred detection method ligase detection reaction (LDR) is used together with a microarray method. Another preferred detection method is quantitative PCR (QRT-PCR).

5 Furthermore, the oligonucleotides of this invention can be used in detecting toxin- producing cyanobacteria together with a detection method using oligonucleotides designed to be specific for any other *mcy* gene, such as mcyA or *mcyD* gene.

One highly preferred embodiment of this invention is the use of the oligonucleotides of this
10 invention together with oligonucleotides designed to be specific for 16S rRNA gene. Cyanobacterial genera can be identified based on the 16S rRNA gene. When oligonucleotides designed to be specific for *mcyE* (or some other mcy gene, such as mcyD) and for 16S rRNA gene are used together for example in the microarray method, it is possible to detect and identify both toxin- and non-toxin-producing genera. It is of great advantage that the
15 oligonucleotides designed to be specific for *mcyE* and for 16S rRNA gene can be used under the same conditions. The LDR can be carried out under the same conditions and the hybridization in microarray on the same slide. This makes the monitoring of non-toxin cyanobacteria and toxin-producing cyanobacteria technically easy and much more useful.

20 The detection method of the present invention can also be combined with a detection method measuring microcystin concentration, cell number, cell density or biomass. For example, *mcyE* copy number can be determined together with microcystin concentration and cell density and the main putative microcystin producers can be indicated.

25 One object of this invention are fragments of *mcyE* gene which are responsible for adding Adda and D-glutamate to the immature synthesis product in microcystin synthesis. In particular, the fragments are responsible for adding Adda-D-glutamate dipeptide to dehydroalanine. Such fragments are or are located in the sequences selected from the group comprising SEQ ID NO. 1 to SEQ ID NO: 34 as shown in Figure 19 A to H or comprising
30 sequences SEQ ID NO: 35 to SEQ ID NO: 39 as shown in Figure 15 A to C.

One object of this invention are furthermore oligonucleotides designed to be specific for any of the above mentioned fragments of *mcyE* gene, in particular for sequences selected from the group comprising SEQ ID NO. 1 to SEQ ID NO: 34 as shown in Figure 19 A to H or

sequences SEQ ID NO: 35 to SEQ ID NO: 39 as shown in Figure 15 A to C or for fragments of said sequences.

Preferred oligonucleotides are primers mcyE-F2 (SEQ ID Nos : 64), AnamcyE-12R (SEQ ID NO: 65) and MicmcyE-R8 (SEQ ID NO:66) which can be used for example in amplifying target (or sample) nucleic acid by PCR.

Preferred oligonucleotides are discriminating probes of SEQ ID NO: 40 to SEQ ID NO: 45 and common probes of SEQ ID NO: 46 to SEQ ID NO: 51, which can be used for example in the ligase detection reaction.

One object of this invention is furthermore the *mcyE* gene from the *Anabaena* genus encoding the amino acid sequence of SEQ ID NO: 67 or a sequence having at least 80 % identity, preferably 90 %, more preferably 95 % identity to said sequence, or a fragment of said sequence having polymorphic sites which make possible of designing oligonucleotides to be specific for the fragment.

One further object of this invention is *mcyE* gene from *Anabaena* genus having the nucleic acid sequence SEQ ID NO: 68 or a sequence having at least 80 % identity, preferably 90 %, more preferably 95 % identity to said sequence, or a fragment of said sequence having polymorphic sites which make possible of designing oligonucleotides to be specific for the fragment.

One object of this invention is furthermore the *mcyD* gene from the *Anabaena* genus encoding the amino acid sequence of SEQ ID NO: 69 or a sequence having at least 80 % identity, preferably 90 %, more preferably 95 % identity to said sequence, or a fragment of said sequence having polymorphic sites which make possible of designing oligonucleotides to be specific for the fragment.

One further object of this invention is *mcyD* gene from *Anabaena* genus having the nucleic acid sequence SEQ ID NO: 70 a sequence having at least 80 % identity, preferably 90 %, more preferably 95 % identity to said sequence, or a fragment of said sequence having polymorphic sites which make possible of designing oligonucleotides to be specific for the fragment.

One object of this invention are fragments of *mcyD* gene. Such fragments are or are located in the sequences selected from the group comprising SEQ ID NO. 131 to SEQ ID NO: 149 as shown in Figure 38 A to F.

One further object of this invention are oligonucleotides which can be used as discriminating probes and which are selected from the group comprising SEQ ID NO: 71 to SEQ ID NO: 90, and common probes which are selected from the group comprising SEQ ID NO: 91 to SEQ ID NO: 110. These primers and probes can be used for example in the ligation detection reaction.

Still further object of this invention is a kit for the detection of toxic cyanobacteria by the microarray method. The kit preferably comprises

- discriminating and common probes designed to be specific for *mcyE* gene;
- DNA or RNA zip and complementary zip codes assigned to be specific for selected cyanobacterial genera.

One still further object of this invention is a kit for detection of toxic cyanobacteria by hybridization. The kit preferably comprises

- primers designed to be specific for the *mcyE* gene;
- probes designed to be specific for selected cyanobacterial genera.

In the kit may be used alternatively or in addition probes and primers designed to be specific for *mcyD* gene or other *mcy* gene.

According to a highly preferred embodiment the kit comprises in addition to probes and primers designed to be specific for mcy gene (such as mcyE and/or mcyD) also probes and primers designed to be specific for 16 S rRNA gene.

Other features, aspects and advantages of the present invention will become apparent from the following description and appended claims.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1. The microcystin synthetase gene cluster of *Anabaena* strain 90, biosynthetic model for the formation of microcystin-LR and the general structure of microcystins. The symbols for the domains are: A, adenylation; C, condensation; T, thiolation; NMT, *N*-methyltransferase; EP, epimerase; TE, thioesterase; KS, β-ketoacyl synthase; AT, acyltransferase; CM, *C*-methyltransferase; DH, dehydratase; KR, β-ketoacyl reductase; ACP, acyl carrier protein; AMT aminotransferase. Adda is 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid, X and Z are variable amino acids. The arrows point to three methyl groups, which are putatively introduced by the *C*-methyltransferase domains. The way of cyclization of the microcystin precursor is shown with an arrow on the right of the picture.

Figure 2. A. Comparison of the putative C-methyltransferase domains in McyG, McyD and McyE of *Anabaena* 90 with three bacterial C-methyltranferase domains in the region of the conserved motifs:
1. (VIL)(LV)(DE)(VI)G(GC)G(TP)G; 2. (PG)(QT)(FYA)DA(IVY)(FI)(CVL) and
3. LL(RK)PGG(RIL)(LI)(LFIV)(IL) (Kagan and Clarke, 1994). EpoE is the polyketide synthase in epothilone biosynthesis of *Sorangium cellulosum* (AF217189), HMWP1 is the high-molecular-weight-protein in yersiniabactin biosynthesis coded by irp1 of *Yersinia enterocolitica* (Y12527) and ECUbiE is *Escherichia coli* C-methyltransferase, UbiE (P27851). Residues in bold letters (in the boxed areas) are identical to the consensus amino acids of the motifs. Amino acids (outside of the boxed areas), which are identical in at least five of the six sequences, are shaded.
B. Alignment of the aminotransferase domain of *Anabaena* 90 McyE, AmcyEamt, with other known aminotransferase domains and with two aminotransferases of *Escherichia coli*. McyEamt and PmcyEamt are from McyE of *Microcystis aeruginosa* PCC7806 (AF183408) and of *Planktothrix agardhii* CYA126 (AJ441056), respectively. ItuAamt is from iturin synthetase of *Bacillus subtilis* RB14 (AB050629) and MycAamt from mycosubtilin synthetase of *Bacillus subtilis* ATCC6633 (AF184956). EcGSA is glutamate-1-semialdehyde aminotransferase (F90648) and EcArgD is ArgD, acetylornithine aminotransferase (P18335). The conserved pyridoxal-5'-phosphate-binding residues (Mehta et al., 1993), an aspartate and a lysine, are marked with the asterisks. Amino acids, which are the same in at least five of the seven proteins, are shaded.

Figure 3.Motif sequence alignments of (A) dehydratase (DH) and (B) ketoreductase (KR) domains of *Anabaena* 90 microcystin synthetase, AMCD-DH2, AMCD-DH3, AMCG-KR1, AMCG-KR2 and AMCD-KR3, with rifamycin synthase, RifE-DH10 and RifE-KR10 (*Amycolatopsis mediterranei*; AF040570), and rapamycin synthase, RapA-DH4, RapB-DH10,
5 RapA-KR4and RapB-KR10, (*Streptomyces hygroscopicus*; X86780). The conserved residues of (A) the active site motif $H(X)_3G(X)_4P$ (Aparicio et al., 1996) and of (B) the NAD cofactor binding site, $GXGXX(G/A)(X)_3(G/A)$, (Scrutton et al., 1990) are marked with asterisks. Amino acids which are invariant in all proteins, are in bold letters (A) and (B). The numbers of the domains refer to the module of the particular synthase.

10

Figure 4. Comparison of the motifs in acyltransferase (AT) domains of the microcystin synthetases with the consensus sequences of malonyl and methylmalonyl loading AT domains described by Ikeda et al. (1999). AT domains (AT1-AT4) are from *Anabaena* 90, AMcyG, AMcyD and AMcyE, from *Microcystis aeruginosa*, MMcyG, MMcyD and MmcyE
15 (AF183408) and from *Planktothrix agardhii*, PMcyG, PMcyD and PmcyE (AJ441056). Bold letters indicate the amino acids, which are significantly specific to malonyl loading domains, and underlined, bold letters point out the residues, which are specific to methylmalonyl loading domains. Serines of the active site are marked with an asterisk.

20 Figure 5.Alignments of the β-ketoacyl synthase (KS) (A) and acyl carrier protein (ACP) (B) domains of *Anabaena* 90 microcystin synthetase with the KS and ACP domains of rapamycin synthase, RapA-KS1, RapA-ACP1 and RapC-ACP11 (*Streptomyces hygroscopicus,* X86780) and of rifamycin synthase, RifA-KS1 and RifA-ACP1 (*Amycolatopsis mediterranei,* AF040570) near the active sites. (A) AMCG-KS, AMCD-KS1, AMCD-KS2 and AMCE-KS
25 are from the KS domains of *Anabaena* 90 McyG, McyD and McyE, respectively. An asterisk marks the active site cysteines. The identical amino acids are in bold letters. The two histidine residues, which are invariant in PKS and fatty acid synthases (Aparicio et al., 1996) are underlined. (B) AMCG-ACP, ACD-ACP1, AMCD-ACP2 are from the ACP domains of *Anabaena* 90 McyG, McyD and McyE. The active site motif, which frequently is LGxDS, is
30 underlined . The serine residues, which bind phospho-pantetheine, are indicated by an asterisk.

Figure 6. The general structure of microcystins and nodularin. Microcystin is a cyclic peptide containing seven amino acids D-Ala-X-D-MeAsp-Z-Adda-D-Glu-Mdha, where X and Z

represent variable L-amino acids, D-Me-Asp is D-*erythro*-β-methylaspartic acid, Mdha is N-methyldehydroalanine, and Adda is the β-amino acid, 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid. Nodularin differs from microcystins by lacking the amino acids D-Ala and X, and having N-methyldehydrobutyrine (Mdhb) in place of Mdha. The dashed line indicates the two amino acids absent in nodularins.

Figure 7. Congruence between the 16S rRNA and *rpo*C1 data set and the microcystin synthetase gene data set. (A) A maximum-likelihood tree based on the 16S rRNA and *rpo*C1 data set (-lnL=8004.26493). Branch lengths are proportional to sequence change. Maximum likelihood and maximum parsimony bootstrap values from 1000 bootstrap replicates are given above and below the line respectively. (B) A maximum-likelihood tree based on the *mcyA*, *mcyD* and *mcyE* data set (-lnL=8781.50660). Branch lengths are proportional to sequence change. Maximum likelihood and maximum parsimony bootstrap values from 1000 bootstrap replicates are given above and below the line respectively.

Figure 8. A maximum-likelihood tree based on the 16S rRNA gene showing the sporadic distribution of cyanobacterial genera known to produce microcystins. Strains of the genera *Planktothrix*, *Microcystis*, *Anabaena* and *Nostoc* produce microcystins while strains of the genus *Nodularia* produce nodularins. Toxic strains are indicated by bold font.

Figure 9. Cycle treshold (Ct) values obtained by microcystin synthetase E (*mcy*E) quantitative real-time PCR (QRT-PCR) with external A) *Anabaena* standard strains of *Anabaena* 90 (O), *Anabaena* 315 (□), and *Anabaena* 202A1 (Δ) as well as with B) those of *Microcystis* strains *Microcystis* GL 260735 (O), *Microcystis* PCC 7806 (□), and *Microcystis* PCC 7941 (Δ) as a function of *mcy*E copy numbers. Error bars, which are almost hidden by the symbols, give the standard deviation for three independent amplifications.

Figure 10. Microcystin concentration (X) (μg l^{-1}) determined with ELISA and *Anabaena* as well as *Microcystis* microcystin synthetase E (*mcy*E) copy numbers (copies ml^{-1}) obtained with quantitative real-time PCR using Lake Tuusulanjärvi water samples collected during summer 1999. Gene *mcy*E copy numbers were calculated with the external standards of *Anabaena* 202A1 (■), *Anabaena* 315 (□), *Microcystis* PCC 7806 (○) and *Microcystis* PCC 7941 (●).

Figure 11. Microcystin concentration (X) (µg l^{-1}) determined with ELISA and *Anabaena* as well as *Microcystis* microcystin synthetase E (*mcy*E) copy numbers (copies ml^{-1}) obtained with quantitative real-time PCR using lake water samples collected from different water depths of four Lake Hiidenvesi basins on 15th of August 2001. Gene *mcy*E copy numbers were calculated with the external standards of *Anabaena* 202A1 (■), *Anabaena* 315 (□), *Microcystis* PCC 7806 (○) and *Microcystis* PCC 7941 (●).

Figure 12. The cell numbers of the most dominant cyanobacterial genera in Lake Tuusulanjärvi in 1999 by light microscopy. The most dominant cyanobacterial genera were *Anabaena* (□), *Microcystis* (O) and *Aphanizomenon* (Δ).

Figure 13. The cell numbers of the most dominant cyanobacterial genera in Lake Hiidenvesi on 15th of August 2001 by light microscopy. The most dominant cyanobacterial genera were *Anabaena* (□), *Microcystis* (O) and *Aphanizomenon* (Δ. The samples were taken from different water depths at the four basins of Lake Hiidenvesi..

Figure 14. Clusters of group-specific *mcyE* gene consensus sequences.

Figure 15. A, B, C. 800 bp consensus sequence of *mcyE* from *Anabaena, Microcystis, Nodularia, Nostoc, Oscillatoria/Planktothrix* (SEQ ID NOs 35 to 39).

Figure 16. The principle of the DNA-chip (Microarray) method.

Figure 17. Deposition scheme of the mcyE probes. Deposition scheme obtained using a non-contact dispensing system. Each zip code was spotted ten times. The deposition quality of the Zip Code oligonucleotides on the slides has been checked by means of hybridisations with Cy3 labelled poly(dT) complementary to the poly$(dA)_{10}$ sequence of each Zip Code.

Figure 18. Hybridization results obtained using PCR amplified mcyE gene coming either from pure strains or from environmental samples as template in LDR .

Figure 19 A - H. Alignment of 800 bp of nucleic acid sequences from 30 strains (+ 4 consensus sequences) from *Anabaena, Microcystis, Nodularia, Nostoc, and Oscillatoria/Planktothrix* genera (SEQ ID NOs 1 to 34).

Figure 20. List of polymorhism positions, group-specific probes (discriminating probes SEQ ID NOs 40 to 45 and common probes 46 to 51) and their correspondent Zip Codes and complementary Zip Codes SEQ ID NOs 52 to 57 and 58 to 63.

5 Figure 21. Amino acid sequence encoded by *Anabaena mcyE* gene (SEQ ID NO 67).

Figure 22 A-D. Nucleic acid sequence of *Anabaena mcyE* gene (SEQ ID NO 68).

Figure 23 A, B. Amino acid sequence encoded by *Anabaena mcyD* gene (SEQ ID NO 69).

10

Figure 24 A-D. Nucleic acid sequence of *Anabaena mcyD* gene (SEQ ID NO 70).

Figure 25A. The cyanobacterial phylogenetic tree constructed using the NJ algorithm, according to a central database of processed sequences. ARB cyanobacterial 16S rRNA gene
15 database we used contained 281 sequences from public databases and 59 from this study.
Figure 25B. Updated ARB tree with Snowella sequences.
Figure 25C. Updated ARB tree with subclustering of Anabaena and Aphanizomenon groups.

Figure 26. Main features of LDR method coupled to a Universal Microarray.
20 Panel A: After the hybridization of a discriminating probe and a common probe to the target sequence (16s rRNA gene), ligation occurs only if there is perfect complementarity at the junction between the two probes. The reaction is thermally cycled.
Panel B: The LDR product is hybridized to an addressable Universal Microarray, where unique Zip code sequences have been spotted.

25

Figure 27 A. Deposition scheme obtained using a contact dispensing system. Each Zip code was spotted four times, except universal Zip code (twelve times) and the Zip code corresponding to hybridization control (eight times).The deposition quality of the Zip Code oligonucleotides on the slides has been checked by means of hybridisations with Cy3 labelled
30 poly(dT) complementary to the poly(dA)$_{10}$ sequence of each Zip Code.
Figure 27B. Deposition scheme of Universal Array for the detection of toxic and non-toxic cyanobacteria. The Universal Array is made of 8 subarray per slide. Each subarray is made of 208 spots including zipcodes for hybridization control, cyanobacterial universal probes, 16S rRNA gene specific probe, mcyE specific probe and empty spot as a negative control. Each

specific zip code for the recognition of cyanobacteria universal probe, 16S RNA gene probe and mcyE gene probe is spotted in quadruplicate. The LDR positive control (zipcode n°63) is replicated 6 times, while the hybridization positive control (zipcode n°66) is replicated 8 times.

5

Figure 28. Some results obtained using as LDR template PCR amplified 16S rRNA gene coming either from pure strains (both axenic and isolated in this study) or from cloned rDNA sequences.

Panel A: Aphanizomenon sp. 202; Panel B: Calothrix marchica Bai 71-96; Panel C: Leptolyngbya OBB19S12; Panel D: Lyngbya OBB32S04; Panel E: Microcystis 1BB 38S; Panel F: Nodularin sp. PCC73104/1; Panel G: Plankthotrix 1LT27S08; Panel H: Spirulina subsalsa PCC6313; Panel I: Synechococcus Heg 74-30; Panel J: Woronichinia OES46; Panel K: Cylindrospermum stagnale PCC7417; Panel L: Synechocystis PCC 6905; Panel M: Nostoc sp. 152; Panel N: Anabaena; Panel O: Cyanothece PCC 7418.

15

Figure 29. Hybridization results obtained using LDR artificial mixes with unbalanced amounts of PCR products derived from the following cyanobacterial samples: Aphanizomenon sp. 202, Microcystis OBB 34S, Sprirulina subsalsa PCC6313, Calothrix sp. PCC7714, Woronichinia OES46 clone. Different ratios have been used: 100:1, 50:1, 100:5, 50:5, in which Aphanizomenon sp. 202 and Microcystis OBB 34S have been the more concentated samples.

Panel A: Unbalanced 100:1 LDR mix, Panel B: 50:1 LDR mix; Panel C: 100:5 LDR mix; Panel D: 50:5 LDR mix; Panel E: unbalanced LDR mix performed with 500 fmol of the amplicon derived from Microcystis OBB 34S and 5 fmol of the PCR fragment obtained from Woronichinia OES46 clone.

Figure 30A. Comparison of the results obtained using two LDR unbalanced mixes 100:1 (100 fmol of Microcystis OBB 34S and 1 fmol each of Spirulina, Woronichinia and Calothrix).

Panel A: The LDR unbalanced mix was prepared using 4U of Pfu DNA ligase.

Panel B: 8U of the enzyme was added in the same LDR unbalanced mix described above.

Figure 30B. 16S and mcyE detection onto universal Array. Example of quantification.

Figure 31. Linear correlation between signal intensity and template concentration

Figure 32. List of the group-specific 16S rRNA gene probes and their correspondent Complementary zip codes (SEQ ID NOs 111 to 130) (discriminating probes SEQ ID NOs 71 to 90, common probes SEQ ID NOs 91 to 110).

5 Figure 33A, B. Cyanobacterial strains used to validate the LDR probes.

Figure 34. Clones of 16S rRNA gene libraries obtained from environmental samples and used in the LDR reaction.

Figure 35. PCR amplification from genomic DNA using 16S cyano primers and mcyE
10 primers; primer F = mcyE-F2 and primer R = mcyE-R4; amplification protocol: 1x(3', 95°C), 30x(30'', 94°C; 30'', 56°C; 1', 72°C), 1x(10', 72°C).

Figure 36. Ligation Detection Reaction for toxic and non-toxic cyanobacteria recognition.

15 Figure 37. Hybridization on DNA chip.

Figure 38 A to F mcyD sequence fragments from different cyanobacteria genera (SEQ ID Nos 131 – 149). In SEQ ID Nos 137, 138 and 139 N is T.

20 Figure 39. List of the group-specific 16S rRNA gene probes (discriminating probes SEQ ID NOs 150 to 156) (common probes SEQ ID NOs 157 to 163) and C-zip Code sequences (SEQ ID Nos 164 to 170).

DETAILED DESCRIPTION OF THE INVENTION

25

Definitions

By "nucleic acid from a biological sample" is in this invention meant any target or sample nucleic acid, which originates from an environmental sample, such as water, soil,
30 cyanobacterial bloom, cyanobacterial culture, mixed population of cyanobacteria and other microbes etc. Nucleic acid is usually DNA, but in can be also RNA. The nucleic acid is usually extracted from the sample by conventional means known for the skilled artisan, but may also be liberated by repeated freeze-thawing to disrupt cellular integrity, or cells are used directly from the sample.

These techniques may also comprise the step of amplifying the nucleic acid before analysis. Amplification techniques are known to those of skill in the art and include, but are not limited to cloning, polymerase chain reaction (PCR), ligase chain reaction (LCR), nested polymerase chain reaction, self sustained sequence replication (Guatelli, J. C. et al., 1990, Proc. Natl. Acad. Sci. USA 87:1874-1878), transcriptional amplification system (Kwoh, D. Y. et al., 1989, Proc. Natl. Acad. Sci. USA 86:1173-1177), and Q-Beta Replicase (Lizardi, P. M. et al., 1988, Bio/Technology 6:1197).

The oligonucleotides of this invention are brought into contact with the target or sample nucleic acid under suitable conditions, which depend on the chosen molecular biology method, such as hybridization, PCR, LDR etc.

By "an oligonucleotide designed to be specific for the *mcyE* gene" it is meant that by using nucleic acid sequence data from several cyanobacterial genera and from several species of the genera, an oligonucleotide is designed to be specific for the *mcyE* gene of the microcystin synthetase operon. The length of an oligonucleotide may be 10 to 150 nucleotides depending on the detection method used. An oligonucleotide for hybridization is at least 20 bp, for PCR at least 10 bp and for LDR at least 15 bp.

Any probe or primer can be prepared according to methods well known in the art and described, e.g., in Sambrook, J. Fritsch, E. F., and Maniatis, T. (1989(Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. For example, discrete fragments of the DNA can be prepared and cloned using restriction enzymes. Alternatively, probes and primers can be prepared using the Polymerase Chain Reaction (PCR) using primers having an appropriate sequence.

Primers and probes (RNA, DNA) described herein may be labeled with any detectable reporter or signal moiety including, but not limited to radioisotopes, enzymes, antigens, antibodies, spectrophotometric reagents, chemiluminescent reagents, fluorescent and any other light producing chemicals. Additionally, these probes may be modified without changing the substance of their purpose by terminal addition of nucleotides designed to incorporate restriction sites or other useful sequences.

These probes may also be modified by the addition of a capture moiety (including, but not limited to para-magnetic particles, biotin, fluorescein, dioxigenin, antigens, antibodies) or attached to the walls of microtiter trays to assist in the solid phase capture and purification of these probes and any DNA or RNA hybridized to these probes. Fluorescein may be used as a signal moiety as well as a capture moiety, the latter by interacting with an anti-fluorescein antibody.

By "a fragment of the *mcyE* gene" is meant principally any fragment of the *mcyE* gene which makes it possible to prepare oligonucleotides capable of identifying the *mcyE* gene from all of the microcystin producing genera and on the other hand is capable of discriminating different cyanobacterial genera from each other. The fragment is preferably related to the region of *mcyE* gene responsible for adding Adda and D-glutamate to the immature synthesis product. In particular, the fragment is related to the region catalyzing a peptide synthesis between Adda-D-glutamate and dehydroalanine and to the adenylating region. More specifically, the fragment is related to the region encoding the end part of the adenylation domain, the phospho-pantetheine binding site and the beginning of the domain which catalyses a peptide bond between D-glutamate and dehydroalanine. The length of the fragment may be between about 500 to 1000 nucleotides, which makes the alignment of nucleic acid sequence data from several cyanobacterial genera and species moderate to handle.

Examples of suitable fragments are the sequences of SEQ ID NO. 1 to SEQ ID NO: 34 as shown in Figure 19 A to H or the consensus sequences SEQ ID NO: 35 to SEQ ID NO: 39 as shown in Figure 15 A to C.

By "a fragment of the *mcyD* gene" is meant principally any fragment of the *mcyD* gene which makes it possible to prepare oligonucleotides capable of identifying the *mcyD* gene from all of the microcystin producing genera and on the other hand is capable of discriminating different cyanobacterial genera from each other.

Examples of suitable mcyD fragments are the sequences of SEQ ID NO. 131 to SEQ ID NO: 149 as shown in Figure 38 A to F.

By "a suitable molecular biology method" is meant the chosen molecular biology method suitable for the purposes of detecting toxic cyanobacteria. The method may be selected from the group comprising hybridization, PCR, QRT-PCR, LCR, LDR and minisequencing.

5 PCR refers to the method for increasing the concentration of a segment of a target sequence in a mixture of genomic DNA without cloning or purification. This process for amplifying the target sequence consists of introducing a large excess of two oligonucleotide primers to the DNA mixture containing the desired target sequence, followed by a precise sequence of thermal cycling in the presence of a DNA polymerase. The two primers are complementary to
10 their respective strands of the double stranded target sequence. To effect amplification, the mixture is denatured and the primers then annealed to their complementary sequences within the target molecule. Following annealing, the primers are extended with a polymerase so as to form a new pair of complementary strands. The steps of denaturation, primer annealing, and polymerase extension can be repeated many times (i.e., denaturation, annealing and extension
15 constitute one "cycle"; there can be numerous "cycles") to obtain a high concentration of an amplified segment of the desired target sequence. The length of the amplified segment of the desired target sequence is determined by the relative positions of the primers with respect to each other, and therefore, this length is a controllable parameter. By virtue of the repeating aspect of the process, the method is referred to as the "polymerase chain reaction" (hereinafter
20 "PCR"). Because the desired amplified segments of the target sequence become the predominant sequences (in terms of concentration) in the mixture, they are said to be "PCR amplified." In addition to genomic DNA, any oligonucleotide or polynucleotide sequence can be amplified with the appropriate set of primer molecules. In particular, the amplified segments created by the PCR process itself are, themselves, efficient templates for subsequent
25 PCR amplifications. With PCR, it is possible to amplify a single copy of a specific target sequence in genomic DNA to a level detectable by the device and systems of the present invention.

PCR oligonucleotide primers or probes may be derived from either strand of the duplex DNA.
30 The primers or probes may consist of the bases A, G, C, or T or analogs and they may be degenerated at one or more chosen nucleotide position(s). The primers or probes may be of any suitable length and may be selected anywhere within the DNA sequences from selected sequences which are suitable. In order to produce primers to a mcyE PCR, the mcyE gene(s) is typically examined using a computer algorithm, which starts at the 5' or at the 3' end of the

nucleotide sequence. Typical algorithms will then identify oligomers in pairs of defined length that are unique to the gene, have a GC content within a range suitable for hybridization, and lack predicted secondary structure that may interfere with hybridization. The number of oligonucleotide pairs may range from two to one million.

5 Minisequencing reaction refers to a type of single base extension sequencing reaction using sequence terminators. In certain embodiments, minisequencing reactions are performed in the substantial absence of free single nucleotides, to minimize or prevent polymerization of nucleic acid beyond the single nucleotide sequenced by the sequence terminator. In certain embodiments, sequence terminators are labeled with fluorescent dyes, so that each nucleotide
10 (A, G, T, or C) is identifiable by the color of the fluorescent label.

QRT-PCR or quantitative real-time PCR method involve measuring the amount of amplification product formed during an amplification process. Fluorogenic nuclease assays are one specific example of a real time quantitation method that can be used to detect and
15 quantitate transcripts of present invention. In general such assays continuously measure PCR product accumulation using a dual-labeled fluorogenic oligonucleotide probe, an approach frequently referred to in the literature simply as the "TaqMan"method. The probe used in such assays is typically a short (ca. 20-25 bases) polynucleotide that is labeled with two different fluorescent dyes. The 5" terminus of the probe is typically attached to a reporter dye and the
20 3" terminus is attached to a quenching dye, although the dyes can be attached at other locations on the probe as well. For measuring a mcyE transcript, the probe is designed to have at least substantial sequence complementarity with a probe binding site on a mcyE transcript. Upstream and downstream PCR primers that bind to regions that flank mcyE are also added to the reaction mixture for use in amplifying the mcyE polynucleotide. When the probe is
25 intact, energy transfer between the two fluorophors occurs and the quencher quenches emission from the reporter. During the extension phase of PCR, the probe is cleaved by the 5" nuclease activity of a nucleic acid polymerase such as Taq polymerase, thereby releasing the reporter dye from the polynucleotide-quencher complex and resulting in an increase of reporter emission intensity that can be measured by an appropriate detection system.

30

Hybridization is used in reference to the pairing of complementary nucleic acids. Hybridization and the strength of hybridization (i.e., the strength of the association between the nucleic acids) is impacted by such factors as the degree of complementary between the

nucleic acids, stringency of the conditions involved, the Tm of the formed hybrid, and the G:C ratio within the nucleic acids. For example stringent hybridization conditions are defined in Sambrook et al. 1989.

5 Ligation Detection Reaction LDR is based on the discriminative properties of the DNA ligation reaction. It requires the design of two probes specific for each target sequence, as described by Barany and co-workers (1999). One oligonucleotide brings a fluorescent label or other detection label and the other a unique sequence named complementary Zip Code (cZip Code). Ligated fragments, obtained in the presence of a proper template by the action of a 10 DNA ligase, are addressed to the location on the microarray where the Zip Code sequence has been spotted. Such an array is therefore "Universal" being unrelated to a specific molecular analysis.

When two complementary pairs of probe elements are utilized, the process is referred to as 15 the ligase chain reaction which achieves exponential amplification of target sequences (F. Barany, "The Ligase Chain Reaction (LCR) in a PCR World," PCR Methods and Applications, 1:5-16 (1991)).

As used herein "Arrays" or "Microarrays" refers to an array of distinct polynucleotides or oligonucleotides synthesized on a substrate, such as paper, nylon or other type of membrane, 20 filter, chip, glass slide, or any other suitable solid support. The microarray can be prepared and used according to the methods described, for example in Lockhart, D. J. et al. (1996; Nat. Biotech. 14: 1675-1680) and Schena, M. et al. (1996; Proc. Natl. Acad. Sci. 93: 10614-10619).

The microarray or detection kit is preferably composed of a large number of unique, single-25 stranded nucleic acid sequences, usually either synthetic antisense oligonucleotides or fragments of cDNAs, fixed to a solid support. The oligonucleotides are preferably about 6-60 nucleotides in length, more preferably 15-30 nucleotides in length, and most preferably about 20-25 nucleotides in length. For a certain type of microarray or detection kit, it may be preferable to use oligonucleotides that are only 7-20 nucleotides in length. The microarray or 30 detection kit may contain oligonucleotides that cover the known 5', or 3', sequence, sequential oligonucleotides which cover the full length sequence; or unique oligonucleotides selected from particular areas along the length of the sequence. Polynucleotides used in the microarray or detection kit may be oligonucleotides that are specific to a mcyE gene or genes of interest.

The nucleotide sequence data can be aligned and clustered according to their phylogenetic lineages so that "group-specific" consensus sequences are yielded: *Anabaena, Microcystis, Nodularia, Nostoc, Oscillatoria/Planktothrix*. Then, "group-specific" probes can be designed using a suitable database, such as ARB database named "Probe design". Among the set of probes, discriminating probes with 3' position unique to each group in order to obtain ligase discrimination can be selected. After hybridization of a discriminating probe and a common probe to the target sequence, ligation occurs only if there is perfect complementarity at the junction between the two oligos. Common probes are designed immediately 3' to the discriminating oligo from the group-specific consensus and the detection is made by microarray method.

Zip code sequences can be selected randomly from those described by Chen and co-workers, 2000. Each Zip code is randomly assigned to a single cyanobacterial group. Each common probe is synthesized to have the complementary Zip code (cZip code) affixed to its 3' end.

Examples of discriminating probes are SEQ ID NO: 40 to SEQ ID NO: 45 and of common probes SEQ ID NO: 46 to SEQ ID NO: 51 designed to be specific for mcyE gene.

Examples of LDR zip codes are zip codes SEQ ID NO: 52 to SEQ ID NO:57.

Furthermore, examples of discriminating probes are SEQ ID NO: 71 to SEQ ID NO: 90 and of common probes SEQ ID NO: 91 to SEQ ID NO: 110 designed to be specific for 16S rRNA gene.

The method of the present invention can be used to detect toxic cyanobacteria at least from the genera *Anabaena*, *Microcystis*, *Planktothrix*, *Nostoc* and *Nodularia*.

The method can be combined if desired with a detection method using oligonucleotides designed specific for any other *mcy* genes or for 16S rRNA gene. A method based on 16S rRNA gene detection is in particular useful, if non-toxic cyanobacteria should be identified in addition to toxic cyanobacteria, when for example the condition of environment is monitored.

The method of this invention can be combined also with methods determining microcystin concentration, cell density, cell number, biomass, biovolume, chlorophyll-a, total RNA/DNA concentrations etc.

5 A kit for the detection of toxic cyanobacteria by microarray method preferably comprises
- discriminating and common probes designed to be specific for the *mcyE* gene;
- DNA or RNA zip and complementary zip codes assigned to be specific for certain cyanobacteria genera.

10 A kit for the detection of toxic cyanobacteria by hybridization preferably comprises
- primers designed to be specific for the *mcyE* gene;
- probes designed to be specific for certain cyanobacteria genera.

In the kit can be in addition to primers or probes designed to be specific for the *mcyE* and/ or
15 *mcyD* gene also primers or probes designed to be specific for 16 S rDNA.

In this invention we have identified and characterized the genes for the biosynthesis of hepatotoxins, microcystins from the filamentous, nitrogen fixing cyanobacterium *Anabaena* strain 90. Microcystin synthetase genes are now known from three different cyanobacterial
20 genera, *Anabaena*, *Microcystis* and *Planktothrix*, which are the main producers of the microcystins. The arrangement of the genes is different between these species. The order of the domains, which are coded by two sets of the genes, is co-linear with the hypothetical sequence of the enzymatic reactions for microcystin biosynthesis only in *Anabaena* 90.

25 These genes provide extensive sequence information for the design of primers to be used in PCR-based methods for the sensitive detection, identification and quantification of producers of hepatotoxic microcystins and nodularins.

Identifying the most potent microcystin producer in a lake could be valuable knowledge e.g.
30 in designing lake restoration strategies. In connection of this invention we identified the microcystin producing genera and quantified the microcystin synthetase gene E (*mcy*E) copy numbers in two lakes (Lake Tuusulanjärvi and Lake Hiidenvesi) by quantitative real-time PCR. Microcystin concentrations and cyanobacterial cell densities of these lakes were also determined. The main microcystin producer in Lake Tuusulanjärvi was *Microcystis* sp., since

average *Microcystis mcy*E copy numbers were over 30 times more abundant than those of *Anabaena*. Lake Hiidenvesi seemed to contain both nontoxic and toxic *Anabaena* as well as toxic *Microcystis* strains. Microcystin concentrations of Lake Tuusulanjärvi and Lake Hiidenvesi correlated positively with *Microcystis mcy*E copy numbers.

McyE seqences from *Anabaena, Microcystis, Nodularia, Nostoc* and *Oscillatoria/Planktothrix* were used for detecting polymorphic positions useful for detecting cyanobacterial strains using several different biomolecular techniques. These unique features were used for designing probes for cyanobacterial detection and identification by LDR in combination with a microarray.

The molecular classification of cyanobacteria is based on 16S rRNA gene sequences obtained from pure cultures (Wilmotte & Herdmann, 2001). Using this molecular information, several techniques can be used to determine the cyanobacterial composition of an environmental sample. The most widely used method is the 16S rRNA gene amplification with cyanobacterial specific PCR primers, cloning, sequencing and phylogenetic reconstruction (Giovannoni et al., 1988). This strategy is very time consuming and therefore is not suited to large scale screenings. Recently, DGGE and TGGE have been widely applied to molecular ecological research (Muyzer, 1999). However, the excision of bands, reamplification and sequencing are necessary to obtain a precise diversity analysis.

Oligonucleotide microarrays (microchips) have a major role in genomics and have gained wide attention in molecular diagnostics. Microarray technology has a great potential in environmental diagnostics. In fact, the DNA microarray technology has already been applied for microbial diversity detection. Microarrays have been used for quantitation of target microbial populations for environmental analysis (Guschin et al., 1997).

Rudi and coworkers (2000) designed a small cyanobacterial specific microarray for *Microcystis, Planktothrix, Anabaena, Aphanizomenon, Nostoc* and *Phormidium.*

DNA microarray and the magnetic-capture hybridization technique have been combined to form a new technology named MAG-microarray. Bacterial magnetic particles (BMPs) on a MAG-microarray have been used for the identification of cyanobacterial DNA (Matsunaga et al., 2001). Genus-specific oligonucleotides probes for the detection of *Anabaena* spp.,

Microcystis spp., *Nostoc* spp., *Oscillatoria* spp. and *Synechococcus* spp. have been designed from the variable region of the cyanobacterial 16S rRNA gene of 148 strains. These probes have been immobilized on BMPs via streptavidin-biotin conjugation and employed for magnetic-capture hybridization against digoxigenin-labeled cyanobacterial 16SrRNA gene. 5 Bacterial magnetic particles have been magnetically concentrated, spotted in a microwell on MAG-microarray and detected. The entire process of hybridization and detection has been automatically performed and all the five cyanobacterial genera have been successfully discriminated.

10 Recently, we have presented a Universal DNA Array approach to discriminate some groups of bacteria (Busti et al., 2002). This procedure, based on the discriminative properties of the DNA ligation reaction, requires the design of two probes specific for each target sequence, as described by Gerry and co-workers (1999). One oligonucleotide brings a fluorescent label and the other a unique sequence named complementary Zip Code (cZip Code). Ligated fragments, 15 obtained in presence of a proper template by the action of a DNA ligase, are addressed to the location on the microarray where the Zip Code sequence has been spotted. Such an array is therefore "Universal" being unrelated to a specific molecular analysis.

Here we present the Universal DNA Array approach applied to the detection of cyanobacterial 20 diversity. We designed probes specific for 19 different cyanobacterial groups (phylogenetic lineages including *Anabaena/Aphanizomenon, Calothrix, Cylindrospermopsis, Cylindrospermum, Gloeothece, Halotolerants, Leptolyngbya, Lyngbya, Microcystis, Nodularia, Nostoc, Oscillatoria/Planktothrix, Phormidium, Prochlorococcus, Spirulina, Synechococcus, Synechocystis, Trichodesmium, Woronichinia*) identified from the 25 phylogenetic tree obtained from the ARB database constructed in this study.

13 axenic strains from culture collection, 38 isolated culture strains and 44 clonal fragments recovered from environmental samples were used for validation purposes with excellent results demonstrating a high discriminative power. The proposed approach is extremely 30 sensitive (down to 1 fmol of PCR amplified 16S gene region are detectable) allowing for the analysis of unbalanced environmental samples. LDR coupled to Universal Microarray performed on PCR samples containing 100:1 ratios of different amplicons yielded the correct identification of the starting strains. This approach is therefore amenable to the analysis of complex environmental samples.

The Universal array was used for the detection of toxic and non-toxic cyanobacteria by using probes designed to detect both the 16 rRNA and mcyE genes. In the presence of the proper DNA template of both 16S rRNA and mcyE genes, the Universal Array functioned very well: only group specific spots, universal spots and the spots corresponding to the hybridization control showed positive.

Genes coding for the synthesis of microcystins in Anabaena

The order of the genes in the microcystin synthetase gene cluster is different in the cyanobacterial species

The arrangement of the genes is different in the gene clusters of microcystin biosynthesis from the strains of three species. In *Anabaena* strain 90, *Microcystis aeruginosa* (Tillett et al., 2000; Nishizawa et al., 2000) and in *Planktothrix agardhii* CYA126 (Christiansen et al., 2003) the NRPS genes, *mcyA*, *mcyB* and *mcyC* have the same order, but the organization of the other genes is different. In *Anabaena* strain 90 and in *M. aeruginosa* the *mcy*-genes are in two clusters, which are transcribed in opposite directions, whereas in *P. agardhii* they are in one cluster transcribed in the same direction (except *mcyT*, which was not found in *Anabaena* and *Microcystis*). The arrangement of the genes from *mcyD* to *mcyH* in *Microcystis* is almost identical in *Planktothrix* (*mcyF* is missing in *Planktothrix*), but it differs from the order in *Anabaena*. In *Planktothrix*, compared to *Microcystis*, the part containing *mcyD*, *mcyE*, *mcyF*, *mcyG*, *mcyH*, *mcyI* and *mcyJ* is reversed. In this rearrangement, *mcyF* and *mcyI* were lost from the cluster and *mcyJ* was relocated after *mcyC*.

The biosynthesis of microcystins

In *Anabaena,* the order of the domains coded by the genes in the two sets is co-linear with the hypothetical sequence of the enzymatic reactions for microcystin biosynthesis (Fig. 1). The progression of the biosynthetic reactions follows the order of the functions coded first by *mcyG* and continuing with the activities coded by *mcyD*, *mcyJ*, *mcyE*, *mcyF*, *mcyI*, *mcyA*, *mcyB* and *mcyC*.

Phenyl acetate is the assumed starting unit in the biosynthesis of Adda (Moore et al., 1991). It is activated by the adenylating domain identified in the N-terminus of McyG, and transferred onto the subsequent thiolation (phosphopantetheine binding) site. Polyketide synthesis reactions are followed (Fig. 1). All four extension units are malonyl-CoA molecules
5 according to the substrate specificity of the AT domains (Fig. 4). In McyG there is a KS domain to catalyse the first condensation reaction between phenylacetate and malonyl-CoA.

The reductive reactions needed to fashion the polyketide chain are putatively catalysed by KR and DH domains of McyD and McyE. The KR domain of McyG is in the right position to
10 reduce the carbonyl group of the putative starter molecule. The methyltransferase domains of McyG, McyD and McyE are the obvious candidates to introduce three methyl groups into the carbon frame of Adda. It was recently verified with a knockout mutant (Christiansen et al., 2003) that the incorporation of the fourth methyl, which is seen in the methoxy group of Adda, is catalysed by McyJ. The amino transferase domain of McyE most likely adds the
15 amino group, which participates in the peptide bond with the glutamate residue.

There are two condensation domains of peptide synthetases in McyE. The first one logically catalyses the peptide bond between Adda and glutamate, which is activated by the adenylation domain of McyE. The signature sequence, which was also determined as
20 DPRHSGVVG for McyE of both *M. aeruginosa* and *P. agardhii*, has no precedents in the databases (Table 2). The synthetases of other peptides, which contain glutamyl residues are known for bacitracin, fengycin and surfactin (accession numbers: AF007865, AF023464, AF087452 and D13262). In these compounds the standard α-carboxyl of glutamate is part of the peptide bond, while in microcystins it is the γ-carboxyl. This is analogous to the activation
25 of aspartate/methylaspartate by the second adenylation domain of McyB. The β-carboxyl of aspartate/methylaspartate instead of the α-carboxyl is engaged in the peptide bond formation. This must have impact on the compositions of the glutamate and aspartate/methylaspartate binding pockets in the adenylation domains.

30 McyA has two adenylation domains for the activation of serine and alanine, respectively. The signature sequences of these domains have models and are almost identical in *Anabaena* 90, *M. aeruginosa* and *P. agardhii* (Table 2). The dehydration of serine supposedly takes place after the activation by adenylation and is catalysed by McyI, which is similar to phosphoglycerate dehydrogenases.

There is only one, internal, condensation domain in McyA, which most likely links dehydroserine and D-alanine. The bond between glutamate and dehydroserine is putatively catalysed by the C-terminal condensation domain of McyE. There is a methyltransferase domain in the first module of McyA for N-methylation of dehydroserine. The epimerase domain at the C-terminus of McyA converts L-alanine to the D-form.

Two modules of McyB and one module of McyC logically activate, and add three residues to the nascent peptide chain: L-leucine or L-arginine, methylaspartate or aspartate and L-arginine, respectively (Fig 1). The amino acids activated by the adenylation domains of McyC and by the first module of McyB (McyB-1) vary most frequently in microcystins. *M. aeruginosa* PCC7806 and *M. aeruginosa* K-139 produce mainly Mcyst-LR, and the substrate specificity conferring sequences in McyB-1 of these strains are identical with the signature sequence for leucine (Table 2). *M. aeruginosa* UV027 and *P. agardhii* CYA126 produce mostly Mcyst-RR, which is also produced by *Anabaena* 90 together with Mcyst-LR. Their signature sequences in McyB-1 are different and have no precedents in the databases (Table 2). In *M. aeruginosa* UV027 the specificity codes of McyB-1 and McyC are almost identical (DVWTIGAVE / DWTIGAVD) and match with the codes of McyC from *M. aeruginosa* K-139 and *M. aeruginosa* PCC7806, respectively (Table 2). Accordingly McyB-1 of *M. aeruginosa* UV027 and McyC activate arginine.

There is no epimerase domain in McyB of *Anabaena* 90 or in the other sequenced versions of McyB, though in microcystins, the aspartyl or methylaspartyl moiety is in the D-form. The epimerization in this position and in the glutamyl residue is putatively catalysed by McyF, which in a BLAST search was similar to aspartate racemases, and was shown by Nishizawa et al., (2001) to complement a D-glutamate deficient mutant of *Escherichia coli*. The C-terminal thiosterase domain of McyC, as generally in nonribosomal peptide synthesis, (Kohli et al., 2001) catalyzes the final step in microcystin biosynthesis, the cyclization of the linear peptide (Fig. 1).

McyH is probably not needed for the synthesis of microcystins but it may participate in the transport of microcystins.

In connection of this invention we obtained DNA sequences of three microcystin synthetase

genes: *mcy*A, *mcy*E and *mcy*D. The *mcy*A gene fragment encodes part of the condensation domain, which catalyses a condensation reaction to form a peptide bond between the growing peptide and D-alanine. The fragment of the *mcy*E gene codes for a partial adenylation domain and a phospho-pantetheine-binding site, the region, which activates glutamic acid.
5 The region of the *mcy*D gene encodes parts of both the β-ketoacyl synthase and the acyltransferase domains. We sampled representative producers of microcystins and nodularins (Table 1) in the genera *Anabaena, Microcystis, Planktothrix, Nostoc*, and *Nodularia*. Individual topologies generated from *mcy*A, *mcy*E and *mcy*D were rooted with homologues identified in BLAST searches. These topologies were congruent with
10 one another (data not shown) and thus the data from all three genes were concatenated in order to increase the amount of information available in phylogenetic analyses.

Phylogenetic evidence for the early evolution of microcystin synthesis

15 In order to investigate the role of horizontal gene transfer in the distribution of microcystin synthetase genes amongst cyanobacteria we assembled a data set comprised of 16S rRNA and *rpo*C1 sequences from the same set of taxa. These genes are conserved and widely used as tools for phylogenetic classification. No incongruence between the 16S rRNA and *rpo*C1 topologies could be found and the sequence data of these two genes was concatenated. We
20 analysed these two data sets separately with maximum parsimony and maximum likelihood optimisation criteria. Bootstrap analyses were conducted to measure the stability of the observed phylogenetic patterns and revealed two well-supported topologies (Fig. 7). The two maximum-likelihood topologies were perfectly congruent (Fig. 7). The bootstrap support for the monophyly of the genera *Anabaena*, *Nodularia* and *Nostoc* was lower in the microcystin
25 synthetase gene data set than in the 16S rRNA and *rpo*C1 data set (Fig. 7). Likewise the bootstrap support for the monophyly of the genera *Planktothrix* and *Microcystis* was lower in the 16S rRNA and *rpo*C1 data set than in the microcystin gene data set (Fig. 7). However, no conflicting nodes received bootstrap support above 45% in any analysis. Individual trees generated from *mcy*A (26 taxa), *mcy*E (30 taxa) and *mcy*D (19 taxa) all consistently supported
30 the reciprocal monophyly of each genus (data not shown). In no instance was support for a lateral transfer recovered. The high degree of congruence between the microcystin synthetase gene data set and 16S rRNA and *rpo*C1 data set is consistent with an ancient origin of microcystins (Fig. 7). This indicates that the phylogenetic marker genes and the microcystin synthetase genes have co-evolved for the entire length of the evolutionary history of this

toxin. The sporadic distribution of microcystin synthetase genes in modern cyanobacteria suggests that the ability to produce the toxin has been lost repeatedly in the more derived lineages of cyanobacteria. Microcystins are one of the few known natural examples of combined polyketide synthase and peptide synthetase systems. Little is known about the evolution of these mixed polyketide and peptide synthetases and it is unclear whether the combination of these two systems is of recent origin. Congruence between the polyketide and peptide portions of the gene cluster as well as the 16S rRNA and *rpo*C1 data set demonstrates that the combination of these two systems is an ancient collaboration in the production of this toxin. Our results do not rule out the possibility that parts of the sequences of the microcystin synthetase gene cluster are of more recent origin. Indeed, the existence of many microcystin variants implies a fast evolution of certain gene domains.

Similarities in the chemical structures and biological action of microcystins and nodularins indicate that these compounds are closely related (Sivonen and Jones,1999). However, the exact relationship between nodularins and microcystins remains ambiguous. Recent studies have suggested that the genes encoding microcystin synthetase have evolved from the genes encoding nodularin synthetase (Christiansen, 2003). Our data rejects the idea that nodularin synthesis predates microcystin synthesis(Christiansen, 2003 or that nodularin synthetase genes are a sister group to microcystin synthetases genes (Moffitt et al. 2001). Instead, our results suggest that nodularin synthetase genes are derived from microcystin synthetase genes and that nodularins should now be regarded as structural variants of microcystins. It is anticipated here that nodularin synthetase genes were formed from the ancestral microcystin synthetase gene set through a relatively recent deletion of the last *mcy*A module and the first *mcy*B module and by mutation changing the substrate specificity coded by the first module of *mcy*A. This finding is consistent with the production of nodularins by a single cyanobacterial genus and the limited structural variation of nodularins in comparison to microcystins Sivonen and Jones,1999). Microcystins are commonly believed to have evolved in response to grazing pressure by zooplankton (DeMott et. al. 1991). Fossils of filamentous akinete-forming cyanobacteria are dated to 2000 million years ago (Amard et al., 1997).

This means that the *Anabaena*, *Nostoc*, and *Nodularia* genera and thus, the common ancestor of microcystin producing cyanobacteria are at least this old. Molecular clocks set a divergence time of 1576 million years ago for the crown eukaryotic lineages (Heckman, D.S. *et al.*, 2001). Metazoans such as copepods and cladocerans are often envisaged as target organisms of

microcystins (DeMott and Moxter,1991). However, microcystin production predates all metazoans. If microcystins evolved as a chemical defense against zooplankton then the targets of the toxin must have been the early branching eukaryotes (Moon-van der Staay, S-Y. et al., 2001 and Brocks et al., 1999).

Protozoans are an underappreciated component of the zooplankton and may have been overlooked as the likely targets for the evolution of chemical defense in this case. It is not clear that microcystins evolved as a chemical defense and other proposed functions for microcystins include siderophobic scavenging of trace metals such as iron (Utkilen and Gjolme, 1995) and a role in signalling and gene regulation (Dittmann *et al,* 2001).

Microcystins and nodularins are highly toxic to eukaryotic cells and pose a serious health risk to water users. Also the genera *Arthrospira* and *Aphanizomenon* are commonly used in health food supplements (Gilroy et al., 2000). Our study demonstrates that the ability to make microcystins has been lost repeatedly throughout the diversification of cyanobacteria. This means that toxin-producing strains may be found unexpectedly.

Quantification of microcystin synthetase E copy numbers of *Microcystis* and *Anabaena* in lakes by quantitative real-time -PCR

In this invention a novel method to indicate the main putative microcystin producer of a lake is provided. The dominant putative microcystin producer was *Microcystis* in Lake Tuusulanjärvi and in the Basin of Kiihkelyksenselkä of Lake Hiidenvesi based on *mcy*E copy number quantification. This method enables to study *in situ* the responses of environmental factors on the growth of microcystin producing genera and could be used to observe the possible changes in cyanobacterial assemblages prior, during, and after lake restoration in order to find out, if the genus targeted lake restoration succeeded.

The main microcystin producers

In Lake Tuusulanjärvi *Microcystis* spp. was the main putative microcystin producer, since average *Microcystis mcy*E copy numbers were clearly higher than those of *Anabaena* and thus, this result was in agreement with the higher cell numbers of *Microcystis* observed

compared to those of *Anabaena*. Microcystin concentrations or hepatotoxicities have also presiously correlated positively with *Microcystis* spp. biomass in Lake Tuusulanjärvi (Ekman-Ekebom et al. 1992, Lahti et al. 1997). *Microcystis* spp. were also the main putative microcystin producers in the Basin of Kiihkelyksenselkä of Lake Hiidenvesi, although *Anabaena* cell numbers were higher than those of *Microcystis*. This indicates that majority of the *Anabaena* cells were nontoxic and *Microcystis* cells toxic in this basin. In the Basins of Mustionselkä, Nummelanselkä and Kirkkojärvi of Lake Hiidenvesi the main microcystin producer could not be assessed, since in the Basins of Mustionselkä and Nummelanselkä, the *Anabaena* and *Microcystis mcy*E copy numbers were quite similar and in the Basin of Kirkkojärvi the *Anabaena* and *Microcystis mcy*E copy numbers were below the detection limit. The low *mcy*E copy numbers detected in Kirkkojärvi were in agreement with the low microcystin concentrations measured from this basin. Microcystin concentration correlated positively with *Microcystis mcy*E copy numbers with all studied samples whereas no significant correlation was found between microcystin concentrations and *Microcystis* and *Anabaena* cell numbers with all studied samples. Therefore, with microscope analysis it is not possible to determine reliably the most potent microcystin producer of a lake. Gene *mcy*E copy numbers, microcystin concentrations, and cyanobacterial cell densities were lower in Lake Hiidenvesi than in Lake Tuusulanjärvi. In Lake Tuusulanjärvi and in surface water of the Basins Nummelanselkä and Kiihkelyksenselkä of Lake Hiidenvesi WHO microcystin concentration guideline value for drinking water quality, 1 $\mu g\ l^{-1}$, (Falconer et al., 1999.) was exceeded.

Microcystis and *Anabaena mcy*E copy numbers were one to over 200 times higher than the cell numbers observed with microscopy in Lake Tuusulanjärvi and Lake Hiidenvesi. In Lake Tuusulanjärvi *Microcystis mcy*E copy numbers increased after August in contrast to the cell density, which decreased. The explanation could be that after August cells had more genome copies or that the DNA of the lysed cells was present in the lake water and followed through the cell concentration and DNA extraction processes to the final DNA sample. Additional explanations for the high *mcy*E copy number and cell density ratio might be that the cell numbers detected with microscope were too low or the genome sizes of the external standard strains were underestimated. Even with the knowledge that cyanobacteria may have several genome copies in a cell (Becker, et al. 2002, Herdman et al., 1979, Labarre et al., 1989), it seems that the obtained *mcy*E copy numbers were too high. The genome sizes estimated for the *Anabaena* standard strains were 5.15 Mb according to the published data of *Anabaena*

PCC 6309 and PCC 7122 (Castenholz, 2001.). These *Anabaena* strains are nontoxic (Lyra, et al. 2001) and lack the microcystin synthetase genes, the sizes of which are not more than 53 or 55 kb (Christiansen et al., 2003, Nishizawa et al., 2000 and Nishizawa et al. 1999 and Tillett et al. 2000 and Example 1). For *Microcystis* standard strains the genome size of 4.70 Mb was used according to the genome size of one of the external standard strains, *Microcystis* PCC 7941 (Castenholz, 2001).

In general, nontoxic strains do not contain *mcy* genes (Neilan et al., 1999 and Tillett et al. 2001). However, some strains may have fragments of microcystin synthetase genes or mutations within these genes (Kaebernick et al. 2001, Neilan et al. 1999 and Tillett et al. 2001). These strains can be amplified with *mcy* primers, although they are not able to produce toxins. However, the significant positive correlation between *Microcystis mcy*E copy numbers and microcystin concentration indicated that such nontoxic strains were probably not present in Lake Tuusulanjärvi and in Lake Hiidenvesi.

Amplification efficiency. *Microcystis mcy*E QRT-PCR amplification efficiencies with Lake Tuusulanjärvi water samples (0.78 – 0.99) were similar to those of *Microcystis* standards (0.86 – 0.94) and those of *Anabaena* standards (0.96 – 0.99), which is a prerequisite for correct *mcy*E copy number quantification of the lake water samples. These similar QRT-PCR amplification efficiencies also ensured that no PCR-inhibiting contaminants were present in the Lake Tuusulanjärvi DNA samples. However, *Anabaena mcy*E QRT-PCR amplification efficiencies with Lake Tuusulanjärvi water samples were higher than one. This result can be explained by competition for primer annealing sites between primers and homologous sequences (Becker et al. 2000, Suzuki et al. 1996, Wawrik et al. 2002) and this competition may lead to suppression of the target DNA (Suzuki et al. 1996). This phenomenon has been shown to occur not only in conventional PCR (Suzuki et al. 1996) but also in QRT-PCR (Becker et al. 2000, Wawrik et al. 2002), although quantification is achieved during the early logarithmic phase of the amplification (Heid et al., 1996). *Anabaena* and *Microcystis mcy*E sequences are homologous (Example 2) . Since in Lake Tuusulanjärvi the concentration of competing *Microcystis mcy*E genes was higher than that of *Anabaena mcy*E genes, it is possible that the *Anabaena mcy*E copy numbers were underestimated. In addition, the *mcy*E-F2 forward primer amplified *Anabaena* as well as *Microcystis* sequences and increased the amount of competing homologous sequences.

Detection range of *mcy*E copy number quantification. The *mcy*E QRT-PCR amplification was log-linear in a range of three to four orders of magnitude. With high DNA template concentration, 6.6 x 10^6 *mcy*E copies in a reaction, amplification was inhibited with the DNAs of *Anabaena* 90, *Anabaena* 202A1, *Microcystis* GL 260735, and *Microcystis* PCC 7941
5 strains, since obtained Ct values were lower than they should have been according to the regression equation or Ct values could not be detected at all. The inhibition was probably caused by contaminants that co-extracted with DNA during the DNA extraction and purification as shown previously (Wintzingerode et al. 1997). The lowest detection limit of *Anabaena* and *Microcystis mcy*E QRT-PCR amplification was 660 *mcy*E copies in a reaction.
10 The error of the Ct values in QRT-PCR has been shown to be higher with low DNA template concentrations than with high template concentrations (Grüntzig et al. 2001). However, in this study the lowest *mcy*E copy number concentrations of the external standards had the same CV% as the other concentrations, 0.1 – 3.6%.

15 **The utilization of the *mcy*E copy number results.** In this study, putative microcystin producing *Anabaena* and *Microcystis* were detected in both studied lakes. In Lake Tuusulanjärvi and in the Basin of Kiihkelyksenselkä of Lake Hiidenvesi the dominant putative microcystin producer was *Microcystis* based on *mcy*E quantification. Reduction of nutrient loading and resuspension (Boers et al. 1991, Chorus and 1999, Reynolds,, 1997)
20 could be successful strategies to decrease the density of *Microcystis*, since these may decrease nitrogen as well as phosphorus concentrations of the water. In addition, lower nutrient concentrations could favor the growth of nontoxic *Microcystis* strains instead of toxic, since the biomass of nontoxic *Microcystis* strains has been demonstrated to be higher than that of toxic strains with low nutrient concentrations at the end of a laboratory experiment (Vézie et
25 al. 2002). Lake Hiidenvesi seemed to have nontoxic and toxic *Anabaena* strains as well as toxic *Microcystis* strains. However, *mcy*E copy numbers should be monitored during the whole growth period in order to have a better understanding of the population dynamics of this lake. A reduction of the external phosphorus loading could affect the mass occurrences of nitrogen-fixing cyanobacteria negatively. It is however not known how the reduction of
30 nitrogen fixing-cyanobacteria would affect the growth of toxic *Microcystis* strains. At least, the presence of toxic *Microcystis* strains should be taken into account in land use management of the catchment area of Lake Hiidenvesi.

Oligonucleotides for detection and identification of toxic cyanobacteria

In this invention was developed the identification on *mcyE* gene region of polymorphisms specific for different toxic cyanobacterial groups identified from the phylogenetic tree
5 obtained from 34 toxic cyanobacterial sequences. The polymorphic positions were used for designing probes for PCR, hybridization, primer extension, ligation and LDR. Probes for ligation have been used in combination with randomly chosen tag sequences appended 5' to the so called common primers in order to be used in the universal array approach. Validation against different samples demonstrate the robustness of the proposed
10 polymorphisn and probes.

Molecular analysis of cyanobacterial diversity by microarrays on "PCR-amplified " 16SrRNA gene

15 We aimed at designing and testing a microarray based system for cyanobacterial diversity identification. We selected a molecular strategy based on the amplification of the 16S rRNA gene region using cyanobacteria specific primers (Edwards et al. 1989, Lepère et al. 2000) followed by group discrimination based on a multiplexed ligation detection reaction performed employing proper probes. Ligated fragments characteristics of each group were
20 demultiplexed on a Universal array. This approach, originally proposed by Gerry et al (1999) has found several application. We used the ARB database including 281 public sequences belonging to the 19 phylogenetic lineages we decided to target (*Anabaena/Aphanizomenon, Calothrix, Cylindrospermopsis, Cylindrospermum, Gloeothece, Halotolerants, Leptolyngbya, Lyngbya, Microcystis, Nodularia, Nostoc, Oscillatoria/Planktothrix, Phormidium,*
25 *Prochlorococcus, Spirulina, Synechococcus, Synechocystis, Trichodesmium, Woronichinia).* Not all of these groups are present in the environmental samples from the lakes involved in the MIDI-CHIP project but all them were included in order to allow for future research studies. Sequences were clustered as shown in figure 25. For each group we calculated a consensus sequence with a cutoff of 75%. The resulting consensuses were aligned and group
30 specific probes were searched along the entire 16S rRNA gene region. Following the LDR approach (figure 26) we identified two unique probes for every group (a common probe and a discriminating probe). Selected probes were tested against the set of sequences of the corresponding group in order to verify the perfect match, in particular around the site of ligation. Then probe sequences were tested against the remaining cyanobacterial sequences in

order to verify their selectivity. Selected probes are spread all over the entire 16S amplicon. Selected common probes were then randomly combined to a set of cZipCode sequences previously proposed for the Universal array approach (Gerry et al 1999, Chen et al 2000). Potential cross hybridization was checked by BLAST analysis of each common and
5 discriminating probe against all others. Probes were then synthesized, HPLC purified and tested by mass spectrometry. This stringent quality assurance procedure is mandatory to achieve expected results in LDR. Ordinary PCR quality probes yielded poor performance due to low phosphorilation or Cy3 labeling and exceedingly high failure sequences. Similar quality controls were performed on the 5' amino-modified ZipCode sequences spotted by
10 contact printing on Codelink Slides. We generated 8 subarrays per slide (96 spots per subarray including zipcodes for a hybridization control (eight spots at corners), cyanobacterial universal probes (12 spots in the middle and at corners) and 19 lineage-specific ZipCodes spotted in quadruplicate. Slides were batch-tested by hybridization using a labeled polyT probe matching the polyA tail appended in 5 ' to every ZipCode probe. In order to validate
15 the designed probes we run a blank (no template) LDR. No signals were detected demonstrating that no false ligation occurred (this problem is often encountered when performing minisequencing (Lindroos, 2002). Then 51 strains of known 16S rRNAsequence belonging to 13 phylogenetic groups (Figure 33) were used to test the proposed system. Figure 28 clearly illustrates LDR specificity when using 100 fmol of each single template
20 independently reacted against the complete set of probes. Six out of 19 groups were not included in the test panel due to their unavailability but their corresponding LDR probes were present in the LDR mix and did not generate any false positive result. It should be noted that, although not identical, the LDR/Universal array efficiency was very similar among all probes. Comparing the intensity between the cyanobacterial universal probe and each lineage specific
25 probe, we found a ratio very close to 1 for most groups. (Here a graph showing this comparison could be more clear that the following description). Probes for *Lyngbya, Nodularia, Anabaena and Cyanothece* (Figure 28 D, F, N, O respectively) consistently yielded higher efficiency. However the similarity of results using very different sequences having very close thermodynamic properties is a distinctive feature of this approach.
30 Hybridization based arrays Loy, 2002; Rudy K. 2000) depend heavily on local sequence characteristics. When hybridization is performed in high salt buffers in a single stringency condition, large variability in signal intensity can be expected (Loy, 2002). On the contrary, using the exquisite sequence specificity of the ligation reaction (Gerry, 1999) and the very high annealing temperatures required during cycling, a very homogeneous behaviour is found.

Very little influence of the sequence context has been demonstrated. Our results in a different sequence context, the highly polymorphic HLA region (Consolandi, 2003) further confirm these findings. Another distinctive feature of the LDR approach is related to the excellent sensitivity gained by means of a cycling procedure based on thermostable ligases. We were able to detect down to 1 fmol (around 2 ng) of PCR amplified material thanks to the linear amplification gained through LDR. Figure 31 show the results we obtained using a serial dilution of Planktothrix 16S amplicon from 100 fmol to 1. A good linear relationship was found plotting the signal intensity against the concentration in a log scale.

The Universal array was used for the detection of toxic and non-toxic cyanobacteria designed to detect both the 16 rRNA and mcyE gene ligated probes. The ligation detection reaction was carried out under the same conditions by using an oligo mix containing both the probes for 16S rRNA gene and the probes for the mcyE gene. Finally the hybridization was carried on the same Universal Array where the 16S rRNA LDR product and, mcyE LDR product were detected.

EXAMPLES

Example 1

Genes coding for the synthesis of hepatotoxic heptapeptides (microcystins) in the cyanobacterium *Anabaena* strain 90

Bacterial strains and culture conditions

The cyanobacterial strain *Anabaena* 90 was isolated from Lake Vesijärvi, Finland and purified axenic (Sivonen et al., 1992; Rouhiainen et al., 1995). It was shown to produce three microcystins (MCYST-LR, MCYST-RR and D-Asp-MCYST-LR (Sivonen et al., 1992). *Anabaena* strain 90 was grown in Z8 medium (Kotai, 1972) without nitrate at ~22^0C with continuous illumination of 20-25 μmol $m^{-2}s^{-1}$. *Escherichia coli* strain DH5 α, which was used as a host for DNA cloning and sequencing, was cultured in Luria Broth at 37 °C.

DNA manipulations, sequencing, screening and mapping of cosmids

Extraction of cyanobacterial DNA and the preparation of genomic library has been described earlier (Rouhiainen et al. 2000). The genomic library was screened by colony hybrdization (Sambrook et al., 1989). The probe labelled with [^{32}P]-dCTP was a 2.5 kb fragment from mcyA of *Microcystis aeruginosa* provided by Dr. Elke Dittmann (Humboldt University, Berlin). A total of about 6,000 colonies were tested. The insert DNA of 29 positive cosmid clones was mapped with *Hind*III, *Eco*RI and *Spe*I. The ends of 18 inserts were sequenced with SP6 and T7 primers, and the cosmid clones for sequencing the microcystin synthetase genes were selected. DNA of the cosmid clones was digested with restriction enzymes *BstE*II, *Hind*III, *Eco*RI, *Sca*I, *Spe*I or *Xba*I and ligated to pBluescript SK(+). Nested deletions and other DNA manipulations were performed according to Sambrook et al., (1989). Sequencing was carried out mainly by the University of Chicago Cancer Research Center DNA Sequencing Facility. Gaps were filled by amplifying chromosomal DNA in PCR with DyNAzyme™ EXT Polymerase (Finnzymes), the sequencing reactions were done with the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems) and analyzed on the ABI 310 Genetic Analyzer. The standard T3 and T7 primers and oligonucleotides derived from already determined sequences were employed.

Sequence analysis

Analysis and comparisons of sequences were performed with the Sequence analysis software package, version 8.0., University of Wisconsin Genetics Computer Group and with EMBOSS (European Molecular Biology Open Software Suite). CAP program (http://bioweb.pasteur.fr/seqanal/ interfaces/ cap.html) was used for sequence assembly. Sequence similarity searches in databases were done with BLAST through the website of the National Center for Biotechnology Information http://www.ncbi.nlm.nih.gov/BLAST). Searches for conserved domains and motifs were accomplished with the CD-Search program (http://www.ncbi.nlm.nih.gov/Structure/ cdd/ cdd.shtml) and with the Motif Scan program (http://hits.isb-sib.ch/cgi-bin/PFSCAN?). Clustal W was applied for multiple sequence alignments (http://npsa-pbil.ibcp. fr/cgi-bin/npsa_automat.pl?page= npsa_clustalw.html).

Organization of the microcystin synthetase genes

Microcystin synthetase genes in *Anabaena* strain 90 (*mcyA-J*) are organized in three putative operons (Fig. 1) with a total size of 55.4 Kb. The first operon (*mcyA-mcyB-mcyC*) is
5 transcribed in the opposite direction compared to the second (*mcyG-mcyD-mcyJ-mcyE-mcyF-mcyI*) and the third operon (*mcyH*). The ORFs *mcyA* and *mcyG* are separated by 1275 bp; *mcyI* and *mcyH* by 297 bp (Fig. 1). The putative promoter regions were identified in front of *mcyA* (the -10 sequence, TAAATT, 315 bp and the –35 sequence, TTGTAT, 339 bp upstream from the translation start codon, ATG, of *mcyA*) and in front of *mcyG* (the -10 sequence,
10 TATAAG, 145 or 223 bp and the –35 sequence, TTGACA, 172 or 250 bp upstream from the potential translation starts of *mcyG*). The promoter region was also identified before *mcyH* (the -10 sequence, TATAAA, 57 or 216 bp and the –35 sequence, TTGATA, 79 or 238 bp from the suggested translation initiation codons). Transcriptional starts prior to *mcyD* (distance 93 bp from *mcyG*), *mcyE* (37 or 95 bp from *mcyJ*), *mcyF* (42 bp from *mcyE*) and
15 before *mcyI* (51 bp from *mcyF*) cannot be ruled out, although no transcription stop loops were identified following the preceding genes, and no Pribnow box could be identified in front of *mcyD*.

Characterization of the peptide synthetase genes

20

In the first operon there are three open reading frames (ORFs) named *mcyA*, *mcyB*, and *mcyC*. We suggest that the translation of *mcyA* starts with the ATG codon preceded (3 bp) by a potential ribosome binding site (RBS) GGAGAAG. The next ORF, *mcyB*, begins with an ATG codon 18 bp downstream from the previous stop codon (TAA) and 12 bp from a
25 potential RBS AGAGGA. *mcyC* is overlapped by *mcyB* with one base pair. A putative RBS (ACGACAAG) is found 5 bp before the start codon ATG of *mcyC*. The lengths of *mcyA*, *mcyB*, and *mcyC* are 8364, 6399 and 3852 bp and they encode polypeptides with predicted masses of 315,663, 243,072, and 146,877 Da, respectively. The sequence analysis of *mcyA*, *mcyB*, and *mcyC* revealed a typical modular structure for nonribosomal peptide synthetase
30 (NRPS) genes (Marahiel et al., 1997) (Fig.1). *mcy*A contains two putative adenylation and thiolation domains, a condensation, an *N*-methyltransferase, and an epimerization domain. In *mcyB* there are two modules, both include condensation, adenylation, and thiolation domains. *mcyC* is composed of one module, containing a condensation, an adenylation, a thiolation, and a thioesterase domain (Fig. 1).

Identification of the polyketide synthase genes

The second operon contains six ORFs named *mcyG-mcyD-mcyJ-mcyE-mcyF-mcyI.* A
5 suggested translation start codon (ATG) of *mcyG* is located 8 bp downstream of a probable
RBS (ACAGGA) giving an ORF (7827 bp), which could code for a protein of 2609 amino
acids with a predicted mass of 289,859 Da. Another possible initiation is at an ATG, 75 bp
upstream from the previously proposed start and 5 bp after a putative RBS (AAGGCA). This
ORF (7905 bp) possibly encodes a protein of 2635 amino acids, 292,851 Da. The ORFs *mcyG*
10 and *mcyD* are separated by 96 bp. The translation of *mcyD* starts probably at an ATG codon 6
bp after a potential RBS (GGAAGGAG), consequently the size of this large ORF is 11,607
bp, encoding 3869 amino acids. Following the stop codon TAG of *mcyJ* there are 36 bp prior
to a presumed ATG initiation codon of *mcyE*, which is preceded (5 bp) by a possible RBS
(GCGGACAA). An alternative ATG start codon for *mcyE* is 57 bp downstream from the
15 previously proposed one and 3 bp from a possible RBS (AATGGAGG). The two versions
(10,446 bp and 10,386 bp) of this large ORF, *mcyE*, could code for polypeptides of 3482
amino acids, 388,755 Da and 3462 amino acids, 386,501 Da, respectively. The ORF *mcyD*
encodes a polypeptide of 3869 amino acids with the predicted mass of 430,216 Da. *mcyD* was
identified as a polyketide synthase (PKS) gene, whereas *mcyG* and *mcyE* have a combined
20 NRPS/PKS gene structure (Fig. 1).

The additional genes

We suggest that the ORF *mcyJ* is initiated with a GTG codon 59 bp downstream of the stop
25 codon (TAA) of *mcyD*, and 5 bp from a putative Shine-Dalgarno sequence AGGAGAG.
There is no ATG codon located nearby. Accordingly, *mcyJ* is predicted to be 930 bp in length.

A small ORF, *mcyF*, (756 bp), following *mcyE*, begins with an ATG codon 42 bp after the
previous stop codon TAG and 6 bp from a putative RBS (GGAGAA). The distance between
30 *mcyF* and the next ORF, *mcyI*, (1011 bp) is 54 bp, and an alleged RBS (AAGGTTAA) is
found 6 bp upstream from the designated start codon ATG of *mcyI.* Downstream (295 bp)
from the stop codon (TAA) of *mcyI* an ORF, *mcyH*, (1776 bp) was found. It presumably is
initiated from the ATG codon 6 bp after a potential RBS (AAGATG). Another possible
translation start codon (ATG) is found 159 bp downstream from the former one and 4 bp from

a putative RBS (AGGCATGG). The sizes of these potential McyH polypeptides of 592 and 539 amino acids are 67,731 Da and 61,754 Da, respectively. *mcyJ*, *mcyF* and *mcyI* encode polypeptides of 310,252 and 337 amino acids with predicted masses of 35,812, 28,426, and 36,750 Da, respectively. McyF is similar to aspartate racemases, McyJ belongs to methyltransferases, and McyI is related to D-3-phosphoglycerate dehydrogenases. McyH contains a membrane spanning and an ATP-binding domain of ABC transporters. A BLAST search of McyH found 75% identity (in 589 aa) to NosG from *Nostoc* sp. GSV224 (AF204805) and 39% identity (in 543 aa) to the hypothetical ABC transporter ATP-binding protein SLL0182 of *Synechocystis* sp. PCC 6803 (Q55774).

Comparison of microcystin synthetase genes

The microcystin synthetase genes were previously sequenced from *M. aeruginosa* strains PCC7806 (*mcyA-mcyJ*, Tillett et al., 2000), K-139 (*mcyA-mcyI*, Nishizawa et al., 2000) and UV027 (*mcyA-mcyC*, Raps et al., unpublished, accession no. AF458094), and from *Planktothrix agardhii* CYA126 (Christiansen et al., 2002). When *Anabaena* 90 sequences were compared to *M. aeruginosa* sequences, they revealed 65 to 75 (*mcyJ* 80 %) percent identities at the amino acid level and 69 to 75 (*mcyJ* 79 %) percent identities at the nucleotide level (Table 1). The arrangement of the microcystin synthetase genes from *mcyD* to *mcyJ* in *Anabaena* 90 is different from the organization in *M. aeruginosa* PCC7806, in *M. aeruginosa* K-139 (known from *mcyD* to *mcyI*) and in *Planktothrix agardhii* CYA126.

TABLE 1. Percentage identity of the microcystin synthetase genes/polypeptides from Anabaena strain 90 with the genes/polypeptides sequenced from other cyanobacteria and the mol% G+C of the genes.

	mcy/Mcy[a]									
	A	**B**	**C**	**D**	**E**	**F**	**G**	**H**	**I**	**J**
M. aeruginosa PCC7806	69/68	72/69	74/73	72/69	75/74	71/65	74/71	74/70	74/71	79/80
mol% G+C	41	39	37	40	39	38	38	35	40	39

M. aeruginosa K-139	69/68	71/69	74/73	72/69	75/75	71/65	74/71	74/70	74/72	
mol% G+C	41	39	37	40	39	37	38	36	39	
M. aeruginosa UV027	69/68	73/71	74/73							
mol% G+C	41	39	37							
P. agardhii CYA126/8	67/66	72/70	80/79	77/73	78/77		77/74	78/75		81/82
mol% G+C	45	39	35	38	38		38	35		37
Anabaena 90 mol% G+C	41	38	37	40	38	34	39	36	38	39

a. References for the sequences: *Microcystis aeruginosa* PCC7806, Tillett *et al.*, 2000; *M. aeruginosa* K-139, Nishizawa, *et al.*, 2000; *M. aeruginosa* UV027, Raps *et al.*, unpublished, AF458094; *Planktothrix agardhii* CYA126/8, Christiansen *et al.*, 2003.

When the microcystin synthetase genes were compared to the anabaenopeptilide synthetase genes of *Anabaena* 90, the highest similarity, 54 %, was between *mcyC* and *apdD.*

In the genome databases of *Anabaena* 7120 (http://www.kazusa.or.jp/cyano/ Anabaena/ search. html) and *Nostoc punctiforme* (http://www.jgi.doe.gov/JGI_microbial/html/nostoc/ nostoc_homepage.html) no genes were found with more than 50% identity to the microcystin synthetase genes at the amino acid level. There are two sequences in the genome database of *Anabaena/Nostoc* 7120 named "microcystin synthetase B" on account of similarity to *mcyB* of *Microcystis aeruginosa* (AY034602): all2643 (ID:3312, 3309 bp) and all2647 (ID:3317, 3261 bp), (identity: 47.0 %, positive: 65.5 % and identity: 43.9 %, positive: 61.9 %, respectively). The matches of these sequences with *mcyB* of *Anabaena* 90 are 53 % and 51 % at the gene level. The translated peptides are 49 %/66 % and 43 %/61 % identical/similar, respectively.

The G+C content of the microcystin synthetase gene cluster (56 kb) from *Anabaena* 90 is 39 %, is lower than the value, 43 %, for the region of the anabaenopeptilide synthetase (39 kb) (Rouhiainen et al., 2000). These figures are in the limits of the mol% G+C values 43.9, 39.1
5 and 42.3 for the type strains *Anabaena cylindrica* (PCC 7122), *Anabaena flos-aquae* (PCC 9332) and for the reference strain of *Anabaena* cluster 2 (PCC 7108), respectively (Rippka et al., 2001).

Substrate specificity of the adenylation domains

10

The substrate specificity-conferring amino acids in the adenylation domains of the microcystin synthetases of *Anabaena* 90, *P. agardhii* CYA126, *M. aeruginosa* PCC7806, K-139, and UV027 were assessed according to Stachelhaus et al., (1999) (Table 2). The substrate specificity codes of the modules McyA-1, McyA-2, McyB-2 and of the
15 nonribosomal peptide synthetase (NRPS) modules in McyG and McyE are identical or nearly identical in all the sequenced microcystin synthetases (Table 2).

TABLE 2. Specificity-conferring amino acids (signature sequences) of the adenylation domains in the microcystin synthetases from different cyanobacterial strains.

20

Module	Strain	Signature sequence[a]	Precedent SS	Activated amino acid	Reference template
McyA	*Anabaena* 90	**DVWHISLID**	**DVWHLSLID**	Ser	SyrE (1, 2)[b]
	M. aeruginosa 7806	**DVWHFSLID**	**DVWHFSLVD**		EntF, MycC (1, 2 3)[b]
	M. aeruginosa K-139	**DVWHFSLID**			
	M. aeruginosa UV027	**DVWHFSLID**			
	P. agardhii CYA 126/8	**DVWHISLID**			
McyA 2	*Anabaena* 90	**DLFNNALTY**			
	M. aeruginosa 7806	**DLFNNALTY**			
	M. aeruginosa K-139	**DLFNNALTY**	**DLFNNALTY**	Ala	BlmIX,MxA (4, 5)[c]
	M. aeruginosa UV027	**DLFNNALTY**			
	P. agardhii CYA 126/8	**DLFNNALSY**			

25
30
35

McyB 1	*Anabaena* 90	DVWFFGLVD			
	M. aeruginosa 7806	DAWFLGNVV	DAWFLGNVV	Leu	BacA,LicA,LicB,
	M. aeruginosa K-139	DAWFLGNVV			SrFA (1)[b]
	M. aeruginosa UV027	DVWTIGAVE		(Arg)	
	P. agardhii CYA 126/8	DALFFGLVD			
McyB 2	*Anabaena* 90	DARHVGIFV			
	M. aeruginosa 7806	DARHVGIFV			
	M. aeruginosa K-139	DARHVGIFV	no precedents	(Asp/MeAsp)	
	M. aeruginosa UV027	DARHVGIFV			
	P. agardhii CYA 126/8	DPRHVGIFI			
McyC	*Anabaena* 90	DVWCFGLVD			
	M. aeruginosa 7806	DVWTIGAVD			
	M. aeruginosa K-139	DVWTIGAVE	no precedents	(Arg)	
	M. aeruginosa UV027	DVWTIGAVD			
	P. agardhii CYA 126/8	DPWGFGLVD			
McyG	*Anabaena* 90	GAFWVAASG			
	M. aeruginosa 7806	GAFWVAASG	no precedents		
	M. aeruginosa K-139	GAFWVAASG			
	P. agardhii CYA 126/8	GAFWVAASG			
McyE	*Anabaena* 90	DPRHSGVVG			
	M. aeruginosa 7806	DPRHSGVVG	no precedents	(Glu)	
	M. aeruginosa K-139	DPRHSGVVG			
	P. agardhii CYA 126/8	DPRHSGVVG			

a. Nine variable amino acids of the signature sequences determined as described by Stachelhaus *et al.*, 1999.

Bold letters indicate the residues, which are identical with the amino acids of the signature sequence from *Anabaena* 90.

b. 1. Stachelhaus *et al.*, 1999, 2. Challis *et al.*, 2000, 3. Duitman *et al.*, 1999.

c. 4. Du *et al.*, 2000, 5. Silakowski *et al.*, 2001.

There are, however, more differences in the specificity codes of variable amino acids activating McyB-1 and McyC module. The substrate specificity regions of the adenylation domains (corresponding amino acids 235-331 of GrsA, Stachelhaus et al., 1999) in McyA,

McyB and in McyC from *Anabaena* 90, *P. agardhii* and from *M. aeruginosa* were compared by using the algorithm of Smith and Waterman in the EMBOSS program package. The substrate specificity regions of McyA, of the second module of McyB (McyB-2) and of McyC are highly conserved. In *Anabaena* 90 and *M. aeruginosa*, the identity/similarity values are 80 /90 % for McyA, 86/92 % for McyB-2 and 70/80 % for McyC. Between *Anabaena* 90 and *P. agardhii* the identity/similarity for the substrate specificity region of McyC is higher, 85/88 %, but lower for the second module of McyA, 73/83 %. The substrate specificity region of McyB-1 is considerably less conserved between *Anabaena* 90 and *M. aeruginosa* PCC7806, 29/53 % than between *Anabaena* 90 and *M. aeruginosa* UV027, or *P. agardhii*, 66/80 %.

Activities encoded by mcyG, mcyD and mcyE of Anabaena 90

Motif scan at Prosite (Database of protein families and domains) and at Pfam (Protein families) database (http://hits.isb-sib.ch/cgi-bin/PFSCAN) and Conserved Domain (CD) search at NCBI (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) were used to discover the putative functions of McyG, mcyD and McyE. In the N-terminal part of McyG a NRPS module was identified, which contains an adenylation domain and a thiolation (phosphopantetheine carrier) domain. Next to this, toward the C-terminus there are four polyketide synthase (PKS) domains: β-ketoacyl synthase (KS), acyltransferase (AT), ketoreductase (KR) and acylcarrier protein (ACP), in this order. Between AT and KR domains there is a C-methyltransferase, (MeT/CM) domain (Fig. 1). McyD contains two modules of the type I polyketide synthases. The first module consists of KS, AT, dehydratase (DH), MeT (CM) (Fig. 1), KR and ACP domains; and module two has KS, AT, DH, KR and ACP domains, in the presented orders. McyE is the other mixed PKS/NRPS, including PKS domains KS, AT, ACP and MeT (CM) (Fig. 1; Fig. 2A). These are followed by a unique aminotransferase domain (AMT) (Fig. 1; Fig. 2B) found in other microcystin synthetases (Tillet et al., 2000; Christiansen et al., 2003), and also in the synthetases of mycosubtilin (Duitman et al., 1999) and iturin (Tsuge et al., 2001) of *Bacillus subtilis*. At the N-terminal region, subsequently there is a NRPS module comprising of two condensation domains, an adenylation and a thiolation (peptidyl carrier) domain (Fig. 1).

Ketoreductase and dehydratase domains

The activity of the KR domains of McyG (one) and McyD (two) can be predicted from the microcystin synthetases structure, and they have the NAD cofactor binding motif, GXGXX(G/A)$(X)_3$(G/A)$(X)_6$G, common to oxidoreductases (Scrutton et al., 1990). (Fig. 3B) The DH domains in the modules of McyD (AMCD-DH2 and AMCD-DH3) contain the active site motif H$(X)_3$D$(X)_4$P and H$(X)_3$G$(X)_4$P, respectively (Fig. 3A). The motif in AMCD-DH3 is identical to the consensus sequence (Aparicio et al., 1996). The motif H$(X)_3$D$(X)_4$P, where Gly is substituted by Asp, is also found in the active DH domain of module 10 in rifamycin synthase (Tang et al., 1998) (Fig. 3). This supports the conclusion based on the microcystin structure, that the DH domains in McyD are functional.

Specificity of the acyl transferase domains

From the structure of the microcystins it is possible to conclude that the single AT domains of McyG and McyE, and the first AT domain of McyD, load methylmalonyl-CoA. But the presence of methyltransferase domains in McyG, McyD and McyE (Fig. 1, Fig. 2A) suggests that the loading unit can be malonyl-CoA. Regions have been identified in AT domains, where the sequences are different depending on the specificity for either malonyl-CoA or methylmalonyl-CoA (Fig. 4) (Ikeda et al., 1999). By analysing the sequences of the acyltransferase domains (Fig. 4) and comparing them with the AT domains of soraphen and rapamycin synthases, which utilize malonyl subunits, we concluded that all the AT domains of microcystin synthetase load malonyl units. The methyltranferase domains of McyG, McyD and McyE carry out three methylations in the positions indicated with arrows (Fig. 1). The CD search relates these domains to the UbiE/COQ5 C-methyltranferase family.

Ketosynthase and acylcarrier protein domains

The active site cysteine and the two histidine residues which are present in polyketide synthases (Aparicio et al., 1996) were found in the KS domains of McyG, McyD and McyE (Fig. 5A). The only ACP domain of McyG and the first ACP domain of McyD have the active site sequence MGXDS, where a methionine residue replaces the commonly identified leucine residue (Fig. 5B). There are also variations in this position of the rifamycin synthase (Tang et al., 1998). The ACP domain from the second module of McyD has the active site motif

LGLNS (Fig. 5B), where Asn takes the place of the generally found Asp as in the module 11 of the rapamycin synthase (Aparicio et al., 1996).

The order of the genes in the microcystin synthetase gene cluster is different in the
5 *cyanobacterial species*

The arrangement of the genes is different in the gene clusters of microcystin biosynthesis from the strains of three species. In *Anabaena* strain 90, *Microcystis aeruginosa* (Tillett et al., 2000; Nishizawa et al., 2000) and in *Planktothrix agardhii* CYA126 (Christiansen et al.,
10 2003) the NRPS genes, *mcyA*, *mcyB* and *mcyC* have the same order, but the organization of the other genes is different. In *Anabaena* strain 90 and in *M. aeruginosa* the *mcy*-genes are in two clusters, which are transcribed in opposite directions, whereas in *P. agardhii* they are in one cluster transcribed in the same direction (except *mcyT*, which was not found in *Anabaena* and *Microcystis*). The arrangement of the genes from *mcyD* to *mcyH* in *Microcystis* is almost
15 identical in *Planktothrix* (*mcyF* is missing in *Planktothrix*), but it differs from the order in *Anabaena*. In *Planktothrix*, compared to *Microcystis*, the part containing *mcyD*, *mcyE*, *mcyF*, *mcyG*, *mcyH*, *mcyI* and *mcyJ* is reversed. In this rearrangement, *mcyF* and *mcyI* were lost from the cluster and *mcyJ* was relocated after *mcyC*.

20 *The biosynthesis of microcystins*

In *Anabaena,* the order of the domains coded by the genes in the two sets is co-linear with the hypothetical sequence of the enzymatic reactions for microcystin biosynthesis (Fig. 1). The progression of the biosynthetic reactions follows the order of the functions coded first by
25 *mcyG* and continuing with the activities coded by *mcyD*, *mcyJ*, *mcyE*, *mcyF*, *mcyI*, *mcyA*, *mcyB* and *mcyC*..

Phenyl acetate is the assumed starting unit in the biosynthesis of Adda (Moore et al., 1991). It is activated by the adenylating domain identified in the N-terminus of McyG, and transferred
30 onto the subsequent thiolation (phosphopantetheine binding) site. Polyketide synthesis reactions are followed (Fig. 1). All four extension units are malonyl-CoA molecules according to the substrate specificity of the AT domains (Fig. 4). In McyG there is a KS domain to catalyse the first condensation reaction between phenylacetate and malonyl-CoA.

The reductive reactions needed to fashion the polyketide chain are putatively catalysed by KR and DH domains of McyD and McyE. The KR domain of McyG is in the right position to reduce the carbonyl group of the putative starter molecule. The methyltransferase domains of McyG, McyD and McyE are the obvious candidates to introduce three methyl groups into the carbon frame of Adda. It was recently verified with a knockout mutant (Christiansen et al., 2003) that the incorporation of the fourth methyl, which is seen in the methoxy group of Adda, is catalysed by McyJ. The amino transferase domain of McyE most likely adds the amino group, which participates in the peptide bond with the glutamate residue.

There are two condensation domains of peptide synthetases in McyE. The first one logically catalyses the peptide bond between Adda and glutamate, which is activated by the adenylation domain of McyE. The signature sequence, which was also determined as DPRHSGVVG for McyE of both *M. aeruginosa* and *P. agardhii*, has no precedents in the databases (Table 2). The synthetases of other peptides, which contain glutamyl residues are known for bacitracin, fengycin and surfactin (accession numbers: AF007865, AF023464, AF087452 and D13262). In these compounds the standard α-carboxyl of glutamate is part of the peptide bond, while in microcystins it is the γ-carboxyl. This is analogous to the activation of aspartate/ methylaspartate by the second adenylation domain of McyB. The β-carboxyl of aspartate/methylaspartate instead of the α-carboxyl is engaged in the peptide bond formation. This must have impact on the compositions of the glutamate and aspartate/methylaspartate binding pockets in the adenylation domains.

McyA has two adenylation domains for the activation of serine and alanine, respectively. The signature sequences of these domains have models and are almost identical in *Anabaena* 90, *M. aeruginosa* and *P. agardhii* (Table 2). The dehydration of serine supposedly takes place after the activation by adenylation and is catalysed by McyI, which is similar to phosphoglycerate dehydrogenases.

There is only one, internal, condensation domain in McyA, which most likely links dehydroserine and D-alanine. The bond between glutamate and dehydroserine is putatively catalysed by the C-terminal condensation domain of McyE. There is a methyltransferase domain in the first module of McyA for N-methylation of dehydroserine. The epimerase domain at the C-terminus of McyA converts L-alanine to the D-form.

Two modules of McyB and one module of McyC logically activate, and add three residues to the nascent peptide chain: L-leucine or L-arginine, methylaspartate or aspartate and L-arginine, respectively (Fig 1). The amino acids activated by the adenylation domains of McyC and by the first module of McyB (McyB-1) vary most frequently in microcystins. *M. aeruginosa* PCC7806 and *M. aeruginosa* K-139 produce mainly Mcyst-LR, and the substrate specificity conferring sequences in McyB-1 of these strains are identical with the signature sequence for leucine (Table 2). *M. aeruginosa* UV027 and *P. agardhii* CYA126 produce mostly Mcyst-RR, which is also produced by *Anabaena* 90 together with Mcyst-LR. Their signature sequences in McyB-1 are different and have no precedents in the databases (Table 2). In *M. aeruginosa* UV027 the specificity codes of McyB-1 and McyC are almost identical (DVWTIGAVE / DWTIGAVD) and match with the codes of McyC from *M. aeruginosa* K-139 and *M. aeruginosa* PCC7806, respectively (Table 2). Accordingly McyB-1 of *M. aeruginosa* UV027 and McyC activate arginine.

There is no epimerase domain in McyB of *Anabaena* 90 or in the other sequenced versions of McyB, though in microcystins, the aspartyl or methylaspartyl moiety is in the D-form. The epimerization in this position and in the glutamyl residue is putatively catalysed by McyF, which in a BLAST search was similar to aspartate racemases, and was shown by Nishizawa et al., (2001) to complement a D-glutamate deficient mutant of *Eschericia coli*. The C-terminal thiosterase domain of McyC, as generally in nonribosomal peptide synthesis, (Kohli et al., 2001) catalyzes the final step in microcystin biosynthesis, the cyclization of the linear peptide (Fig. 1).

McyH is probably not needed for the synthesis of microcystins but it may participate in the transport of microcystins.

Example 2

Taxon sampling, amplification and sequencing

Genomic DNA from 36 strains of *Anabaena*, *Microcystis*, *Planktothrix*, *Nodularia*, and *Nostoc* was extracted. We chose three regions of the microcystin synthetase gene cluster to study the evolution of this biosynthetic system in cyanobacteria. A fragment of 291-297 bp from the *mcy*A gene was amplified with mcyA-Cd 1R (5'-aaaagtgttttattagcggctcat-3') and mcyA-Cd 1F (5'-aaaattaaaagccgtatcaaa-3') primers and sequenced as described earlier

(Hisbergues et al. 2003). An 818 bp region of the *mcy*D gene was amplified with mcyDF (5'-gatccgattgaattagaaag-3') and mcyDR (5'-gtattccccaagattgcc-3') primers. An 809-812 bp region of the *mcy*E gene was amplified with the *mcy*E-F2 (5'-gaaatttgtgtagaaggtgc-3') and mcyE-R4 (5'-aattctaaagcccaaagacg-3') primers. The *mcy*E PCR products of *Nodularia* sp.
5 strains were cloned with the TOPO TA cloning kit (Invitrogen) according to the manufacturer's instructions. The *rpo*C1 gene fragment of 750 bp was amplified with degenerate primers RF (5'-tggggghgaaagnacaytncctaa-3') and RR (5'-gcaaancgtccnccatcyaaytgba-3'). PCR reactions for *mcy*E, *mcy*D and *rpo*C1 were performed in a 20 µl final volume containing 1 µl of DNA, 1 x DynaZyme II PCR buffer, 250 µM of each
10 deoxynucleotide, 0.5 µM of both PCR primers, and 0.5 U of DynaZyme II DNA polymerase (Finnzymes, Espoo, Finland). The following protocol was used: 95°C, 3 min; 30 x (94°C, 30 sec; 56°C, 30 sec; 72°C, 1 min); 72°C, 10 min. A region containing the 16S rRNA gene and the internal transcribed spacer 1 (ITS1) was amplified using primers and conditions described earlier (Lepére et al., 2000) from strains, for which the 16S rRNA sequence data was not
15 available. The *mcy*D and *mcy*E gene products were sequenced directly with primers used for amplification except for the cloned *mcy*E sequences of *Nodularia* sp. strains, which were sequenced with primers anchored in the pCR2.1-TOPO vector, M13F (-20) and M13R. The *rpo*C1 gene products were sequenced with the amplification primers and with two additional internal sequencing primers RintF (5'-gatatgcccctgcgggatgt-3') and RintR (5'-
20 acatcccgcaggggcatatc-3'). The 16S rRNA gene region of the amplified PCR products was sequenced directly using sets of internal primers (Edwards et al., 1989).

Sequencing of the *mcy*D, *mcy*E and 16S rRNA genes was performed by Genome Express (France). The *rpo*C1 products were sequenced with ABI PRISM 310 Genetic Analyzer.
25 The *mcy*A sequences were assembled as described by Hisbergues *at al.*. The chromatograms of *mcy*D, *mcy*E, *rpo*C1 and 16S rRNA gene sequences were checked and edited with Chromas 2.2 program (Technelysium Pty Ltd.). Contig assembly and alignment of the sequences were performed with BioEdit Sequence Alignment Editor (Hall et al., 1999).

30 **Phylogenetic analyses**

Primer sequences and ambiguous regions of the alignments were excluded. The aligned data sets were the following lengths: *mcyA* (99 amino acids), *mcyD* (286 amino acids), *mcyE* (270 amino acids), *rpoC1* (750 bp) and 16S rRNA (1455 bp). These sequences were

combined with the sequence available from *Microcystis aeruginosa* PCC 7806 (Tillett et al. ,2000). and *Planktothrix agardhii* NIVA-CYA 126/8 (Christiansen et al.,2003).

Outgroups for each of the three microcystin synthetase genes were identified with BLAST searches (Supplementary Information). We aligned *mcy*A, *mcy*E, and *mcy*D and the top three hits in BLAST searches with BioEdit (Hall et al. 1999).

Only conserved and reliably aligned sequence regions from the outgroup sequences were used in order to minimise potential phylogenetic reconstruction artefacts derived from the use of distant outgroups Swofford et al. 1996).. In order to assess the stability of the ingroup tree topology, which could be influenced by the addition of outgroup lineages due to long branch attraction, the phylogenetic trees were analysed with and without the chosen outgroups. Phylogenetic analyses were performed with PAUP (Swofford, 2001) and PHYLIP (Felsenstein, 1993).. Maximum likelihood and maximum parsimony analyses were used to reconstruct trees from each *mcy* gene fragment, and to compare the tree topologies of the separate and concatenated *mcy* gene sets and the 16S rRNA and *rpo*C1 genes. 16S rRNA sequences of 53 cyanobacterial strains and three outgroup species were used to construct a maximum-likelihood tree, to which the distribution of microcystin and nodularin producing cyanobacteria among other cyanobacteria was mapped (Fig. 8).

Table 3. Accession numbers for sequences used in phylogenetic reconstruction. A solid line denotes unsuccessful attempts to amplify this region from the three strains of the genus *Nodularia* used in this study. A dash indicates cases where no attempt was made to obtain sequence data.

Taxon	*mcy*A	*mcy*E	*mcy*D	16S rRNA	*rpo*C1
Microcystis sp. HUB 5-2-4	AJ515451	-	-	-	-
Microcystis aeruginosa NIES 89	AJ515459	AY382530	AY424988	U03403	-
Microcystis sp. 199	AJ515452	-	-	AJ133172	-
Microcystis sp. GL260735	AJ515454	AY382531	-	AY439282	-
Microcystis sp. GL280646	AJ515455	AY382532	-	-	-
Microcystis sp. IZANCYA5	AJ515456	AY382533	-	-	-
Microcystis sp. IZANCYA25	-	AY382534	-	-	-
Microcystis sp. TuM7C	AJ515458	-	-	-	-
Microcystis viridis NIES 102	AJ515457	AY382535	AY424991	U40332	AY425001
Microcystis aeruginosa PCC 7941	AJ515460	AY382536	AY424989	U40340	-
Microcystis aeruginosa PCC 7806	AF183408	AF183408	AF183408	AF139299	AY425000

Microcystis sp. 98	-	AY382537	-	-	-
Microcystis sp. 205	AJ515453	AY382538	AY424990	AY439281	-
Nostoc sp. 152	AJ515475	AY382539	AY424984	AJ133161	AY424997
Nodularia spumigena HEM	————	AY382540	AY424985	AF268005	AY424999
Nodularia spumigena BY1	————	AY382541	AY424987	AF268004	-
Nodularia sp. F81	————	AY382542	AY424986	AY439283	AY424998
Anabaena sp. 66A	AJ515462	AY382543	AY424983	AJ133157	-
Anabaena sp. 66B	AJ515463	-	-	-	-
Anabaena flos-aquae NIVA-CYA83/1	AJ515466	AY382544	-	AJ133158	-
Anabaena sp. 202A1/35	AJ515464	AY382545	AY424980	AJ133159	-
Anabaena lemmermannii 202A2	AJ515465	AY382546	AY424981	AJ293104	AY424995
Anabaena sp. 90	AJ515461	AJ536156	AJ536156	AJ133156	AY424996
Anabaena sp. PH256	-	AY382547	-	-	-
Anabaena sp. 315	-	AY382548	-	-	-
Anabaena sp. 318	-	AY382549	-	-	-
Anabaena sp. 299	-	AY382550	AY424982	AJ293106	-
Planktothrix sp. HUB 076	AJ515472	-	-	-	-
Planktothrix sp. PCC7821	AJ515473	-	-	-	-
Planktothrix sp. NIVA-CYA34	AJ515474	-	-	-	-
Planktothrix sp. 49	AJ515470	AY382551	AY424992	AJ133167	AY425003
Planktothrix sp. 97	AJ515471	AY382552	-	-	-
Planktothrix sp. NIVA-CYA126	AJ441056	AJ441056	AJ441056	AJ133166	-
Planktothrix sp. NIVA-CYA127	AJ515468	AY382553	AY424993	AJ133168	AY425002
Planktothrix sp. NIVA-CYA128/R	AJ515469	AY382554	AY424994	AJ133169	-
Oscillatoria sp. 213	-	AY382555	-	-	-
Oscillatoria sp. 226	-	AY382556	-	-	-

Table 4. Accession numbers of sequences used to root the microcystin gene data set in figure 7. The outgroup sequences identified by BLAST searches were fused together to form three outgroup sequences in the *mcyA*, *mcyD* and *mcyE* concatenated gene data set.

Gene	Outgroup	Accession	Organism	Gene	Function
*Mcy*A	Outgroup 1	AF210249	*Streptomyces verticillus*	*blm*X	Bleomycin biosynthetic gene
	Outgroup 2	AE004755	*Pseudomonas aeruginosa*	PA3327	Probable non-ribosomal peptide synthetase
	Outgroup 3	X97860	*Amycolatopsis mediterranei*	*aps*	Peptide-synthetase
*Mcy*D	Outgroup 1	AF395828	*Aphanizomenon ovalisporum*	*aoa*C	Polyketide synthase
	Outgroup 2	AJ421825	*Stigmatella aurantiaca*	*sti*H	Stigmatellin biosynthetic gene
	Outgroup 3	AP003590	*Nostoc sp. PCC 7120*	*alr*2680	Polyketide synthetase
*Mcy*E	Outgroup 1	D29676	*Bacillus brevis*	*Grs*2	Gramicidin S synthetase 2
	Outgroup 2	X70356	*Bacillus subtilis*	*srf*A1	Surfactin synthetase
	Outgroup 3	AF004835	*Brevibacillus brevis*	*tyc*C	tyrocidine synthetase 3

Table 5. Accession numbers for 16S rRNA sequences used to construct the maximum-likelihood tree presented in figure 8.

Species	Strain	16S rRNA
Cyanobacteria		
Subsection I Chroococcales		

Cyanobium gracile	PCC 6307	AF001477
Cyanothece sp.	PCC 7424	AF132932
Gloeobacter violaceus	PCC 7421	AF132790
Gloeothece membranacea	PCC 6501	X78680
Microcystis aeruginosa	PCC 7806	U03402
Microcystis aeruginosa	PCC 7941	U40340
Microcystis wesenbergii	NIES 104	AJ133174
Synechococcus elongatus	PCC 6301	X03538
Synechococcus leopoliensis	PCC 7942	AF132930
Synechococcus sp.	PCC 7002	AJ000716
Synechococcus sp.	PCC 6716	AF216942
Synechococcus sp.	WH 8103	AF311293
Synechocystis sp.	PCC 6803	D64000
Thermosynechococcus elongatus	BP-1	AP005376
Prochlorococcus marinus	MED 4	AF001466
Prochlorococcus marinus	MIT 9313	AF053399
Subsection II Pleurocapsales		
Chroococcidiopsis sp.	SAG 2023	AJ344552
Chroococcidiopsis thermalis	PCC 7203	AB039005
Myxosarcina sp.	PCC 7312	AJ344561
Myxosarcina sp.	PCC 7325	AJ344562
Pleurocapsa minor	SAG 4.99	AJ344564
Pleurocapsa sp.	PCC 7516	X78681
Xenococcus sp.	PCC 7305	AF132783
Subsection III Oscillatoriales		
Arthrospira sp.	PCC 8005	X70769
Leptolyngbya sp.	PCC 7375	AF132786
Leptolyngbya sp.	PCC 7104	AB039012
Limnothrix redekei	NIVA-CYA 227/1	AB045929
Lygnbya aestuarii	PCC 7419	AJ000714
Oscillatoria rosea	IAM-220	AB003164
Oscillatoria sancta	PCC 7515	AF132933
Planktothrix agardhii	NIVA-CYA 126	AJ133166
Planktothrix sp.	2	AJ133185
Planktothrix sp.	49	AJ133167
Pseudanabaena sp.	PCC 6903	AF132778
Spirulina major	PCC 6313	X75045
Spirulina subsalsa	IAM-223	AB003166
Trichodesmium erythraeum	IMS101	Unpublished*
Prochlorothrix hollandica	-	AF132792
Subsection IV Nostocales		
Anabaena sp.	66A	AJ133157
Anabaena sp.	90	AJ133156
Anabaenopsis circularis	NIES 21	AF247595
Anabaenopsis sp.	PCC 9215	AY038033
Aphanizomenon flos-aquae	NIES 81	AJ293131
Cyanospira rippkae	PCC 9501	AY038036
Cylindrospermum stagnale	PCC 7417	AF132789
Nodularia spumigena	BY1	AF268004
Nodularia sp.	F81	AY439283
Nodularia spumigena	PCC 73104	AF268023
Nostoc sp.	PCC 7120	X59559
Nostoc punctiforme	PCC 73102	AF027655
Nostoc sp.	152	AJ133161
Nostoc sp.	PCC 9709	AF027654
Scytonema hofmannii	PCC 7110	AF132781
Subsection V Stigonematales		
Chlorogloeopsis sp.	PCC 7518	X68780
Fischerella muscicola	PCC 7414	AF132788
Outgroups		
Bacillus subtilis	BS62	AB016721

Chlorobium tepidum	-	M58468
Escherichia coli	K12	AE000129

* Unpublished 16S rRNA obtained from *Trichodesmium erythraeum* IMS101 on the Joint Genome Institute webpage (www.jgi.doe.gov).

5 **Example 3**

Primer design and specificity testing. General microcystin synthetase E forward primer (mcyE-F2) and genus specific reverse primers for *Anabaena* (AnamcyE-12R) as well as for *Microcystis* (MicmcyE-R8) (Table 6) were designed with *mcy* gene sequences of *Anabaena*
10 90 (see Example 1), by using BLAST (1) and BioEdit (Hall 1999).

Specificity of these primers was tested with 14 *Anabaena*, 13 *Microcystis*, 8 *Planktothrix* strains and with one *Nostoc* strain (Table 7). *Microcystis* and *Planktothrix* strains were grown in Z8 medium (Kotai 1972), whereas *Anabaena* and *Nostoc* strains were grown in a modified
15 Z8 medium without nitrogen. The strains were grown under continuous light (20 μmol $m^{-2}s^{-1}$) at 20 ± 2°C.

PCR reaction was carried out with 1 μl of extracted DNA, 1 x DynaZyme II PCR buffer [10 mM Tris-HCl, pH 8.8 at 25°C, 1.5 mM $MgCl_2$, 50 mM KCl, 0.1% Triton X-100,
20 (Finnzymes)], 250 μM dNTPs (Finnzymes), 0.5 μM of primers (Sigma-Genosys Ltd.) and 0.5 U of DyNAzyme II DNA polymerase (Finnzymes) in a volume of 20 μl. The PCR amplification was performed with initial denaturation at 95°C for 3 min followed by either 30 (*Anabaena*) or 25 (*Microcystis*) cycles at 94°C for 30 s, at 58°C for *Anabaena* and at 60°C for *Microcystis* for 30 s and at 72°C for 60 s, followed by 10 min final extension at 72°C.
25 Presence or absence of the *mcy*E product was determined using 20 μl of amplification product and 1.5% agarose gel electrophoresis.

Lake water samples. Water samples were collected at Lake Tuusulanjärvi from 0 to 2 m depth every second or third week during summer period 1999. For DNA extraction one liter
30 of lake water was concentrated to less than 2 ml by centrifugation and stored at –70°C. Lake Hiidenvesi consists of several natural basins representing a transition from hypertrophy to mesotrophy. Water samples were collected from 3 to 5 different depths from basins of Kirkkojärvi (3.5 m deep at the sampling site), Mustionselkä (4 m), Nummelanselkä (6 m), and

Kiihkelyksenselkä (30 m) on 15th of August in 2001. For DNA extraction 100 ml of lake water was filtered through 3 μm pore size Poretics® polycarbonate disc filter (47 mm), (Osmonics Inc.) and cells were stored with lysis buffer at –20°C (14). For microcystin concentration analysis, 5 ml of lake water was stored in a glass vial at –20°C. Cyanobacterial cell densities were determined using the inverted microscope technique (Utermöhl, 1958) from the samples which were preserved with acid Lugol's solution (Willén, 1962) and stored in darkness at 4°C.

Isolation and purification of DNAs. Genomic DNAs of the *Anabaena*, *Microcystis*, *Planktothrix* and *Nostoc* strains and the lake water samples were extracted with a hot phenol-chloroform-isoamylalcohol -method (Giovannoni et al., 1990). Extracted DNAs were purified either once (strains) or twice (lake water samples) with Prep-A-Gene® DNA Purification Systems (Bio-Rad) according to the manufacturer's instructions and eluted in 60 μl.

QRT-PCR. External standards for *mcy*E copy number quantification were prepared using genomic DNAs of strains *Anabaena* 90, 315, and 202A1 as well as those of *Microcystis* GL 260735, PCC 7806, and PCC 7941. Genomic DNA concentration of these DNAs was measured with a spectrophotometer at 260 nm (Beckman DU-7400). Purity was determined by calculating the ratio of the absorbances measured at 260 nm and 280 nm. Approximate genome sizes, *Anabaena* 5.15 Mb and *Microcystis* 4.70 Mb, were used in *mcy*E copy number calculation. These genome sizes were estimated based on the genome sizes of *Anabaena* PCC 6309, *Anabaena* PCC 7122 and *Microcystis* PCC 7941 (Castenholz, 2001). The *mcy*E copy numbers of the standard strains DNAs were calculated using following equation with the assumption that each genome had only one *mcy*E gene and the molecular weight of one bp was 660 g mol^{-1}:

Equation 1.
$$\text{Copies } \mu l^{-1} = \frac{6 \times 10^{23}\ [\text{copies mol}^{-1}] \times \text{DNA concentration } [\text{g } \mu l^{-1}]}{\text{Molecular weight of one genome } [\text{g mol}^{-1}]}$$

Ten-fold dilution series of genomic DNAs of the standard strains were prepared and these dilutions were amplified with *Anabaena* and *Microcystis mcy*E QRT-PCR. Linear regression equations of the obtained cycle threshold values (Ct values, i.e. the first turning points of the

fluorescence curves as a function of cycle numbers) were calculated as a function of known *mcy*E copy numbers.

The QRT-PCR reaction was carried out with 1 μl of DNA of standard strains or lake water samples, 3 mM $MgCl_2$, 0.5 μM of both primers (Sigma-Genosys Ltd.) and 1 μl of hot start reaction mix to a final volume of 10 μl (LightCycler – fastStart DNA master SYBR green I – kit, Roche Diagnostics). Amplification was performed with initial preheating of 10 min at 95°C followed by 45 cycles at 95°C for 2 s, at 58°C for 5 s and at 72°C for 10 s. Generation of the products was monitored after each extension step at 77°C in *Anabaena* and 78°C in *Microcystis* *mcy*E QRT-PCR by measuring fluorescence of double-stranded DNA binding SYBR green 1 dye using LightCycler QRT-PCR (Roche Diagnostics). All lake water samples were amplified three times. The Ct values were determined by the second derivative maximum method of LightCycler software (version 3.5). Copy numbers of *mcy*E gene of the lake water samples were determined by converting obtained Ct values into the *mcy*E copy numbers according to the regression equations of the external standards that gave the highest (*Anabaena* 202A1 and *Microcystis* PCC7941) and lowest (*Anabaena* 315 and *Microcystis* PCC7806) *mcy*E copy numbers (Figs. 9A and B).

Amplification efficiencies, e ($e = 10^{-1/S} - 1$, s = slope of the linear regression), of the *Anabaena* and *Microcystis* *mcy*E QRT-PCR with standard strains were calculated as a function of known *mcy*E copy numbers and with those of Lake Tuusulanjärvi DNA samples as a function of different dilutions of the samples.

In order to determine melting temperatures for the amplification products of the standard strains and of the lake water samples, temperature was raised after QRT-PCR from 65°C to 95°C and fluorescence was detected continuously. Characteristic melting temperatures of the *mcy*E QRT-PCR products were determined with LightCycler software (version 3.5).

Microcystin analysis of the strains and lake water samples. Dry weight of the *Anabaena*, *Microcystis*, *Planktothrix* and *Nostoc* strains was measured and microcystin was extracted by sonication as detailed previously (Repka et al., 2001). Microcystin concentration of the strains was analyzed with an Agilent 1100 Series high performance liquid chromatograph with a diode array detector and Luna 5 μm C18 column (150 x 2 mm, Phenomenex). A mobile phase was 10 mM ammonium acetate and acetonitrile. During 6 to 40 minutes,

concentration of acetonitrile increased from 24% to 60%. Flow rate was 0.2 ml min^{-1} at 40°C, injection volume 20 µl, and detection at 238 nm. Purified microcystin-LR was used as a standard and microcystins were identified by their UV spectra and retention times.

5 Total microcystin of the lake water samples was extracted from 5 ml of lake water using tip sonicator for 5 min (Braun Labsonic-U). Prior measuring microcystin concentration with EnviroGard® microcystins plate kit (Strategic Diagnostics Inc.) and plate spectrophotometer (Labsystems iEMS reader MF) samples were filtered through 0.2 µm Puradisc™ filters (Whatman) to remove the particles.

10

Statistical analysis. Spearman correlation coefficients between microcystin concentration (µg l^{-1}), *mcy*E copy numbers (copies ml^{-1}), and *Anabaena* as well as *Microcystis* cell numbers (cells ml^{-1}) of lake water samples were calculated with SAS® statistical software for Windows (SAS Institute Inc.).

15

Specificity of the primers. The *mcy*E gene primers (Table 6) were both genus and *mcy*E gene specific, since a single amplification product was observed when genomic DNA of microcystin producing *Anabaena* or *Microcystis* strain was used as a template in PCR with *Anabaena* or *Microcystis* genus specific primers (Table 7).

20

Detection range of *mcy*E copy numbers. The QRT-PCR was log-linear from 6.6×10^2 to 6.6×10^5 *mcy*E copies in a reaction when the genomic DNAs of the standard strains *Anabaena* 90, *Anabaena* 202A1, *Microcystis* GL 260735 or *Microcystis* PCC 7941 were used as a template and from 6.6×10^2 to 6.6×10^6 when those of standard strains *Anabaena* 315 or *Microcystis*
25 PCC 7806 were used (Figs. 9A and B). The lowest reliable *mcy*E copy numbers in Lake Tuusulanjärvi were 42, 84, 33, and 63 copies ml^{-1} when calculated with the regression equations of the standards *Anabaena* 315, *Anabaena* 202A1, *Microcystis* 7806, and *Microcystis* 7941. In Lake Hiidenvesi the lowest reliable *mcy*E copy numbers were ten times higher than in Lake Tuusulanjärvi, 420, 840, 330, and 630 copies ml^{-1} when calculated with
30 the same standards, respectively. One ng of genomic DNA of *Anabaena* and *Microcystis* standard strains contained 1.76×10^5 and 1.94×10^5 *mcy*E copies. The purity of these DNAs varied from 1.8 to 1.9.

The *mcy*E copy numbers of lake water. *Microcystis* *mcy*E copy numbers in Lake Tuusulan-

järvi were 11 to 91 times more abundant than those of *Anabaena mcy*E copy numbers calculated as a ratio of the average *mcy*E copy numbers obtained with *Anabaena* 315, *Anabaena* 202A1, *Microcystis* PCC 7941 and *Microcystis* PCC 7806 standards (Fig. 10). *Microcystis mcy*E copy numbers were also more abundant than those of *Anabaena* in the Basin of Kiihkelyksenselkä of Lake Hiidenvesi (Fig. 11). In the Basins of Nummelanselkä and in Mustionselkä *Microcystis* and *Anabaena mcy*E copy numbers were quite similar (Fig. 11). In the Basin of Kirkkojärvi both *Microcystis* and *Anabaena mcy*E copy numbers were below the detection limits determined with the standards (Fig. 11). In Lake Hiidenvesi (Fig. 11) the average *mcy*E copy numbers of *Anabaena* and *Microcystis* as well as microcystin concentrations were lower than in Lake Tuusulanjärvi (Fig 11). Microcystin concentration had a statistically significant positive correlation with *Microcystis mcy*E copy numbers of all studied samples within the *mcy*E copy number detection range determined with the standards (Table 8).

Amplification efficiency. With Lake Tuusulanjärvi water samples the *Microcystis mcy*E QRT-PCR amplification efficiencies (0.78 – 0.99, Table 4) were similar to the amplification efficiencies of the *Microcystis* standards (0.86 – 0.94, Table 4). However, *Anabaena mcy*E QRT-PCR amplification efficiencies with Lake Tuusulanjärvi water samples (1.14 to 2.36, Table 4) were unrealistic high compared to the amplification efficiencies of the *Anabaena* standard strains (0.96 – 0.99, Table 9).

Melting curve analysis. Characteristic melting temperatures of the *mcy*E QRT-PCR products (247 bp) of the three *Anabaena* (average = 79.6°C, CV = 0.4%, n = 38, Table 5) and three *Microcystis* (average = 81.5°C, CV = 0.2%, n = 38, Table 5) standard strains corresponded to the melting temperatures of *Anabaena* (average = 79.3°C, CV = 0.3%, n = 58) and *Microcystis* (average = 81.7°C, CV = 0.2%, n = 63) *mcy*E QRT-PCR products amplified with lake water samples (data not shown). The 1.9°C difference in the average characteristic melting temperatures was due to over 40 nucleotide difference between *Anabaena* and *Microcystis mcy*E sequences.

Primer dimers were detected in *Anabaena* and in *Microcystis mcy*E QRT-PCR with negative controls and in *Anabaena mcy*E QRT-PCR with lake water samples that had low template DNA concentration, although hot start Taq DNA polymerase provided by the manufacturer of the kit was used. The error caused by the primer dimers was avoided by measuring

fluorescence of *Anabaena* and *Microcystis mcy*E QRT-PCR amplification at higher temperature (77°C, 78°C, respectively) than the melting temperature of the primer dimers.

Microcystin concentration and cyanobacterial cell density of lake water. Microcystin concentrations as well as *Anabaena* and *Microcystis* cell densities were highest in Lake Tuusulanjärvi on July and started to decrease thereafter (Figs. 10 and 12). In Lake Hiidenvesi microcystin concentrations and cell densities were lower than those in Lake Tuusulanjärvi (Figs. 11 and 13). According to microscope analysis, *Microcystis* cells were more abundant than *Anabaena* cells in Lake Tuusulanjärvi whereas *Microcystis* cells were observed only occasionally in Lake Hiidenvesi. *Anabaena* was the most dominant genus in the Basins of Kirkkojärvi and Mustionselkä of Lake Hiidenvesi whereas *Aphanizomenon* was the most dominant genus in the Basins of Nummelanselkä and Kiihkelyksenselkä of Lake Hiidenvesi as well as in the Lake Tuusulanjärvi.

Table 6. Primers used in this study.

Primer	Sequence (5' to 3')	
mcyE-F2	GAA ATT TGT GTA GAA GGT GC *	(SEQ ID NO 64)
AnamcyE-12R	CAA TCT CGG TAT AGC GGC	(SEQ ID NO 65)
MicmcyE-R8	CAA TGG GAG CAT AAC GAG	(SEQ ID NO 66)

* Forward primer, mcyE-F2, used in this study, was described in Example 2

Table 7. Specificity of *Anabaena* (mcyE-F2, AnamcyE-12R) and *Microcystis* (mcyE-F2, MicmcyE-R8) microcystin synthetase E (*mcy*E) primers was studied using *Anabaena, Microcystis, Planktothrix*, and *Nostoc* strains. Presence (+) or absence (-) of the *mcy*E product. Microcystin (MC) production (+) or lack of production (-). Accession numbers indicate *mcy*E sequences available in GenBank. Culture collections: PCC, Pasteur Culture Collection, Paris, France; NIVA-CYA, Norwegian Institute for Water Research, Oslo, Norway; NIES, National Institute for Environmental Studies, Tsukuba, Japan.

Genus Strain	MC	*Anabaena* *mcy*E primers	*Microcystis mcy*E primers	Accession No	Reference
Anabaena					
66A	+	+	-	XX	47, b
90	+	+	-	AJ536156	47, a
202A1	+	+	-	XX	47, b
202A2/41	+	+	-	XX	47, b

NIVA-CYA83/1	+	+	-	XX	47,b
315	+	+	-	XX	b
318	+	+	-	XX	b
86	-	-	-		46
123	-	-	-		46
14	-	-	-		46
PCC 6309	-	-	-		43
PCC 7108	-	-	-		43
PCC 73105	-	-	-		43
PCC 9208	-	-	-		43
Microcystis					
98	+	-	+	XX	47, b
205	+	-	+	XX	47, b
GL 260735	+	-	+	XX	55, b
GL 280646	+	-	+	XX	55, b
IZANCYA 5	+	-	+	XX	53, b
IZANCYA 25	+	-	+	XX	53, b
NIES102	+	-	+	XX	29, b
NIES A 89	+	-	+	XX	29, b
PCC 7941	+	-	+	XX	43, b
PCC 7806	+	-	+	AF183408	43, 51
130	-	-	-		44
269	-	-	-		44
GL 060916	-	-	-		55
Planktothrix					
49	+	-	-	XX	47, b
97	+	-	-	XX	47, b
213	+	-	-		47
NIVA-CYA 126	+	-	-	AJ441056	9, 47
NIVA-CYA 127	+	-	-	XX	47, b
NIVA-CYA 128/R	+	-	-	XX	47, b
45	-	-	-		44
PCC 6304	-	-	-		43
Nostoc					
152	+	-	-	XX	48, b

a) Example 1

b) Example 2

(9) Christiansen et al. 2003, (29) Lyra et al., 2001, (43) Rippka and Herdman, 1992, (44) Rouhiainen et al. 1995,(46) Sivonen and Jones, 1999, (47) Sivonen et al. 1989, (48) Sivonen et al. 1995, (53) Vasconcelos et al. , 1995, (55) Vezie et al. 1998,

Table 8. Spearman correlation coefficients between microcystin concentration ($\mu g\ l^{-1}$) and microcystin synthetase E (*mcy*E) copy numbers (copies ml^{-1}) calculated using different standards (*Anabaena* 202A1, *Anabaena* 315, *Microcystis* PCC 7806 and *Microcystis* PCC7941) and cell numbers (cells ml^{-1}) in Lake Tuusulanjärvi and Lake Hiidenvesi. Sum of *Anabaena* and *Microcystis mcy*E copy numbers was counted by adding the average copy numbers calculated using the two *Anabaena* and *Microcystis* standards. Number inside the parenthesis shows the number of samples used to calculate the spearman correlation.

Lake water samples	Anabaena McyE		Microcystis mcyE		Sum of *Anabaena* and *Microcystis* *mcy*E	*Microcystis* cells	*Anabaena* cells	*Microcystis* *Anabaena* cells
	202 A1	315	PCC 7806	PCC 7941				
All samples			0.57*	0.57*	0.52, p=0.10			
	(11)	(11)	(15)	(15)	(11)	(21)	(21)	(21)
Lake	1***	1***						0.86 *
Tuusulanjärvi	(5)	(5)	(6)	(6)	(5)	(7)	(7)	(7)
Lake Hiidenvesi	(6)	(6)	(9)	(9)	(6)	(14)	(14)	(14)

• : p < 0.5, ** : p < 0.1, *** : p < 0.01

Table 9. *Anabaena* and *Microcystis mcy*E QRT-PCR amplification efficiencies, e ($e = 10^{-1/S} - 1$, S = slope of linear regression equation), of the external standard strains calculated as a function of *mcy*E copy numbers and those of Lake Tuusulanjärvi water samples calculated as a function of different dilutions of the samples. r^2 denotes coefficient of determination.

Strain or Sampling date	Amplification efficiency	S	r^2	*mcy*E copy numbers or Dilution factors
Microcystis				
GL 260735	0.86	-3.71	1	6.6×10^2, 6.6×10^3, 6.6×10^4, 6.6×10^5

PCC 7806	0.92	-3.53	1	6.6 x 10^2, 6.6 x 10^3, 6.6 x 10^4, 6.6 x 10^5, 6.6 x 10^6
PCC 7941	0.94	-3.47	1	6.6 x 10^2, 6.6 x 10^3, 6.6 x 10^4, 6.6 x 10^5
12-Jul	0.95	-3.46	1	1, 0.1, 0.05, 0.01, 0.005
2-Aug	0.97	-3.39	1	1, 0.1
23-Aug	0.99	-3.34	1	1, 0.1
7-Sep	0.80	-3.92	1	1, 0.1
20-Sep	0.78	-3.99	1	1, 0.1
6-Oct	0.88	-3.66	1	1, 0.1
Anabaena				
90	0.96	-3.41	1	6.6 x 10^2, 6.6 x 10^3, 6.6 x 10^4, 6.6 x 10^5
315	0.99	-3.34	1	6.6 x 10^2, 6.6 x 10^3, 6.6 x 10^4, 6.6 x 10^5, 6.6 x 10^6
202A1	0.98	-3.36	1	6.6 x 10^2, 6.6 x 10^3, 6.6 x 10^4, 6.6 x 10^5
12-Jul	1.32	-2.74	1	1, 0.1, 0.05
2-Aug	1.14	-3.02	1	1, 0.1, 0.05
23-Aug	1.32	-2.74	1	1, 0.1
7-Sep	2.36	-1.90	0.98	1, 0.1, 0.05

Table 10. Characteristic melting temperatures ($T_m \pm$ CV%) of the microcystin synthetase E quantitative real-time PCR amplification products (247 bp) obtained using LightCycler melting curve analysis. Nucleotide differences were calculated for the 209 bp long sequence between the primer annealing sites. Number of samples is denoted by n.

	$T_m \pm$ CV%	n	Nucleotide differences					
			Anabaena			*Microcystis*		
Strain			90	315	202 A1	GL 26 0735	PCC 7806	PCC 7941
Anabaena								
90	79.7 ± 0.2	12						
315	79.3 ± 0.4	14	0					
202A1	79.7 ± 0.2	12	1	1				
Microcystis								
GL 260735	81.3 ± 0.2	12	45	45	46			
PCC 7806	81.5 ± 0.2	15	47	47	48	2		
PCC 7941	81.5 ± 0.1	11	47	47	48	2	1	

Example 4

We were interested in the *mcyD* gene region as part of an evolutionary study on microcystin synthetase genes from different genera of cyanobacteria.

The *McyD* gene is involved in the formation of the Adda amino acid and this amino acid along with D-glutamate is critical to microcystin toxicity (Goldberg, J., Huang, H-B., Kwon, Y-G., Greengard, P., Nairn, A.C. et al. Three-dimensional structure of the catalytic subunit of protein serine/threonine phosphatase-1. Nature 376, 745-753 (1995). The Adda amino acid is proposed to be assembled by McyG, McyD and McyE (Tillett, D. et al. Structural organization of microcystin biosynthesis in *Microcystis aeruginosa* PCC7806: an integrated peptide-polyketide synthetase system. Chem. Biol. 7, 753-764 (2000). The mcyD gene region we sequenced encodes parts of a beta-ketoacyl synthase and a acyltransferase domain (Tillett et al. 2000). The region we looked at is specifically involved in one round of chain elongation

of the growing Adda amino acid (Tillett et al. 2000).

The 818 bp region of the *mcyD* gene was amplified with the mcyDF (5'-gatccgattgaattagaaag-3') and mcyDR (5'-gtattccccaagattgcc-3') primers. PCR reactions for the mcyD PCR products were performed in a 20 ml final volume containing 1 ml of DNA, 1 x DynaZyme II PCR buffer, 250 mM of each deoxynucleotide, 0.5 mM of both PCR primers, and 0.5 U of DynaZyme II DNA polymerase (Finnzymes, Espoo, Finland). The following thermocycle protocol was used: 95°C, 3 min; 30 x (94°C, 30 sec; 56°C, 30 sec; 72°C, 1 min); 72°C, 10 min. Sequencing of the mcyD PCR products was performed by Genome Express (France).

Example 5

Oligonucleotides for detection and identification of toxic cyanobacteria

Materials and Methods

All chemicals and solvents were purchased from Sigma-Aldrich (Italy) and used without further purification. Oligonucleotides were purchased from Interactiva Biotechnologie GmbH (Germany).

DNA samples

The samples used to validate the probes were *Anabaena* 202A1, *Microcystis* 205, *Planktothrix* 49, *Nostoc* 152 and the environmental samples 0TU35 (>10 um fraction) and 0TU33 (bloom sample).

Ligation probe design

For Ligation Detection Reaction, we designed specific probes for the *mcy*E sequences of five different groups. These groups were identified using a phylogenetic tree obtained from the ARB software, version Beta 011107.

ARB (www.arb-home.de) is a UNIX-based program for aligning a large number of DNA sequences and for constructing phylogenetic trees according to a central database of processed sequences.

5 The *mcy*E sequences were aligned using CLUSTAL W (Thompson et al., 1994) and internal ARB algorithms. The phylogenetic tree was constructed using the neighbor-joining (NJ) algorithm (Saitou and Nei, 1987). The groups are the following: *Anabaena, Microcystis, Nodularia, Nostoc, Oscillatoria/Planktothrix (OP).*

10 From the sequence alignment a "group-specific" consensus sequence was obtained with a cutoff percentage of 95%. This value is compared with the frequency of the residues found at each alignment position. If the residue at a given position occured at a lower frequency than the cutoff percentage, an IUPAC ambigous symbol was displayed in the consensus sequence. Then, group-specific probe design was obtained using a tool on ARB database named "Probe
15 design".

All oligonucleotides were designed to have a melting temperature (T_m) between 64 and 68°C.

Discriminating probes were purchased with a Cy3 label at their 5' terminal position and
20 common probes with a phosphate in the same position.

Universal Array preparation

Microarrays were prepared using CodeLink™ slides (Amersham Biosciences), designed to covalently immobilize NH_2-modified oligonucleotides.

25

5' amino-modified Zip Code oligonucleotides, carrying an additional poly$(dA)_{10}$ tail at their 5' end, were diluted to 25 μM in 100 mM phosphate buffer (pH 8.5). Spotting was performed using a non contact piezo driven dispensing system (Nanoplotter, GeSim, Germany). Printed slides were processed according to the manufacturer's protocols.

30

Quality control of printed surfaces was performed by sampling one slide from each deposition batch. The printed slide was hybridized with 1 μM 5' Cy3 labeled poly$(dT)_{10}$ in a solution containing 5X SSC and 0.1 mg/ml salmon sperm DNA at RT for 2 h, then washed for 15 min

in 1xSSC. The fluorescent signal was controlled by laser scanning following procedures described in "Array hybridization, detection and data analysis".

PCR amplifications from DNA samples.

5

Ligation Detection Reaction.

Ligation Detection Reaction was carried out in a final volume of 20 µl containing 20 mM Tris-HCl (pH 7.5), 20 mM KCl, 10 mM $MgCl_2$, 0.1% NP40, 0.01mM ATP, 1 mM DTT, 2
10 pmol of each discriminating probe, 2 pmol of each common probe and 100 fmol of purified PCR products. The reaction mixture was preheated for 2 min at 94°C and spinned in a microcentrifuge for 1 min; then 1 ul of 4U/ul Pfu DNA ligase (Stratagene, La Jolla, California) was added. Alternatively, 0,5 ul of 50U/ul Tth DNA ligase (ABgene) was used.

15 The LDR was cycled for 30 rounds of 90°C for 30 sec and 60°C for 4 min in the GeneAmp PCR system 9700 thermal cycler (Applied Biosystems, California).

Array hybridization, detection and data analysis.

In a 0.5-ml microcentrifuge tube, the LDR mix (20 µl) was diluted to obtain 65 µl of
20 hybridization mixture containing 5X SSC and 0.1 mg/ml salmon sperm DNA. The mix, after heating at 94°C for 2 min and chilling on ice, was applied onto the slide under a hybridization chamber.

Hybridization was carried out in the dark at 65°C for two hours in a temperature-controlled
25 water bath. After hybridization, the microarray was washed at 65°C for 15 min in pre-warmed 1X SSC, 0.1% SDS. Finally, the slide was spinned at 80 g for 3 min.

The fluorescent signals were acquired at 5 µm resolution using a ScanArray® 4000 laser scanning system (PerkinElmer Life Sciences) with green laser for Cy3 dye (λ_{ex} 543 nm/λ_{em}
30 570 nm). Both the laser and the photomultiplier (PMT) tube power were set at 70-95 %.
To quantitate the fluorescent intensity of the spots we used the QuantArray Quantitative Microarray Analysis software (Perkin Elmer Life Sciences).

Recently, we have presented a Universal DNA Array approach to discriminate some groups of bacteria (Busti et al., 2002). This procedure, based on the discriminative properties of the DNA ligation reaction, requires the design of two probes specific for each target sequence, as described by Barany and co-workers (1999). One oligonucleotide brings a fluorescent label and the other a unique sequence named complementary Zip Code (cZip Code). Ligated fragments, obtained in presence of a proper template by the action of a DNA ligase, are addressed to the location on the microarray where the Zip Code sequence has been spotted. Such an array is therefore "Universal" being unrelated to a specific molecular analysis.

Here we present the Universal DNA Array approach applied to the detection of cyanobacterial *mcy*E gene diversity.

Ligation Probes Design

We used the ARB software to perform the sequence alignment of cyanobacterial *mcy*E sequences. These sequences were aligned and clustered according to their phylogenetic lineages so that 5 "group-specific" consensus sequences were yielded: *Anabaena, Microcystis, Nodularia, Nostoc, Oscillatoria/Planktothrix (OP)* (Figure 14). Then, "group-specific" probes were designed using a tool on ARB database named "Probe design". Among this set of probes, we selected discriminating probes with 3' position unique to each group in order to obtain ligase discrimination. As a matter of fact, after hybridization of a discriminating probe and a common probe to the target sequence, ligation occurs only if there is perfect complementarity at the junction between the two oligos. Common probes were designed immediately 3' to the discriminating oligo from the group-specific consensus.

All the selected probes are described in Figure 20. We selected one probe pair for each group of interest, except for the *Oscillatoria/Planktothrix* group.

Figure 15 shows the alignment of the "group-specific" consensus sequences and the relative discriminating probes.

Zip codes assignment and Quality Control of the Universal Array

We randomly selected 6 Zip code sequences from those described by Chen and co-workers, 2000. Each Zip code was randomly assigned to a single cyanobacterial group. Each common probe was synthesized to have the complementary Zip code (cZip code) affixed to its 3' end

(Figure 20). No significant self-annealing of the common probe-cZip sequences was detected by computer analysis (data not shown).

The Zip codes were deposited using a non contact deposition system. The deposition scheme is shown in Figure 17. In order to verify the deposition quality of the Zip Code oligonucleotides on the slides, we performed hybridisations with Cy3 labelled poly(dT) complementary to the poly$(dA)_{10}$ sequence of each Zip Code. Every controlled slide revealed intense fluorescent signals corresponding the spotted oligonucleotides, as shown in Figure 17. This result indicated a rather uniform deposition of the oligos on the Universal Array.

LRD detection onto Universal Array

1) Probes specificity

The specificity of the probes for *mcy*E cyanobacterial groups was tested using PCR amplified fragment of this gene coming either from pure strains or from environmental samples, as indicated in Materials and Methods.

LDRs were conducted in the presence of the PCR product of each single sample as template and in the presence of all the probes (discriminating probes and common probes).
A negative control of the entire process was performed using double distilled water instead of genomic DNA as PCR substrate. After standard cycling, ten microliters of the reaction mixture were used in the LDR. Following hybridisation on the universal chip, no signal was detected even setting PMT and laser to 95% of their power (data not shown).

In the presence of the proper DNA template, the Universal Array behaved as expected: only group-specific spots showed positive signal. The results are showed in Figure 18.

2) Probe sensitivity

In order to establish the detection limit of the method, we performed the Ligation Detection Reaction starting from 50, 5 and 1 fmol of three different PCR products as substrates. The detected signals progressively decrease and three visible signals were detected up to 1 fmol of the PCR products. No signals were detected using 0,5 fmol of the substrates even setting PMT and laser to 95% of their power (data not shown).

Example 6

Molecular analysis of cyanobacterial diversity by microarrays on "PCR-amplified" 16 rRNA gene

5

All chemicals and solvents were purchased from Sigma-Aldrich (Italy) and used without further purification. Oligonucleotides were purchased from Interactiva Biotechnologie GmbH (Germany).

10 **DNA samples**

The samples used to validate the probes included axenic strains kept in the authors' culture collections, strains isolated from European lakes and a reservoir during this study, and clones of environmental DNA libraries obtained from Lake Esch-sur-Sûre (Luxembourg) and Lake Tuusulanjärvi (Finland). The 16S rRNA gene of the cultured strains and clones was sequenced 15 (unpublished data). In addition, the array was tested with an environmental DNA sample (Lake Tuusulanjärvi), which was isolated with the hot-phenol method. To verify the microarray results, the same environmental sample was analyzed with DGGE and cloning of the 16S rRNA gene.

20 **Ligation probe design**

For Ligation Detection Reaction, we designed specific probes for the 16S rRNA gene sequences of different cyanobacterial groups. These groups were identified using a cyanobacterial 16S rRNA gene tree obtained from the ARB software, version Beta 011107. ARB (www.arb-home.de) is a UNIX-based program for aligning a large number of 16S 25 rRNA gene sequences and for constructing phylogenetic trees according to a central database of processed sequences. ARB cyanobacterial 16S rDNA database we used contained 281sequences from public databases and 57 from this study, in addition to the outgroup *Escherichia coli*. All these sequences were longer than 1400 bp, except the two sequences of Antarctic *Phormidium* (about 1350 bp) and 21 (out of 42) sequences of *Prochlorococcus* 30 *marinus* (about 1250 bp). All sequences were aligned with CLUSTAL W (24) and ARB.. The phylogenetic tree was constructed using the neighbor-joining (NJ) algorithm (Saitou and Nei, 1987). As shown in Figure 25, the selected cyanobacterial groups are the following: *Anabaena/Aphanizomenon, Calothrix, Cylindrospermopsis, Cylindrospermum, Gloeothece, Halotolerants, Leptolyngbya,* Palau *Lyngbya, Microcystis, Nodularia, Nostoc,*

Oscillatoria/Planktothrix, Antarctic *Phormidium, Prochlorococcus, Spirulina, Synechococcus, Synechocystis, Trichodesmium, Woronichinia.*

From the sequence alignment a "group-specific" consensus sequence was obtained with a cutoff percentage of 75%. This value is compared with the frequency of the residues found at each alignment position. If the residue at a given position occured at a lower frequency than the cutoff percentage, an IUPAC ambigous symbol was displayed in the consensus sequence.
Then, the 19 group consensus sequences were imported in GCG Omiga 2.0 (Oxford Molecular Ltd.) for group-specific probe design. The specificity of each probe pair (discriminating probe and common probe) was controlled on the entire bacterial 16S rDNA ARB database. All oligonucleotides were designed to have a melting temperature (T_m) between 64 and 68°C.

Discriminating probes were purchased with a Cy3 label at their 5' terminal position and common probes with a phosphate in the same position.

Universal Array preparation

Microarrays were prepared using CodeLink™ slides (Amersham), designed to covalently immobilize NH_2-modified oligonucleotides.
5' amino-modified Zip Code oligonucleotides, carrying an additional poly$(dA)_{10}$ tail at their 5' end, were diluted to 25 µM in 100 mM phosphate buffer (pH 8.5). Spotting was performed using a contact dispensing system MicroGrid II (BioRobotics). Printed slides were processed according to the manufacturer's protocols. 8 subarrays per slide were generated.
Quality control of printed surfaces was performed by sampling one slide from each deposition batch. The printed slide was hybridized with 1 µM 5' Cy3 labeled poly$(dT)_{10}$ in a solution containing 5X SSC and 0.1 mg/ml salmon sperm DNA at RT for 2 h, then washed for 15 min in 1xSSC. The fluorescent signal was controlled by laser scanning following procedures described in "Array hybridization, detection and data analysis".

PCR amplifications from DNA samples.

The DNA region coding for 16S ribosomal RNA was amplified with a universal primer 16SF27 (5'AGAGTTTGATCMTGGCTCAG 3') (Edwards et al., 1989) and a cyanobacterial

specific primer 23S30R (5'CCTCGCCTCTGTGTGCCTAGGT3') (Lepère et al., 2000) which permitted the amplification of a ca 2000 bp fragment.

PCR amplifications were performed in a GeneAmp PCR system 9700 thermal cycler (Applied Biosystem, California). The reaction mixtures include 500 nM each primer, 200 µM each dNTP, 10 mM Tris-HCl (pH 8.8), 1.5 mM $MgCl_2$, 50 mM KCl, 0.1% (wt/vol) Triton X-100, 1U of DynaZyme DNA polymerase (Finnzymes OY, Espoo, Finland) and 5-8 ng of genomic DNA, in a final volume of 50 µl. Prior to amplification, DNA was denatured for 5 min at 95°C. Amplification consisted of 30 cycles of 94°C for 45 s, 57°C for 45 s and 72°C for 2 min. After the cycles, an extension step (10 min at 72°C) was performed.

The PCR products were purified by GFX PCR DNA purification kit (Amersham Biosciences, Piscataway-NJ), eluted in 50 µl of autoclaved water and quantified by the BioAnalyzer 2100 (Agilent Technologies).

Ligation Detection Reaction

Ligation Detection Reaction was carried out in a final volume of 20 µl containing 20 mM Tris-HCl (pH 7.5), 20 mM KCl, 10 mM $MgCl_2$, 0.1% NP40, 0.01mM ATP, 1 mM DTT, 250 fmol of each discriminating probe, 250 fmol of each common probe, 10 fmol of the hybridization control and 25 fmol of purified PCR products. The reaction mixture was preheated for 2 min at 94°C and spinned in a microcentrifuge for 1 min; then 1 ul of 4U/ul Pfu DNA ligase (Stratagene, La Jolla, California) was added. The LDR was cycled for 30 rounds of 90°C for 30 sec and 60°C for 4 min in the GeneAmp PCR system 9700 thermal cycler (Applied Biosystems, California).

Array hybridization, detection and data analysis.

In a 0.5-ml microcentrifuge tube, the LDR mix (20 µl) was diluted to obtain 65 µl of hybridization mixture containing 5X SSC and 0.1 mg/ml salmon sperm DNA. The mix, after heating at 94°C for 2 min and chilling on ice, was applied onto the slide in the Press-To-Seal Silicone Isolators 1.0 x 9 mm (Schleicher & Schuell).

Hybridization was carried out in a hybridization chamber in the dark at 65°C for two hours in a temperature-controlled water bath. After hybridization, the microarray was washed at 65°C for 15 min in pre-warmed 1X SSC, 0.1% SDS. Finally, the slide was spinned at 80 g for 3 min..

The fluorescent signals were acquired at 5 μm resolution using a ScanArray® 4000 laser scanning system (PerkinElmer Life Sciences) with green laser for Cy3 dye (λ_{ex} 543 nm/λ_{em} 570 nm). Both the laser and the photomultiplier (PMT) tube power were set at 70-95 %.
To quantify the fluorescent intensity of the spots we used the QuantArray Quantitative Microarray Analysis software (Perkin Elmer Life Sciences).

When statistical analyses were performed, we included the fluorescent intensity values obtained from replicated spots (four replicates spot for each group, eight replicates spot for the universal) and replicates experiments sets (three LDR-universal array experiments).

Sequence Analysis of Cyanobacterial 16S rDNA and Ligation Probes Design

We used the ARB software to perform the sequence alignment of cyanobacterial 16S rDNA. The ARB database we used contained 281 cyanobacterial sequences from public databases and 57 from this study. These sequences were aligned and clustered according to their phylogenetic lineages so that 19"group-specific" consensus sequences were yielded (Figure 25).

Then, the 19 group consensi were imported in GCG Omiga 2.0 (Oxford Molecular Ltd.). The Omiga software is a graphically oriented package that permits the identification of "group-specific" nucleotide polymorphisms. Thus, the probes were designed complementary to polymorphic regions on the basis of a final alignment among group-specific consensi. The selection process consisted in several steps. Firstly, we considered the ligase reaction features. As shown in Figure 26, after hybridization of a discriminating probe and a common probe to the target sequence, ligation occurs only if there is perfect complementarity at the junction between the two oligos. For this reason, to obtain ligase discrimination, we selected discriminating probes with 3' position unique to each group. Common probes were designed immediately 3' to the discriminating oligo from the group-specific consensus.

Secondly, among this set of probes, we selected only those pairs of probes, which differed from all representatives of the other groups at least for the 3' terminal position of the discriminating probes, but which were invariant in all members of their group. Examples of probe design procedure are shown in Figure 27.

Finally, in order to discard potentially aspecific probe pairs, we analyzed each probe pair (discriminating probe and common probe) using a tool on ARB database, which permit to verify probes against all the bacterial 16S rRNA gene sequences. Initially, we considered 60 group specific probe pairs, but only 21 of these have been chosen after the selection step described above.

All the selected probes are described in Figure 32. When the consensus sequence contains a degenerate base, we included inosine during oligonucleotide synthesis at these degenerate positions.

Although DNA samples for some of the 19 selected groups (i.e. *Gloeothece, Antarctic Phormidium, Prochlorococcus marinus, Trichodesmium*) were not available because these cyanobacteria are not present in the lakes under scrutiny, all the ARB phylogenetic lineages have been considered in the experimental set-up to allow for future applications of this cyanobacterial microarray.

In order to have a positive control for the Ligation Detection Reaction, a universal probe pair, matching all the cyanobacteria, was designed and the corresponding Zip code was included in the Universal Array. As a positive control for the hybridisation reaction, a Cy3 labelled complementary Zip Code sequence was added in the hybridization mixture and the corresponding Zip code was included in the Universal Array.

Zip Codes assignment and Quality Control of the Universal Array

We randomly selected 21 Zip code sequences from those described by Barany and coworkers and Chen and co-workers . Each Zip code was randomly assigned to a single cyanobacterial group, except Zip code1 which is the positive control for the hybridisation reaction.

Each common probe was synthesized to have the complementary Zip code (cZip code) affixed to its 3' end (Figure 32 and 39). No significant self-annealing of the twenty common probe-cZip sequences was detected by computer analysis (data not shown).

5 The Zip codes were deposited using a contact deposition system generating 8 subarrays per slide. The deposition scheme is shown in Figure 28. In order to verify the deposition quality of the Zip Code oligonucleotides on the slides, we performed hybridisations with Cy3 labelled poly(dT) complementary to the poly$(dA)_{10}$ sequence of each Zip Code.

10 **LDR detection onto Universal Array of Cyanobacterial 16S rDNA Samples**

1) Probes specificity

The specificity of the probes for freshwater cyanobacterial groups was tested using PCR amplified 16S rRNA gene coming either from pure strains (both axenic and isolated in this
15 study) or from cloned rDNA sequences. All pure strains used to validate the LDR probes are described in Figure 33. The sequences obtained from the clones have been aligned in the ARB database with the sequences of pure cyanobacterial strains in order to define their phylogenetic group. The clones used are described in Figure 34.

20 LDRs were conducted in the presence of the PCR product of each single strain or clone as template and in the presence of all the probes (discriminating probes and common probes).

A negative control of the entire process was performed using double distilled water instead of genomic DNA as PCR template. After standard cycling, ten microliters of the reaction
25 mixture were used in the LDR. Following hybridisation on the Universal Array, no signal was detected even setting PMT and laser to 95% of their power (data not shown).

In the presence of the proper DNA template, the Universal Array behaved as expected: only group specific spots, universal spots and the spots corresponding to the hybridization control
30 showed positive signal. Some of the results are shown in Figure 29.

2) Probe sensitivity

In order to establish the detection limit of the method and the correlation between signal

intensity and template concentration, we performed Ligation Detection Reactions starting from 100 to 0,5 fmol of PCR products obtained from Planktothrix 1LT as substrates. The detected signals progressively decrease and a visible signal was detected up to 1 fmol of the PCR product. No signals were detected using 0,5 fmol of the substrates even setting PMT and laser to 95% of their power (data not shown). The linear correlation between signal intensity and template concentration is shown in Figure 31.

3) Use of artificial mixes of PCR products from different strains.

In order to determine the efficiency of the LDR method in presence of complex molecular targets, we used artificial mixes with unbalanced amounts of PCR products derived from the following cyanobacterial samples: Aphanizomenon sp. 202, Microcystis OBB 34S, Spirulina subsalsa PCC6313, Calothrix sp. PCC7714, clone Woronichinia OES46. After separate PCR reactions, the amplified fragments were pooled in unbalanced LDR mixes using different ratios: 100:1, 50:1, 100:5, 50:5. In all these experiments Aphanizomenon sp. 202 and Microcystis OBB 34S were the more concentated samples. Moreover, we mixed also 500 fmol of the amplicon derived from Microcystis OBB 34S with 5 fmol of the PCR fragment obtained from Woronichinia OES46 clone. After the hybridization of the LDR products onto the Universal Array, the signals related to the lower concentated template were not detected in the LDR mixes with these ratio: , 100:1 and 50:1. Only in presence of the LDR products obtained from the mixes with the ratio 100:5 and 50:5 all the expected signals are detected Figure 29. The fluorescent intensity of the spots was quantified and the results are shown in Figure 29. Furthermore, we compared also the resuls obtained using two LDR unbalanced mixes 100:1 (100 fmol of Microcystis OBB 34S and 1 fmol each of Spirulina, Woronichinia and Calothrix), in one of which 8U of Pfu DNA ligase was added, whereas the other was prepared using 4U of the enzyme, as described in Materials and Methods. Hybridization signals of the lower concentated substrates were detected only from the LDR product obtained using 8U of Pfu DNA ligase instead of 4U (Figure 30).

LDR detection onto Universal Array of 16S rDNA and mcyE from environmental samples

We made PCR amplification from genomic DNA using 16S cyanobacteria specific primers. The PCR conditions used are shown in Figure 35. We made also PCR amplification from genomic DNA using mcyE gene primers. The ligation detection reaction was made under the

same conditions by using an oligo mix containing both the probes for 16S rRNA gene and the probes for the mcyE gene as shown in Figure 36. Finally the hybridization was carried on the same Universal Array where the 16S rRNA LDR product and, mcyE LDR product were detected

Example 7

Microarray platform for toxic and non-toxic detection in cyanobacteria.

Materials and methods.

All chemicals and solvents were purchased from Sigma-Aldrich (Italy) and used without further purification. Oligonucleotides were purchased from Interactiva Biotechnologie GmbH (Germany).

Ligation probe design

The mcyE probe design has been previously described in Example 5 in "Ligation probe design". The 16S rRNA gene probe design has been previously described in Example 6 in "Ligation probe design", but was added the probe design for a further cyanobacteria group : Snowella. The Snowella probe design was performed using the updated ARB database containing 281 sequences from public databases and 69 from this study (Figure 25B). The updated database allowed to design specific probe for Aphanizomenon and Anabaena subgroups as shown in figure 25C. The probe design allows the detection of 20 toxic and non-toxic cyanobacteria groups.

Universal Array preparation

Microarrays were prepared using CodeLink™ slides (Amersham), designed to covalently immobilize NH_2-modified oligonucleotides.

5' amino-modified Zip Code oligonucleotides, carrying an additional poly(dA)$_{10}$ tail at their 5' end, were diluted to 25 µM in 100 mM phosphate buffer (pH 8.5). Spotting was performed using a contact dispensing system MicroGrid II (BioRobotics). Printed slides were processed according to the manufacturer's protocols. 8 subarrays per slide were generated.

The Universal array used for the detection of toxic and non-toxic cyanobacteria was designed to detect both the 16 rRNA and mcyE gene ligated probes. For this purpose the deposition scheme was improved as shown in Figure 27B. We generated 8 subarray per slide. Each

subarray is made of 208 spots including zipcodes for hybridization control, cyanobacterial universal probes, 16S rRNA gene specific probe, mcyE specific probe and empty spot as a negative control. Each specific zip code for the recognition of cyanobacteria universal probe, 16Ss RNA gene probe and mcyE gene probe is spotted in quadruplicate. The LDR positive control (zipcode n°63) is replicated 6 times, while the hybridization positive control (zipcode n°66) is replicated 8 times.

Quality control of printed surfaces was performed by sampling one slide from each deposition batch. The printed slide was hybridized with 1 µM 5' Cy3 labeled poly(dT)$_{10}$ in a solution containing 5X SSC and 0.1 mg/ml salmon sperm DNA at RT for 2 h, then washed for 15 min in 1xSSC. The fluorescent signal was controlled by laser scanning following procedures described in "Array hybridization, detection and data analysis".

PCR amplification from DNA samples

The PCR of mcyE gene and 16S rRNA gene were performed separately, using the conditions previously described in Examples 5 and 6 in "PCR amplification from DNA samples".

Ligation Detection Reaction

The Ligation Detection Reaction for toxic and non-toxic cyanobacteria detection was done mixing together the PCR product of 16S rRNA and mcyE gene and the discrimination and common probe specific for both 16s rRNA and mcyE gene, figure 36.

Ligation Detection Reaction was carried out in a final volume of 20 µl containing 20 mM Tris-HCl (pH 7.5), 20 mM KCl, 10 mM $MgCl_2$, 0.1% NP40, 0.01mM ATP, 1 mM DTT, 250 fmol of each discriminating probe, 250 fmol of each common probe, 10 fmol of the hybridization control and 25 fmol of purified PCR products. The reaction mixture was preheated for 2 min at 94°C and spinned in a microcentrifuge for 1 min; then 1 ul of 4U/ul Pfu DNA ligase (Stratagene, La Jolla, California) was added. The LDR was cycled for 30 rounds of 90°C for 30 sec and 60°C for 4 min in the GeneAmp PCR system 9700 thermal cycler (Applied Biosystems, California).

Array hybridization, detection and data analysis

In a 0.5-ml microcentrifuge tube, the LDR mix (20 µl) was diluted to obtain 65 µl of hybridization mixture containing 5X SSC and 0.1 mg/ml salmon sperm DNA. The mix, after

heating at 94°C for 2 min and chilling on ice, was applied onto the slide in the Press-To-Seal Silicone Isolators 1.0 x 9 mm (Schleicher & Schuell).

Hybridization was carried out in a hybridization chamber in the dark at 65°C for two hours in
5 a temperature-controlled water bath. After hybridization, the microarray was washed at 65°C for 15 min in pre-warmed 1X SSC, 0.1% SDS. Finally, the slide was spinned at 80 g for 3 min.

The fluorescent signals were acquired at 5 μm resolution using a ScanArray® 4000 laser
10 scanning system (PerkinElmer Life Sciences) with green laser for Cy3 dye (λ_{ex} 543 nm/λ_{em} 570 nm). Both the laser and the photomultiplier (PMT) tube power were set at 70-95 %.
To quantify the fluorescent intensity of the spots we used the QuantArray Quantitative Microarray Analysis software (Perkin Elmer Life Sciences).

15 When statistical analyses were performed, we included the fluorescent intensity values obtained from replicated spots (four replicates spot for each group, eight replicates spot for the universal) and replicates experiments sets (three LDR-universal array experiments).

Zip codes assignment and Quality Control of the Universal Array

20

We randomly selected 33 Zip code sequences from those described by Chen and co-workers, 2000. Each Zip code was randomly assigned to a single cyanobacterial group. Each common probe, for both 16S rRNA and mcyE gene recognitin, was synthesized to have the complementary Zip code (cZip code) affixed to its 3' end (Figure 20, 32 and 39). No
25 significant self-annealing of the common probe-cZip sequences was detected by computer analysis (data not shown).

The Zip codes were deposited using a contact deposition system. The deposition scheme is shown in Figure 27B. In order to verify the deposition quality of the Zip Code
30 oligonucleotides on the slides, we performed hybridisations with Cy3 labelled poly(dT) complementary to the poly$(dA)_{10}$ sequence of each Zip Code. Every controlled slide revealed intense fluorescent signals corresponding the spotted oligonucleotides, as shown in Figure 27B. This result indicated a rather uniform deposition of the oligos on the Universal Array.

LDR detection onto Universal Array of Cyanobacterial 16S rDNA and mcyE Samples

Probes specificity

The specificity of the probes was tested using PCR amplified 16S rRNA and mcyE gene coming from pure strains (both axenic and isolated in this study.)

A negative control of the entire process was performed using double distilled water instead of genom DNA as PCR template. After standard cycling, ten microliters of the reaction mixture were used in the LDR. Following hybridisation on the Universal Array, no signal was detected even setting PMT and laser to 95% of their power (data not shown).

In the presence of the proper DNA template of both 16S rRNA and *mcyE* genes, the Universal Array functioned very well: only group specific spots, universal spots and the spots corresponding to the hybridization control showed positive signal. Some of the results are shown in Figure 30B.

References

Altschul, S. F., T. L. Madden, A. A. Schäffer, J. Zhang, Z. Zhang, W. Miller, and D. J. Lipman. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic. Acids. Res. 25:3389-3402.

Amard, B. & Bertrand-Sarfati, J. Microfossils in 2000 Ma old cherty stromatolites of the Franceville Group, Gabon. *Precambrian Res.* 81 197-221 (1997).

Aparicio, J.F., Molnár, I., Schwecke, T., König, A., Haydock, S.F., Khaw, L.E., Staunton, J., Leadlay, P.F., 1996. Organization of the biosynthetic gene cluster for rapamycin in *Streptomyces hygroscopicus*: analysis of the enzymatic domains in the modular polyketide synthase. Gene 169, 9-16.

Baker, J. A., B. A. Neilan, B. Entsch, and D. B. McKay. 2001. Identification of cyanobacteria and their toxigenicity in environmental samples by rapid molecular analysis. Environ. Toxicol. 16:472-482.

Baker, J. A., B. Entsch, B. A. Neilan, and D. B. McKay. 2002. Monitoring changing toxigenicity of a cyanobacterial bloom by molecular methods. Appl. Environ. Microbiol. 68:6070-6076.

Becker, S., M. Fahrbach, P. Böger, and A. Ernst. 2002. Quantitative tracing, by *Taq* nuclease assays, of a *Synechococcus* ecotype in highly diversified natural population. Appl. Environ. Microbiol. 68:4486-4494.

Becker, S., P. Böger, R. Oehlmann, and A. Ernst. 2000. PCR bias in ecological analysis: a case study for quantitative *Taq* nuclease assays in analyses of microbial communities.

Appl. Environ. Microbiol.
66:4945-4953.

Boers, P., L. van Ballegooijen, and J. Uunk. 1991. Changes in phosphorus cycling in a shallow
5 lake due to food web manipulations. Freshwater Biol. 25:9-20.

Brocks, J.J., Logan, G.A., Buick, R., & Summons, R.E. Archean molecular fossils and the early rise of eukaryotes. *Science* 285,1033-1036 (1999).

10 Busti E, Bordoni R, Castiglioni B, Monciardini P, Sosio M, Donadio S, Consolandi C, Rossi Bernardi L, Battaglia C, De Bellis G. Bacterial discrimination by means of a universal array approach mediated by LDR (ligase detection reaction). BMC Microbiol 2:27 (2002).

Castenholz, R. W. 2001. Phylum BX. Cyanobacteria, oxygenic photosynhetic bacteria. p.
15 473-599. *In* D. R. Boone, R. W. Castenholz and G. M. Garrity (ed.), Bergey's manual of systematic bacteriology, 2nd edition, vol. 1. Springer-Verlag.

Cavalier-Smith T. Origins of secondary metabolism. *Ciba Found Symp*. 171, 64-80 (1992).

20 Chen, J. et al. A microsphere-based assay for multiplexed single nucleotide polymorphism analysis using single base chain extension. Genome Res. 10, 549-557 (2000).A clear presentation of the concept of generic 'tag' sequences as applied to SNP genotyping.

Chorus I., and L. Mur. 1999. Preventative measures. p. 235-273. *In* I. Chorus and J. Bartram
25 (ed.), Toxic cyanobacteria in water. E & FN Spon. London and New York.

Christiansen, G., Fastner, J., Erhard, M., Börner, T., Dittmann, E., 2003. Microcystin Biosynthesis in *Planktothrix*: Genes, Evolution, and Manipulation. J. Bacteriol. 185, 564-572.

30

DeMott, W. R. & Moxter, F. Foraging on cyanobacteria by copepods: responses to chemical defenses and resource abundance. *Ecology* 72, 1820-1834 (1991).

Dittmann, E., Neilan, B.A., Erhard, M., von Döhren, H., Börner, T., 1997. Insertional
35 mutagenesis of a peptide synthetase gene that is responsible for hepatotoxin production in the cyanobacterium *Microcystis aeruginosa* PCC7806. Mol. Microbiol. 26, 779-787.

Dittmann, E. *et al.* Altered expression of two light-dependent genes in a microcystin-lacking mutant of *Microcystis aeruginosa* PCC 7806. *Microbiol.* 147, 3113-3119 (2001).

40

Du, L., Sanchez, C., Chen, M., Edwards, D.J., Shen, B., 2000. The biosynthetic gene cluster for the antitumor drug bleomycin from *Streptomyces verticillus* ATCC15003 supporting functional interactions between nonribosomal peptide synthesis and a polyketide synthase. Chem. Biol. 7, 623-642.

45

Duitman, E.H., Hamoen, L.W., Rembold, M., Venema, G., Seitz, H., Saenger, W., Bernhard, F., Reinhardt, R., Schmidt, M., Ullrich, C., Stein, T., Leenders, F., Vater, J., 1999. The mycosubtilin synthetase of *Bacillus subtilis* ATCC6633: A multifunctional hybrid between a peptide synthetase, an amino transferase, and a fatty acid synthase. Proc Natl Acad Sci USA
50 96, 13294-13299.

Edwards, U., Rogall, T., Blöcker, H., Emde, M. & Böttger, E.C. Isolation and direct complete nucleotide determination of entire genes. Characterization of a gene coding for 16S ribosomal RNA. *Nucleic Acids Res.* 17, 7843-7853 (1989).

5 Ekman-Ekebom, M., M. Kauppi, K. Sivonen, M. Niemi, and L. Lepistö. 1992. Toxic cyanobacteria in some Finnish lakes. Environ. Toxicol. Wat. Qual. 7:201-213.

Falconer, I., J. Bartram, I. Chorus, T. Kuiper-Goodman, H. Utkilen, M. Burch, and G. A. Codd. 1999. Safe levels and safe practices. p. 155-178. *In* I. Chorus and J. Bartram (ed.),
10 Toxic cyanobacteria in water. E & FN Spon. London and New York.

Fastner, J., Erhard, M., von Döhren H., 2001. Determination of oligopeptide diversity within a natural population of *Microcystis* spp. (cyanobacteria) by typing single colonies by matrix-assisted laser desorption ionization-time of flight mass spectrometry. Appl. Environ.
15 Microbiol. 67, 5069-5076.

Felsenstein, J. *PHYLIP (Phylogenetic Inference Package)*, version 3.5c. (Department of Genetics, Univ. Washington, Seattle, 1993).

20 Fujii, K., Harada, K., Suzuki, M., Kondo, F., Ikai, Y., Oka, H., Carmichael, W. W., Sivonen K., 1996. Occurrence of novel cyclic peptides together with microcystins from toxic cyanobacteria, *Anabaena* species. In: Yasumoto, T., Oshima, Y., Fukuyo, Y. (Eds.), Harmful and Toxic Algal Blooms. Intergovermental Oceanographic Commission of UNESCO, Paris, pp. 559-562.
25
Gerry, N. P. et al. Universal DNA microarray method for multiplex detection of low abundance point mutations. J. Mol. Biol. 292, 251-262 (1999).

Gilroy, D.J., Kauffman, K.W., Hall, R.A., Huang, X. & Chu, F.S. Assessing potential health
30 risks from microcystin toxins in blue-green algae dietary supplements. *Environ. Health Perspect.* 108, 435-439 (2000).

Giovannoni, S. J., E. F. DeLong, T. M. Schmidt, and N. R. Pace. 1990. Tangential flow filtration and preliminary phylogenetic analysis of marine picoplankton. Appl. Environ.
35 Microbiol. 56:2572-2575.

Goldberg, J., Huang, H.B., Kwon, Y.G., Greengard, P., Nairn, A.C., Kuriyan, J., 1995. Three-dimensional structure of the catalytic subunit of protein serine/threonine phosphatase-1. Nature 376, 745-53.
40
Grüntzig, V., S. C. Nold, J. Zhou, and J. M. Tiedje. 2001. *Pseudomonas stutzeri* nitrite reductase gene abundance in environmental samples measured by real-time PCR. Appl. Environ. Microbiol. 67:760-768.

45 Gugger, M., C. Lyra, P. Henriksen, A. Couté, J.-F. Humbert, and K. Sivonen. 2002. Phylogenetic comparison of the cyanobacterial genera *Anabaena* and *Aphanizomenon*. Int. J. Syst. Evol. Microbiol. 52:1867-1880.

Hall, T.A. BioEdit: a user-friendly biological sequence alignment editor and analysis program
50 for Windows 95/98/NT. *Nucl. Acids. Symp. Ser.* 41, 95-98 (1999).

Harada, K.-I., F. Kondo, and L. Lawton. 1999. Laboratory analysis of cyanotoxins. p. 369-405. *In* I. Chorus and J. Bartram (ed.), Toxic cyanobacteria in water. E & FN Spon. London and New York.

5 Heckman, D.S. *et al.* Molecular evidence for the early colonization of land by fungi and plants. *Science* 293, 1129-1133 (2001).

Heid, C. A., J. Stevens, K. J. Livak, and P. M. Williams. 1996. Real time quantitative PCR. Genome Res. 6:986-994.

10

Herdman, M., M. Janvier, R. Rippka, and R.Y. Stanier. 1979. Genome size of cyanobacteria. J. Gen. Microbiol. 111:73-85.

Hisbergues, M., Christiansen, G., Rouhiainen, L., Sivonen, K. & Börner, T. PCR-based
15 identification of microcystin producing genotypes of different cyanobacterial genera: application to environmental samples. *Arch Microbiol.* 180, 402-410 (2003).

Hopwood, D.A. Genetic contributions to understanding polyketide synthases. *Chem. Rev.* 97, 2465-2497 (1997).

20

Ikeda, H., Nonomiya, T., Usami, M., Ohta, T., Omura, S., 1999. Organization of the biosynthetic gene cluster for the polyketide anthelmintic macrolide avermectin in *Streptomyces avermitilis*. Proc. Natl. Acad. Sci. USA 96, 9509-9514.

25 Kaebernick, M., T. Rohrlack, K. Christoffersen, and B.A. Neilan. 2001. A spontaneous mutant of microcystin biosynthesis: genetic characterization and effect on *Daphnia*. Environ. Microbiol. 3:669-679.

Kagan, R.M., Clarke, S., 1994. Widespread occurrence of three sequence motifs in diverse S-
30 adenosylmethionine-dependent methyltransferases suggests a common structure for these enzymes. Arch. Biochem. Biophys. 310,417-427.

Kohli, R.M., Trauger, J.W., Schwarzer, D., Marahiel, M.A., Walsh, C.T., 2001. Generality of peptide cyclization catalyzed by isolated thioesterase domains of nonribosomal peptide.
35 Biochemistry 40, 7099-7108.

Kotai, J., 1972. Instructions for preparation of modified nutrient solution Z8 for algae, publication B-11/69, Norwegian Institute for Water Research. Blindern, Oslo.

40 Kuiper-Goodman, T., Falconer, I. & Fitzgerald, J. in *Toxic Cyanobacteria in Water. A Guide to their Public Health Consequences, Monitoring and Management* (eds Chorus, I. & Bartram, J.) 113-153 (E and FN Spoon, London, 1999).

Kurmayer, R., E. Dittmann, J. Fastner, and I. Chorus. 2002. Diversity of microcystin genes
45 within a population of the toxic cyanobacterium *Microcystis* spp. in Lake Wannsee (Berlin, Germany). Microb. Ecol. 43:107-118.

Labarre, J., F. Chauvat, and P. Thuriaux. 1989. Insertional mutagenesis by random cloning of antibiotic resistance genes into the genome of the cyanobacterium *Synechocystis* strain PCC
50 6803. J. Bacteriol. 171:3449-3457.

Lahti, K., J. Rapala, M. Färdig, M. Niemelä, and K. Sivonen. 1997. Persistence of cyanobacterial hepatotoxin, microcystin-LR in particulate material and dissolved in lake water. Wat. Res. 31:1005-1012.

5 Lee, S. J., M.-H. Jang, H.-S. Kim, B.-D. Yoon, and H.-M. Oh. 2000. Variation of microcystin content of *Microcystis aeruginosa* relative to medium N:P ratio and growth stage. J. Appl. Microbiol. 89:323-329.

Lepére, C., Wilmotte, A. & Meyer B. Molecular diversity of *Microcystis* strains
10 (Cyanophyceae, Chroococcales) based on 16S rDNA sequences. *Syst. Geogr. Pl.* 70, 275-283 (2000).

Lindroos K et al, 2002 Nucleic Acid Research vol 30, no 14e70.

15 Lyra, C., Suomalainen, S., Gugger, M., Vezie, C., Sundman, P., Paulin, L., Sivonen, K., 2001. Molecular characterization of planktic cyanobacteria of *Anabaena*, *Aphanizomenon*, *Microcystis* and *Planktothrix* genera. Int. J. System. Evol.Microbiol. 51, 513-526.

MacKintosh, C., Beattie, K.A., Klumpp, S., Cohen, P., Codd, G.A., 1990. Cyanobacterial
20 microcystin-LR is a potent and specific inhibitor of protein phosphatase 1 and 2A from both mammals and higher plants. FEBS Lett. 264, 187-192.

Marahiel, M.A., Stachelhaus, T., Mootz, H.D., 1997. Modular peptide synthetases involved in nonribosomal peptide synthesis. Chem. Rev. 97, 2651-2673.

25

Mehta, P.K., Hale, T.I., Christen, P., 1993. Aminotranferases: demonstration of homology and division into evolutionary subgroups. Eur. J. Biochem. 214, 549-561.

Moffitt, M.C. & Neilan, B.A. On the presence of peptide synthetase and polyketide synthase
30 genes in the cyanobacterial genus *Nodularia*. *FEMS Microbiol. Lett.* 196, 207-214 (2001).

Moon-van der Staay, S-Y., Wachter, R. & Vaulot, D. Oceanic 18S rDNA sequences from picoplankton reveal unsuspected eukaryotic diversity. *Nature* 409, 607-610 (2001).

35 Moore, R.E., Chen, J.L., Moore, B.S., Patterson, G.M.L., Charmichael, W.W., 1991. Biosynthesis of microcystin-LR. Origin of the carbons in the Adda and Masp units. J. Am. Chem. Soc. 113, 5083-5084.

Namikoshi, M., K. L. Rinehart, R. Sakai, R. R. Stotts, A. M. Dahlem, V. R. Beasley, W. W.
40 Carmichael, and W. R. Evans. 1992. Identification of 12 hepatotoxins from a Homer Lake bloom of the cyanobacteria *Microcystis aeruginosa*, *Microcystis viridis*, and *Microcystis wesenbergii*: nine new microcystins. J. Org. Chem. 57:866-872.

Namikoshi, M., Rinehart, K.L., 1996. Bioactive combounds produced by cyanobacteria. J.
45 Ind. Microbiol. 17, 373-384.

Neilan, B. A., D. Jacobs, T. del Dot, L. L. Blackall, P. R. Hawkins, P. T. Cox, and A. E. Goodman. 1997. rRNA sequences and evolutionary relationships among toxic and nontoxic cyanobacteria of the genus *Microcystis*. Int. J. Syst. Bacteriol. 47:693-697.

50

Neilan, B.A. *et al.* Nonribosomal peptide synthesis and toxigenity of cyanobacteria. *J. Bacteriol.* 181, 4089-4097 (1999).

Nishizawa, T., M. Asayama, K. Fujii, K.-I. Harada, and M. Shirai. 1999. Genetic analysis of the peptide synthetase genes for a cyclic heptapeptide microcystin in *Microcystis* spp. J. Biochem. 126:520-529.

Nishizawa, T., Ueda, A., Asayama, M., Fujii, K., Harada, K., Ochi, K., Shirai, M., 2000. Polyketide synthase gene coupled to the peptide synthetase module involved in the biosynthesis of the cyclic heptapeptide microcystin. J. Biochem. 127, 779-789.

Nishizawa, T., Asayama, M., Shirai, M., 2001. Cyclic heptapeptide microcystin biosynthesis requires the glutamate racemase gene. Microbiology 147, 1235-1241.

Nonneman, D., and P. V. Zimba. 2002. A PCR-based test to assess the potential for microcystin occurrence in channel catfish production ponds. J. Phycol. 38:230-233.

Ohtake, A., M. Shirai, T. Aida, N. Mori, K.-I. Harada, K. Matsuura, M. Suzuki, and M. Nakano. 1989. Toxicity of *Microcystis* species isolated from natural blooms and purification of the toxin. Appl. Environ. Microbiol. 55:3202-3207.

Orr, P.T., and G.J. Jones. 1998. Relationship between microcystin production and cell division rates in nitrogen-limited *Microcystis aeruginosa* cultures. Limnol. Oceanogr. 43:1604-1614.

Otsuka, S. *et al.* Phylogenetic relationships between toxic and non-toxic strains of the genus *Microcystis* based on 16S to 23S internal transcribed spacer sequence. *FEMS Microbiol. Lett.* 172, 15-21 (1999).

Pan, H., L. Song, Y. Liu, and T. Börner. 2002. Detection of hepatotoxic *Microcystis* strains by PCR with intact cells from both culture and environmental samples. Arch. Microbiol. 178:421-427.

Rapala, J., K. Sivonen, C. Lyra, and S.I. Niemelä. 1997. Variation of microcystins, cyanobacterial hepatotoxins in *Anabaena* spp. as a function of growth stimuli. Appl. Environ. Microbiol. 63:2206-2212.

Rapala, J., and K. Sivonen. 1998. Assessment of environmental conditions that favor hepatotoxic and neurotoxic *Anabaena* spp. strains cultured under light limitation at different temperatures. Microb. Ecol. 36:181-192.

Repka, S., J. Mehtonen, J. Vaitomaa, L. Saari, and K. Sivonen. 2001. Effects of nutrients on growth and nodularin production of *Nodularia* strain GR8b. Microb. Ecol. 42:606-613.

Ressom, R. *et al. Health Effects of Toxic Cyanobacteria (Blue-green Algae)* (Australian Government Publishing Service, Canberra, 1994).

Reynolds, C. S. 1997. Vegetation processes in the pelagic: a model for ecosystem theory. Excellence in Ecology, Ecology Institute, Oldendorf/Luhe, 371 p.

Rippka, R., and M. Herdman. 1992. Pasteur culture collection of cyanobacterial strains in axenic culture. Catalogue & taxonomic handbook, volume I: catalogue of strains 1992/1993. Paris, Institut Pasteur. p. 103.

5 Rippka, R., Castenholz, R.W., Iteman, I., Herdman, M., 2001. Form-genus I. *Anabaena* Bory de St. Vincent 1822 *sensu* Rippka, Deruelles, Waterbury, Herdman and Stanier 1979. In: Boone, D.R., Castenholz, R.W. (Eds.), Bergey's Manual of Systematic Bacteriology. Springer-Verlag, New York, pp. 566-568. 2nd edn, vol. 1.

10 Rouhiainen, L., Sivonen, K., Buikema, W.J., Haselkorn, R., 1995. Characterization of toxin-producing cyanobacteria by using an oligonucleotide probe containing a tandemly repeated heptamer. J. Bacteriol. 177, 6021-6026.

Rouhiainen, L., Paulin, L., Suomalainen S., Hyytiäinen, H., Buikema, W., Haselkorn, R.,
15 Sivonen, K., 2000. Genes encoding synthetases of cyclic depsipeptides, anabaenopeptilides, in *Anabaena* strain 90. Mol. Microbiol. 37, 156-167.

Saitou N., Nei M. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol. 4, 406-25 (1987).

20

Sambrook, J., Fritsch, E.F., Maniatis, T., 1989. Molecular cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Plainview, NY, 2nd edn.

Scrutton, N. S., Berry, A., Perham, R. N., 1990. Redesign of the coenzyme specificity of a
25 dehydrogenase by protein engineering. Nature 343, 38-43.

Silakowski, B., Nordsiek, G., Kunze, B., Blöcker, H., Müller R., 2001. Novel features in a combined polyketide synthase/non-ribosomal peptide synthetase: the myxalamid biosynthetic gene cluster of the myxobacterium *Stigmatella aurantiaca* Sga 15. Chem. Biol. 8, 59-69.

30

Sivonen, K., K. Himberg, R. Luukkainen, S. I. Niemelä, G. K. Poon, and G. A. Codd. 1989. Preliminary characterization of neurotoxic cyanobacteria blooms and strains from Finland. Toxic. Assess. 4:339-352.

35 Sivonen, K., W. W. Carmichael, M. Namikoshi, K. L. Rinehart, A. M. Dahlem, and S. I. Niemelä. 1990. Isolation and characterization of hepatotoxic microcystin homologs from the filamentous freshwater cyanobacterium *Nostoc* sp. strain 152. Appl. Environ. Microbiol. 56:2650-2657.

40 Sivonen, K., Namikoshi, M., Evans, W.R., Carmichael, W.W., Sun, F., Rouhiainen, L., Luukkainen, R., Rinehart, K.L., 1992. Isolation and characterization of a variety of microcystins from seven strains of the cyanobacterial genus *Anabaena*. Appl. Environ. Microbiol. 58, 2495-2500.

45 Sivonen, K., M. Namikoshi, R. Luukkainen, M. Färdig, L. Rouhiainen, W. R. Evans, W. W. Carmichael, K. L. Rinehart, and S. I. Niemelä. 1995. Variation of cyanobacterial hepatotoxins in Finland, p. 163-169. *In* M. Munavar and M. Luotola (ed.), The contaminants in the nordig ecosystem: dynamics, processes & fate. Ecovision world monograph series, Amsterdam, the Neatherlands.

50

Sivonen, K., Jones, G., 1999. Cyanobacterial toxins. In: Chorus, I., Bertram, J. (Eds.), Toxic Cyanobacteria in Water: A Guide to their Public Health Consequences, Monitoring and Management. E. & F.N. Spon, London, pp. 41-111.

5 Stachelhaus, T., Mootz, H.D., Marahiel M.A., 1999. The specificity-conferring code of adenylation domains in nonribosomal peptide synthetases. Chem. Biol. 6, 493-505.

Suzuki, M. T., and S. J. Giovannoni. 1996. Bias caused by template annealing in the amplification of mixtures of 16S rRNA genes by PCR. Appl. Environ. Microbiol. 62:625-630.

10

Swofford, D. L., Olsen, G. J., Waddell, P. J. & Hillis, D. M. in *Molecular Systematics* (eds Hillis, D. M., Moritz, C. & Mable, B. K.) 407–514 (Sinauer, Sunderland, Massachusetts, 1996).

15 Swofford, D. L. *PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods)* Version 4.0b8 (Sinaeur, Sunderland, Massachusetts, 2001).

Tang, L., Yoon, Y.J., Choi, C-Y., Hutchinson, C.R., 1998. Characterization of the enzymatic domains in the modular polyketide synthase involved in rifamycin B biosynthesis by
20 *Amycolatopsis mediterranei*. Gene 216, 255-265.

Taton, A., S. Grubisic, E. Brambilla, R. De Wit and A. Wilmotte (2003). Cyanobacterial Diversity in Natural and Artificial Microbial Mats of Lake Fryxell (McMurdo Dry Valleys, Antarctica): a Morphological and Molecular Approach. Appl. Environ. Microbiol. 69, 5157-
25 5169.

Thompson JD, Higgins DG, Gibson TJ. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 22, 4673-80 (1994).

30

Tillett, D., Dittmann, E., Erhard, M., von Döhren, H., Börner, T., Neilan, B.A., 2000. Structural organization of microcystin biosynthesis in *Microcystis aeruginosa* PCC7806: an integrated peptide-polyketide synthetase system. Chem. Biol. 7, 753-764.

35 Tillett, D., Parker, D.L. & Neilan, B.A. Detection of toxigenicity by a probe for the microcystin synthetase A gene (mcyA) of the cyanobacterial genus *Microcystis*: comparison of toxicities with 16S rRNA and phycocyanin operon (phycocyanin intergenic spacer) phylogenies. *Appl. Environ.* Microbiol. 67, 2810-2818 (2001).

40 Tsuge, K., Akiyama, T., Shoda, M., 2001. Cloning, sequencing, and characterization of the iturin A operon. J. Bacteriol. 183, 6265-6273.

Utermöhl, H. 1958. Zur Vervollkommnung der quantitativen phytoplankton-methodik. Mitt. Int. Ver. Limnol. 9:1-38.

45

Utkilen, H. & Gjølme, N. Iron-stimulated toxin production in *Microcystis aeruginosa. Appl. Environ. Microbiol.* 61, 797-800 (1995).

Vasconcelos, V. M., K. Sivonen, W. R. Evans, W. W. Carmichael, and M. Namikoshi. 1995.
50 Isolation and characterization of microcystins (haptapeptide hepatotoxins) from Portuguese strains of *Microcystis aeruginosa* KUTZ. emend ELEKIN. Arch. Hydrobiol. 134:295-305.

Vezie, C., L. Brient, K. Sivonen, G. Bertru, J.-C. Lefeuvre, and M. Salkinoja-Salonen. 1998. Variation of microcystin content of cyanobacterial blooms and isolated strains in Lake Grand-Lieu (France). Microb. Ecol. 35:126-135.

5

Vézie, C., J. Rapala, J. Vaitomaa, J. Seitsonen, and K. Sivonen. 2002. Effect of nitrogen and phosphorus on growth of toxic and nontoxic *Microcystis* strains and on intracellular microcystin concentrations. Microb. Ecol. 43:443-454.

10 Wawrik, B., J.H. Paul, and F.R. Tabita. 2002. Real-time PCR quantification of *rbcL* (ribulose-1,5-bisphosphate carboxylase/oxygenase) mRNA in diatoms and pelagophytes. Appl. Environ. Microbiol. 68:3771-3779.

Willén, T. 1962. Studies on the phytoplankton of some lakes connected with or recently
15 isolated from the Baltic. Oikos 13:169-199.

Wintzingerode F., U. B. Göbel, and E. Stackebrandt. 1997. Determination of microbial diversity in environmental samples: pitfalls of PCR-based rRNA analysis. FEMS Microbiol. Rev. 21:213-229.

20

CLAIMS

1. A method for detecting toxic cyanobacteria, characterized in that the method comprises that nucleic acid from a biological sample is brought into contact with an oligonucleotide designed to be specific for the *mcyE* gene, and the presence or absence of toxic cyanobacteria is detected by a suitable molecular biology method.

2. The method according to claim 1, wherein the oligonucleotide is designed to be specific for a region of the *mcyE* gene responsible for adding Adda and D-glutamate to the immature synthesis product of microcystin.

3. The method according to claim 1 or 2, wherein the oligonucleotide is designed to be specific for a region of the *mcyE* gene comprising two domains, the adenylation domain and the domain which catalyses a peptide bond between Adda- D-glutamate dipeptide and dehydroalanine.

4. The method according to any of claims 1 to 3, wherein the oligonucleotide is designed to be specific for a fragment of the *mcyE* selected from the group of genera *Anabaena*, *Microcystis*, *Planktothrix*, *Nostoc* and *Nodularia*.

5. The method according to any one of the preceding claims, wherein the nucleic acid from a biological sample is DNA or RNA.

6. The method according to any one of the preceding claims, wherein the oligonucleotide is designed to be specific for a fragment of the *mcyE* gene selected from the group of sequences SEQ ID NO. 1 to SEQ ID NO: 34 as shown in Figure 19 A to H or to a fragment of said sequences.

7. The method according to any one of the preceding claims, wherein the oligonucleotide is designed to be specific for a fragment of the *mcyE* gene selected from the group of consensus sequences SEQ ID NO: 35 to SEQ ID NO: 39 as shown in Figure 15 A to C or to a fragment of said sequences.

8. The method according to any one of the preceding claims, wherein the oligonucleotide is selected from the group of mcyE-F2 (SEQ ID Nos : 64), AnamcyE-12R (SEQ ID NO: 65) and MicmcyE-R8 (SEQ ID NO:66).

5 9. The method according to any one of the preceding claims, wherein the oligonucleotide is selected from the group of discriminating probes SEQ ID NO: 40 to SEQ ID NO: 45.

10. The method according to any one of the preceding claims, wherein the the oligonucleotide is selected from the group of common probes SEQ ID NO: 46 to SEQ ID NO: 51.

10

11. The method according to any one of the preceding claims, wherein the detection is combined with a detection method using oligonucleotides designed to be specific for any other mcy gene, such as *mcyA* or *mcyD,* or for 16S rRNA.

15 12. The method according to any one of the preceding claims, wherein detection is combined with a detection method selected from the group of measuring microcystin concentration, determining cell number, cell density or determining biomass.

13. A fragment of the *mcyE* gene, c h a r a c t e r i z e d in that it is on the region of the *mcyE*
20 gene responsible for adding Adda and D-glutamate to the immature synthesis product of microcystin and that it is or is located in any of the sequences selected from the group comprising SEQ ID NO. 1 to SEQ ID NO: 34 as shown in Figure 19 A to H, or is a sequence having at least 80 % identity, preferably 90 % identity to the sequence.

25 14. A fragment of the *mcyE* gene, c h a r a c t e r i z e d in that it is on the region of the *mcyE* gene responsible for adding Adda and D-glutamate to the immature synthesis product of microcystin and that it is or is located in any of the sequences selected from the group comprising consensus sequences SEQ ID NO: 35 to SEQ ID NO: 39 as shown in Figure 15 A to C, or is a sequence having at least 80 % identity, preferably 90 % identity to the sequence.

30

15. An oligonucleotide, c h a r a c t e r i z e d in that it is designed to be specific for the region of the *mcyE* gene responsible for adding Adda and D-glutamate to the immature synthesis product of microcystin that is or is located in any of the sequences selected from the group comprising SEQ ID NO: 1 to SEQ ID NO: 34 as shown in Figure 19 A to H or selected from

the group comprising any of the consensus sequences SEQ ID NO: 35 to SEQ ID NO: 39 as shown in Figure 15 A to C

16. An oligonucleotide selected from the group of mcyE-F2 (SEQ ID Nos : 64), AnamcyE-
5 12R (SEQ ID NO: 65) and MicmcyE-R8 (SEQ ID NO:66).

17. An oligonucleotide selected from the group of discriminating probes of SEQ ID NO: 40 to SEQ ID NO: 45.

10 18. An oligonucleotide selected from the group of common probes of SEQ ID NO: 46 to SEQ ID NO: 51.

19. *mcyE* gene from *Anabaena* genus encoding the amino acid sequence of SEQ ID NO: 67 or a sequence having at least 80 % identity, preferably 90 % identity to the sequence, or a
15 fragment of said sequence having polymorphic sites which make possible of designing oligonucleotides to be specific for the fragment.

20. *mcyE* gene from *Anabaena* genus having the nucleic acid sequence SEQ ID NO: 68 or a sequence having at least 80 % identity, preferably 90 % identity to the sequence, or a
20 fragment of said sequence having polymorphic sites, which make possible of designing oligonucleotides to be specific for the fragment.

21. A fragment of the *mcyD* gene, characterized in that it is on the region of the *mcyD* gene responsible for chain elongation of the growing Adda amino acid in the synthesis of
25 microcystin and that it is or is located in any of the sequences selected from the group comprising sequences SEQ ID NO: 131 to SEQ ID NO: 149 as shown in Figure 38 A to F or is a sequence having at least 85 % identity, preferably 90 % identity to the sequence.

22. *mcyD* gene from *Anabaena* genus encoding the amino acid sequence of SEQ ID NO: 69
30 or a sequence having at least 80 % identity, preferably 90 % identity to the sequence, or a fragment of said sequence having polymorphic sites which make possible of designing oligonucleotides to be specific for the fragment.

23. *mcyD* gene from *Anabaena* genus having the nucleic acid sequence SEQ ID NO: 70 or a sequence having at least 80 % identity, preferably 90 % identity to the sequence or a fragment of said sequence having polymorphic sites which make possible of designing oligonucleotides to be specific for the fragment.

24. An oligonucleotide selected from the group of discriminating probes of SEQ ID NO:71 to SEQ ID NO:90.

25. An oligonucleotide selected from the group of common probes of SEQ ID NO:91 to SEQ ID NO:110.

26. An oligonucleotide selected from the group of discriminating probes of SEQ ID NO:150 to SEQ ID NO:163.

27. An oligonucleotide selected from the group of common probes of SEQ ID NO:157 to SEQ ID NO:163.

28. A kit for detection of toxic cyanobacteria by microarray method, c h a r a c t e r i z e d in that it comprises
- discriminating probes and common probes designed to be specific for *mcy* gene, such as mcyE and D;
- DNA or RNA zip and complementary zip codes assigned to be specific for selected cyanobacteria genera.

29. A kit for detection of toxic cyanobacteria by hybridization, c h a r a c t e r i z e d in that it comprises
- primers designed to be specific for the *mcy* gene, such as mcyE or D;
- probes designed to be specific for selected cyanobacteria genera.

30. The kit according to claim 28 or 29, which comprises in addition to primers and probes designed to be specific for mcy gene also primers and probes for 16S rRNA gene.

31. A method for detecting toxic and non-toxic cyanobacteria, c h a r a c t e r i z e d in that the method comprises that nucleic acid from a biological sample is brought into contact with an

oligonucleotide designed to be specific for one or more mcy genes, such as mcyE or mcyD, and with an oligonucleotide designed to be specific for the 16SrRNA gene, and the presence or absence of toxic cyanobacteria is detected by a suitable molecular biology method.

5 32. The method according to claim 31, wherein the oligonucleotides are designed to be specific for a region of the *mcyE* and for a region of 16SrRNA gene.

33. The method according to any one of claims 1 to 12 or claims 31 or 32, wherein the molecular biology method is selected from the group comprising hybridization, PCR, reverse

10 transcriptase PCR, QTR-PCR, LCR, LDR and minisequencing.

34. The method according to any one of claims 1 to 12 or 31 to 33, wherein the detection is made by microarray method.

Microcystin synthetase gene cluster of *Anabaena* sp. 90

0 10 20 30 40 50 55 kb

mcyH *mcyI* *mcyF* *mcyE* *mcyJ* *mcyD* *mcyG* *mcyA* *mcyB* *mcyC*

C T A C AMT CM ACP AT KS ACP KR DH AT KS ACP KR CM DH AT KS ACP KR CM AT KS T A

A N-MT T C A T EP C A T C A T C A T TE

D-Glu Adda Mdha D-Ala Leu D-Me-Asp Arg

Adda D-Glu Mdha D-Ala Leu D-Me-Asp Arg

Fig. 1

A

```
AMcyG    RILEIGAGTGATTHQILQACA-SRQINYTFTDISPFFLETAKDTFA-HSFIEYKVLDIEK
AMcyD    RILEIGGGTGSSTAAILPHLP-PEQIEYTFTDISSSFLTRAKENFSNYPFFIKYQTLDIEK
AMcyE    QIIEVGGGTGATSEAIVNNLN-LNHTTYFFTELSPVLLNKARQKFKNRHKFNFNQLDIEK
EpoE     SILEIGAGTGATTAAVLPVLL-PDRTEYHFTDVSPLFLARAEQRFRDHPFLKYGILDIDQ
HMWP1    RILEVGGGTGGTTAWLLPELNGVPALEYHFTDISALFTRRAQQKFADYDFVKYSELDLEK
EcUbiE   TVLDLAGGTGDLTAKFSRLVG--ETGKVVLADINESMLKMGREKLRNIGVIG-NVEYVQA
                1

AMcyG    DPEIQGFLPGSYDLIIAANVLHSTRDLQGETLPHIRGLLRPGGHLLLLELT
AMcyD    APISQGFLPSYFDIIIAANVLHATADIN-ETLNNVRSLLAPNAILILLEST
AMcyE    SPVSQGLTAHSYHIVVAANVLHSTRNIT-ETLNNIRELLIPGGYLVLLETV
EpoE     EPAGQGYAHQKFDVIVAANVIHATRDIR-ATAKRLLSLLAPGGLLVLVEGT
HMWP1    EAQSQGFQAQSYDLIVAANVIHATRHIG-RTLDNLRPLLKPGGRLLMREIT
EcUbiE   NAEALPFPDNTFDCITISFGLRNVTDKD-KALRSMYRVLKPGGRLLVLEFS
                       2                   3
```

B

```
AMcyEamt  FIQKLRKLTQQKEIALIFDEIITG-RITPGG QEWFEIEADIVVYGKAIGGGLPISMICGK
McyEamt   FIQKLRQITHKKGITLIFDEIITGF-RIAPGGAQEWFNVEADIVIYGKAIGGGLPISMICGK
PMcyEamt  FIQKLRKLTQEKGIALIFDEIITGF-RIAPGGAQQWFNIEADIVIYGKAIGGGLPISMICGK
ItuAamt   FLKELRAITQQSGTALIMDEIITGF-RIGLGGAQEWFGIQADLVTYGKIIGGGQPLGVVAGK
MycAamt   FLKELRAITQQSGTALIMDEIITGF-RIGLGGAQEWFDIQADLVTYGKIIGGGQPLGIVAGK
EcGSA     FLPGLRALCDEFGALLIIDEVMTGF-RVALAGAQDYYGVEPDLTCLGKIIGGGMPVGAFGGR
EcArgD    FLQGLRELCDQHQALLVFDEVQCGMGRTGDLFAYMHYGVTPDILTSAKALGGGFPISAMLTT
                            *                               *
```

Fig. 2

A

```
AMCD-DH2     ASISQNNPEFLTEHQVFDKPIFPGAAFIEMAL
AMCD-DH3     GEISSEYPDYLEGHKVFGKILFPATGFIETIL
RifE-DH10    SRLSLRSHPWLADHAVRDVVIVPGTGLVELAV
RapA-DH4     GRVSLATHAWLADHAVWGRVLLPGTAFVELVV
RapB-DH10    GRVSLATHAWLADHAVRGSVLLPGTGFVELVV
                       *   *    *
```

B

```
AMCG-KR1     QAQATYLITGGIGHLGLQLARHLV-DLGAKHLILTTR
AMCD-KR2     RQDGFYLISGGTGGLGLATARWMI-EHGACHLVLCSR
AMCD-KR3     SKEGAYLITGGLGKLGLLMAQWL-SQMGSSHLVLCSR
RifE-KR10    KTRGPVLVTGGTGSLGGLVARHLVERHGVRQLVLASR
RapA-KR4     DPDGTVLITGGSGVLAGIAARHLVAERGVRHLLLLSR
RapB-KR10    DPDGTVLITGGSGVLAGIAARHLVAERGVRHLLLLSR
                       * *  *  *
```

Fig. 3

AT1	AMcyG	GQGSQY	TSYTQPALFVVEVALAQLW	GHSL	SHAFH	WIQHL
	MMcyG	GQGSQY	TAYTQPALFLIEVALAQLW	GHSL	AHAFH	WLQHL
	PMcyG	GQGSQY	TSYTQPALFIIEVALAQLW	GHSL	SHAFH	WLQHL
AT2	AMcyD	GEVRVN	PLYVHPTLFALQYALCELW	GNGL	WQEFH	WVDAI
	MMcyD	GEAQSH	SLSVQPPLFAYQYALCELW	GSGL	WEAFH	WINSG
	PMcyD	GEAQSN	PLSVQPILFAFHYALCELW	GSGL	WKGFH	WITSV
AT3	AMcyD	GQGSQY	TQITQPALFSLEYALAKLW	GHSI	SHAFH	WLRHL
	MMcyD	GQGSQY	TQITQPVIFSLEYALAKLW	GHSI	SHAFH	WVAHL
	PMcyD	GQGSQY	TQITQPVLFSFEYALAKLW	GHSI	SHAFH	WVNHL
AT4	AMcyE	GQGACY	TAYAQPAIFALEYSLAMLW	GHSV	NGAFH	WRQQS
	MMcyE	GQGACY	TAYAQPAIFALEYSLTMLW	GHSV	TQAFH	WSKQC
	PMcyE	GQGACY	TAYAQPAIFALEYAVAMLW	GHSV	TQAFH	WRQQC
		H	FT E	V L		
Malonyl		GQGXQR	TXYAQXXXXXXQXALXXXX	GHSI	XXAFH	WXXXX
Methymalonyl		GQGXQW	VDVVQXXXXXXMXSLAXXW	GHSQ	DYASH	WXXNL
			A	*		

Fig. 4

A

```
AMCG-KS1   GPSVNVQTACSTGLVVVHLA - 122aa - VEAHGTGTKLGDPIE - 18 aa - GSVKTNIGHMQIASGIVGLIK
AMCD-KS2   GPSLAIDTACSSSLVAVHLA - 121aa - VEAHGTGTSLGDPIE - 20 aa - GSVKTNLGHLAAAAGISGLIK
AMCD-KS3   GPSMTVDTACSSSLVAVHLA - 121aa - IEAHGTGTALGDPIE - 19 aa - GSVKTNLGHLEGAAGIAGLIK
AMCE-KS4   GPCMSIDAACASSLVALHQA - 121aa - IEAHGTGTSLGDPIE - 17 aa - GSVKTNIGHLEAAAGIAGTIK
RapA-KS1   GPAITVDTACSSSLVALHQA - 121aa - VEAHGTGTTLGDPIE - 18 aa - GSLKSNIGHAQAAAGVSGVIK
RifA-KS1   GPAVTVDTACSSSLVAMHLA - 121aa - VEAHGTGTTLGDPIE - 18 aa - GSLKSNIGHAQAAAGVAGVIK
                   *
```

B

```
AMCG-ACP1   EQGFLEMGIDSLLSIELKNRLEKGLEVALPASLIF
AMCD-ACP2   QQGFFDMGMDSLTSTELRNLLQTDFNCSLPTTIAF
AMCD-ACP3   HTSFLELGLNSLMVLEFKNRLQSNLACTLPTSIIF
AMCE-ACP4   DETLLNLGADSIILTDFVRKIEEKFGVKVKIDQLF
RapA-ACP1   TGAFRDLGVDSLTAVELRNGLAKATGLRLPATLVF
RapC-ACP11  TTAFKDLGINSLTAVELRNSLAKATELRLPATLVF
RifA-ACP1   GRTFKDAGFDSLTAVELRNRLAAATGLTLSPAMIF
                   *
```

Fig. 5

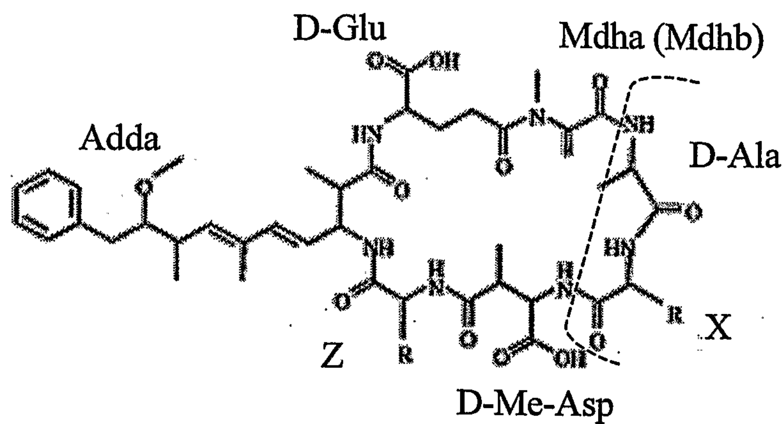
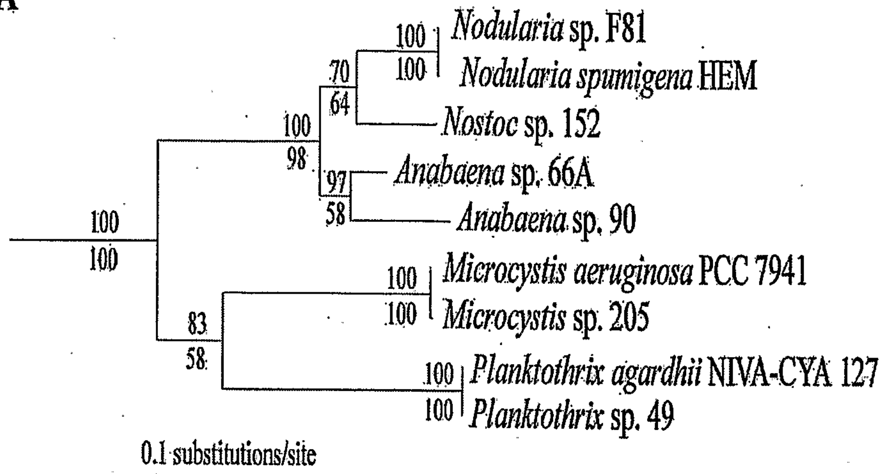


Fig. 6

A



B

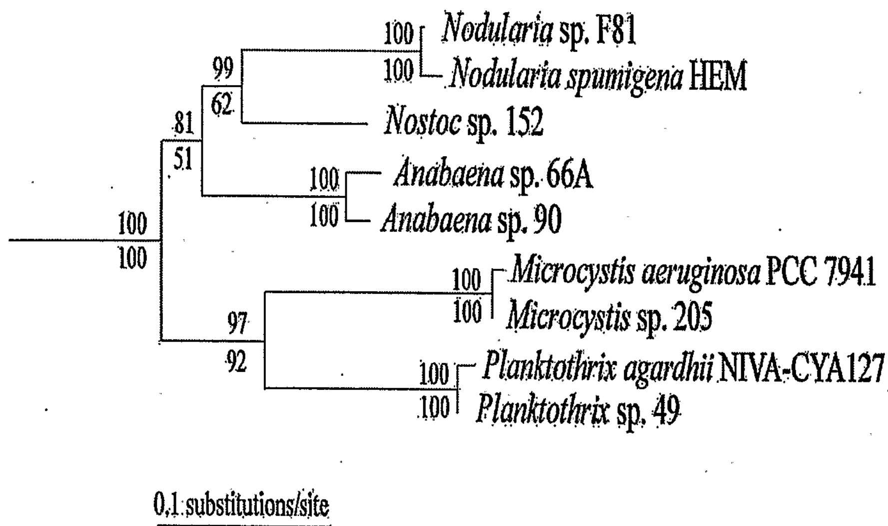


Fig. 7

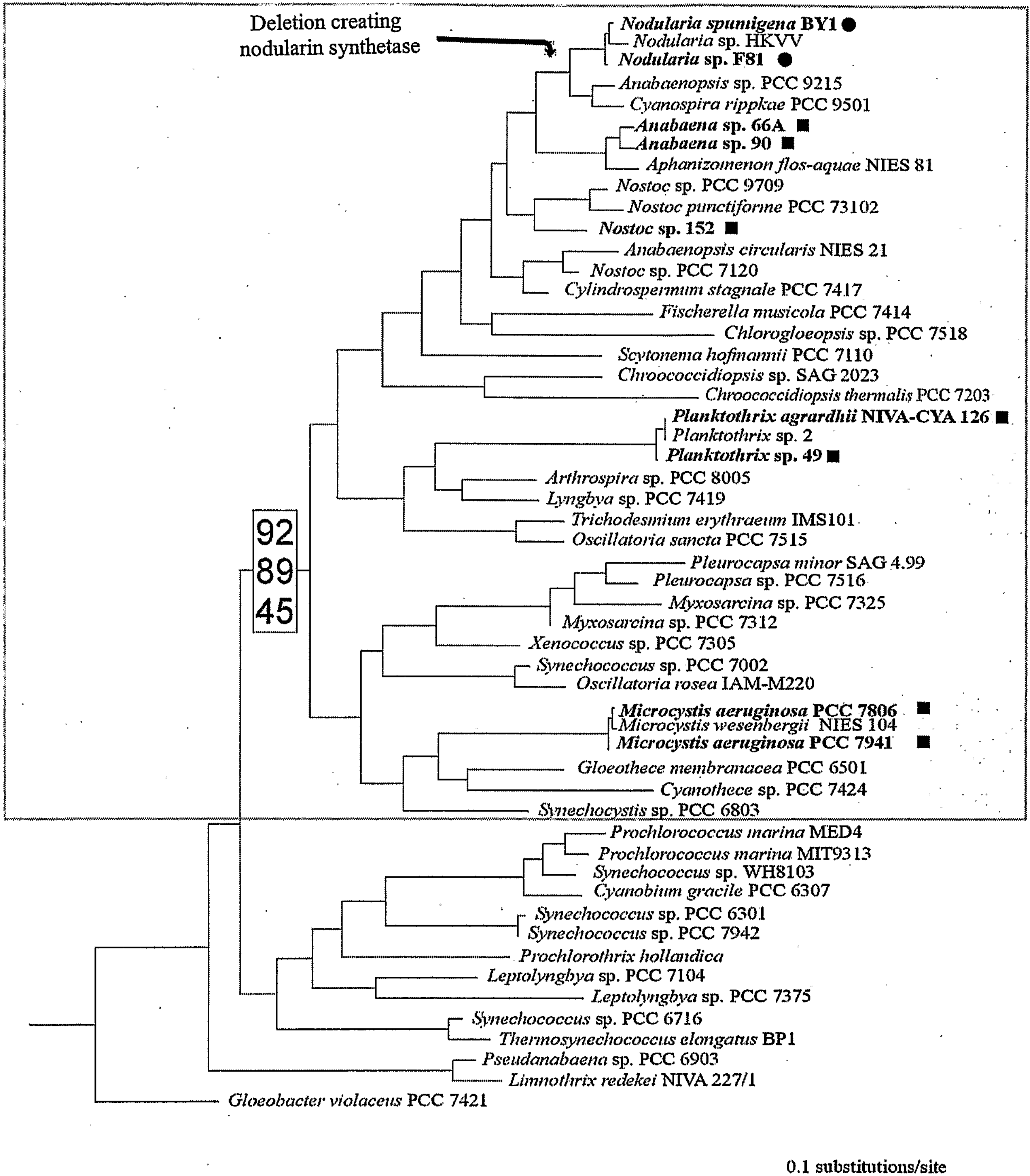


Fig. 8

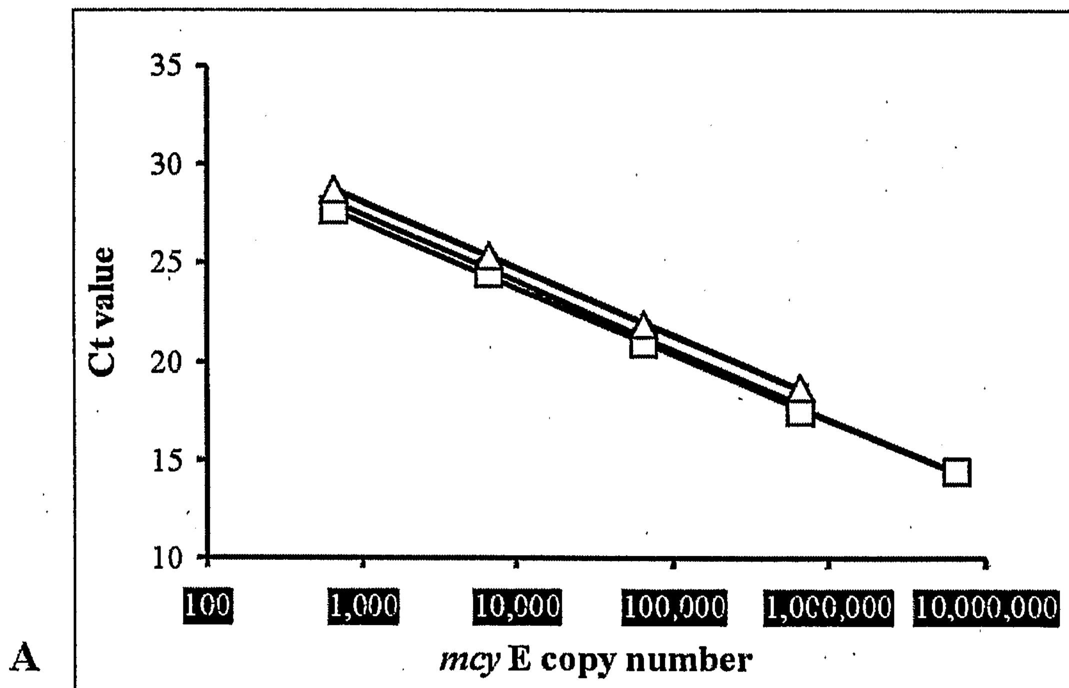


Fig. 9A

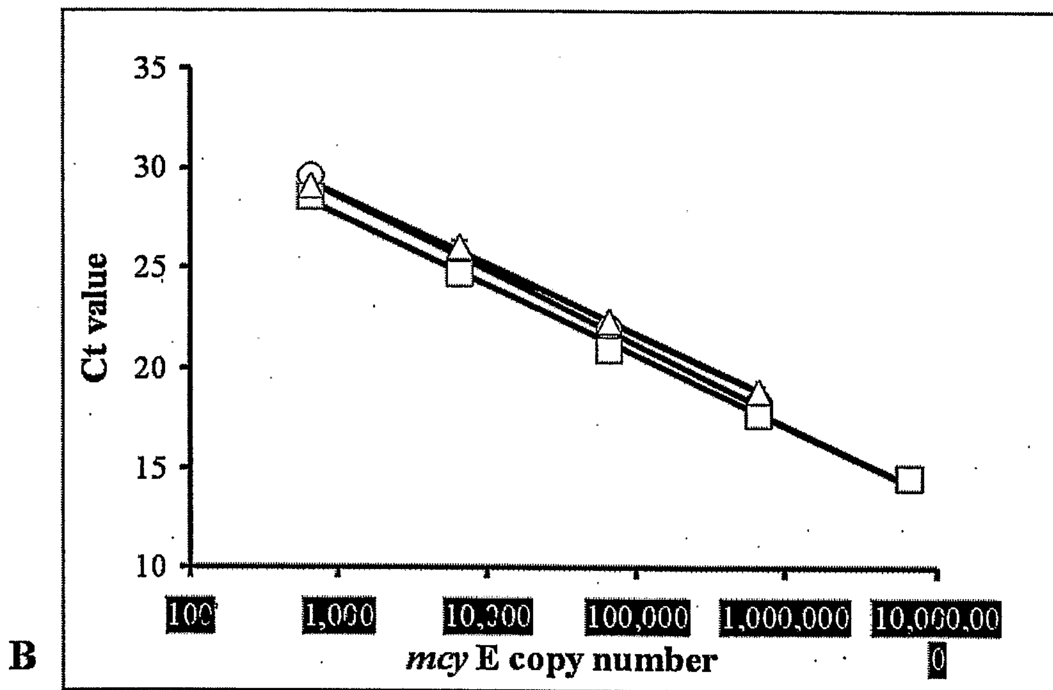


Fig. 9B

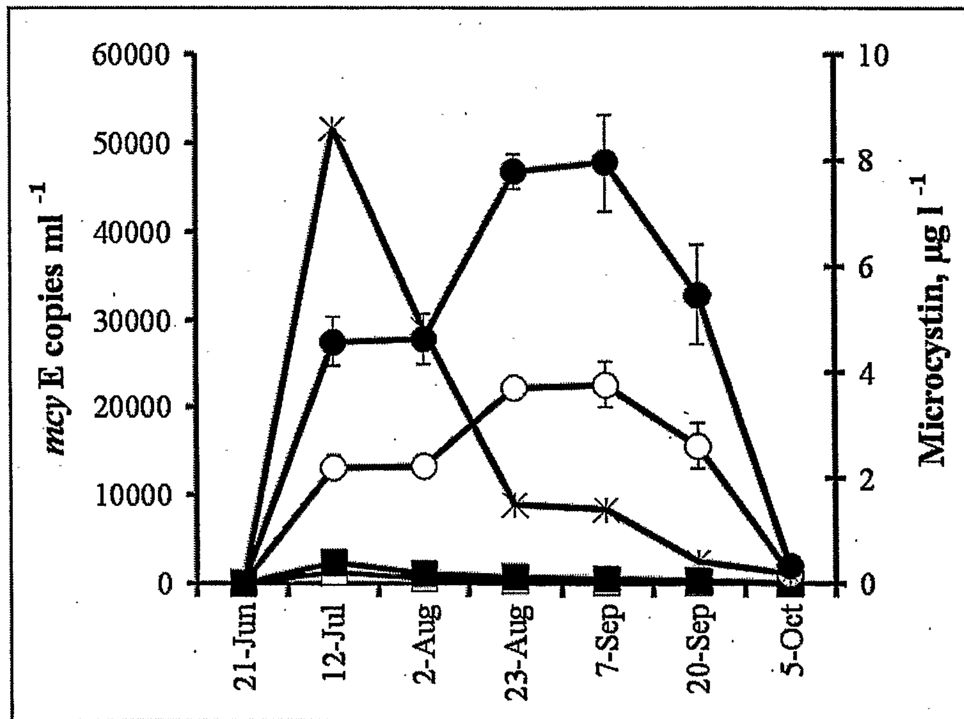


Fig. 10

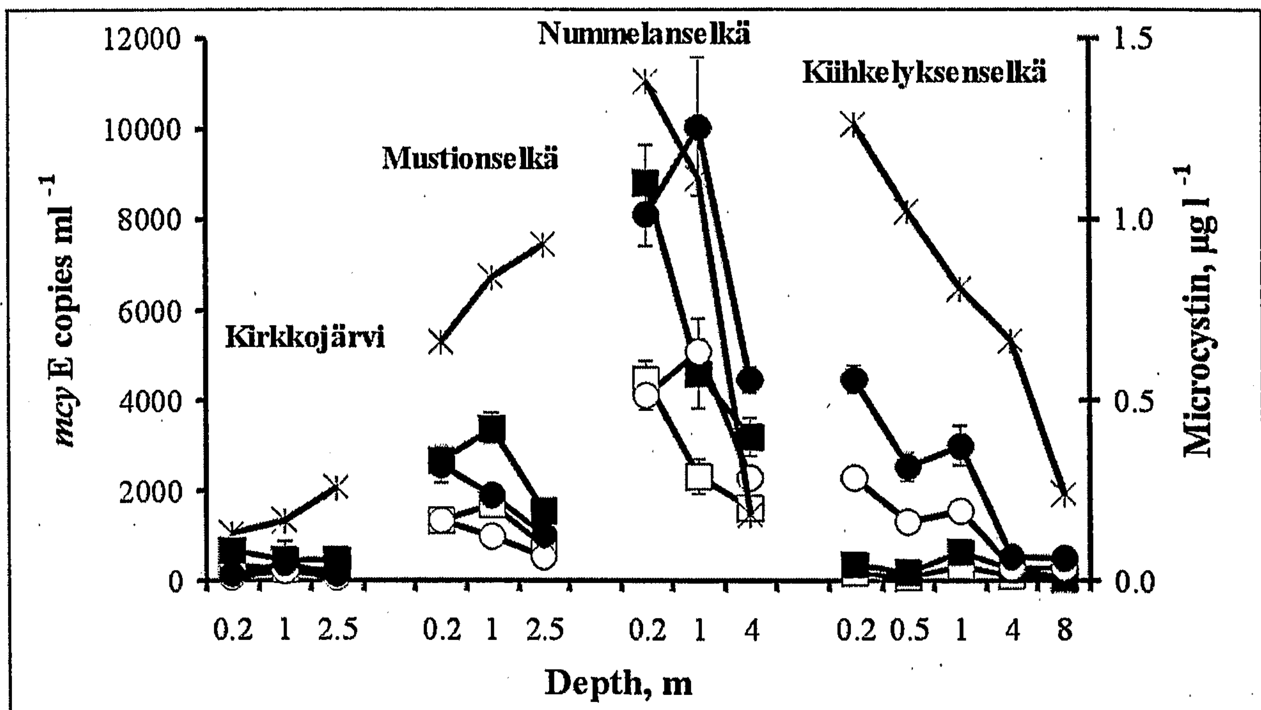


Fig. 11

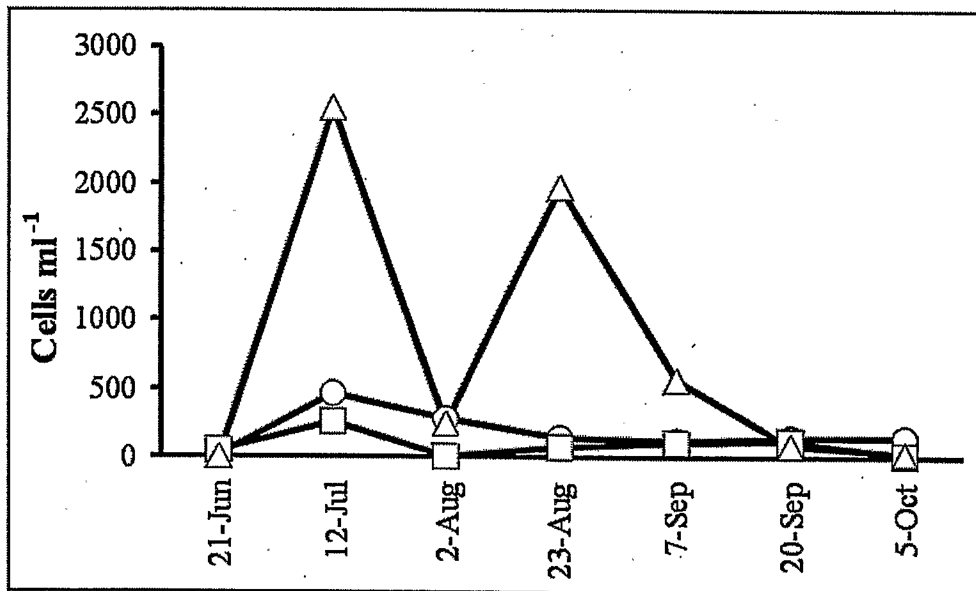


Fig. 12

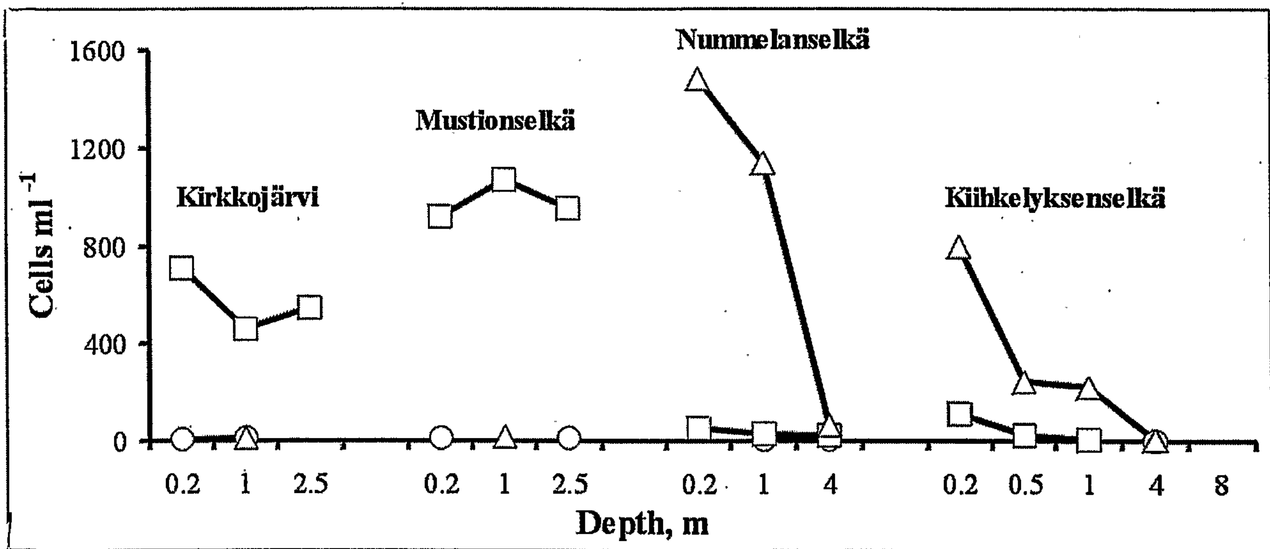


Fig. 13

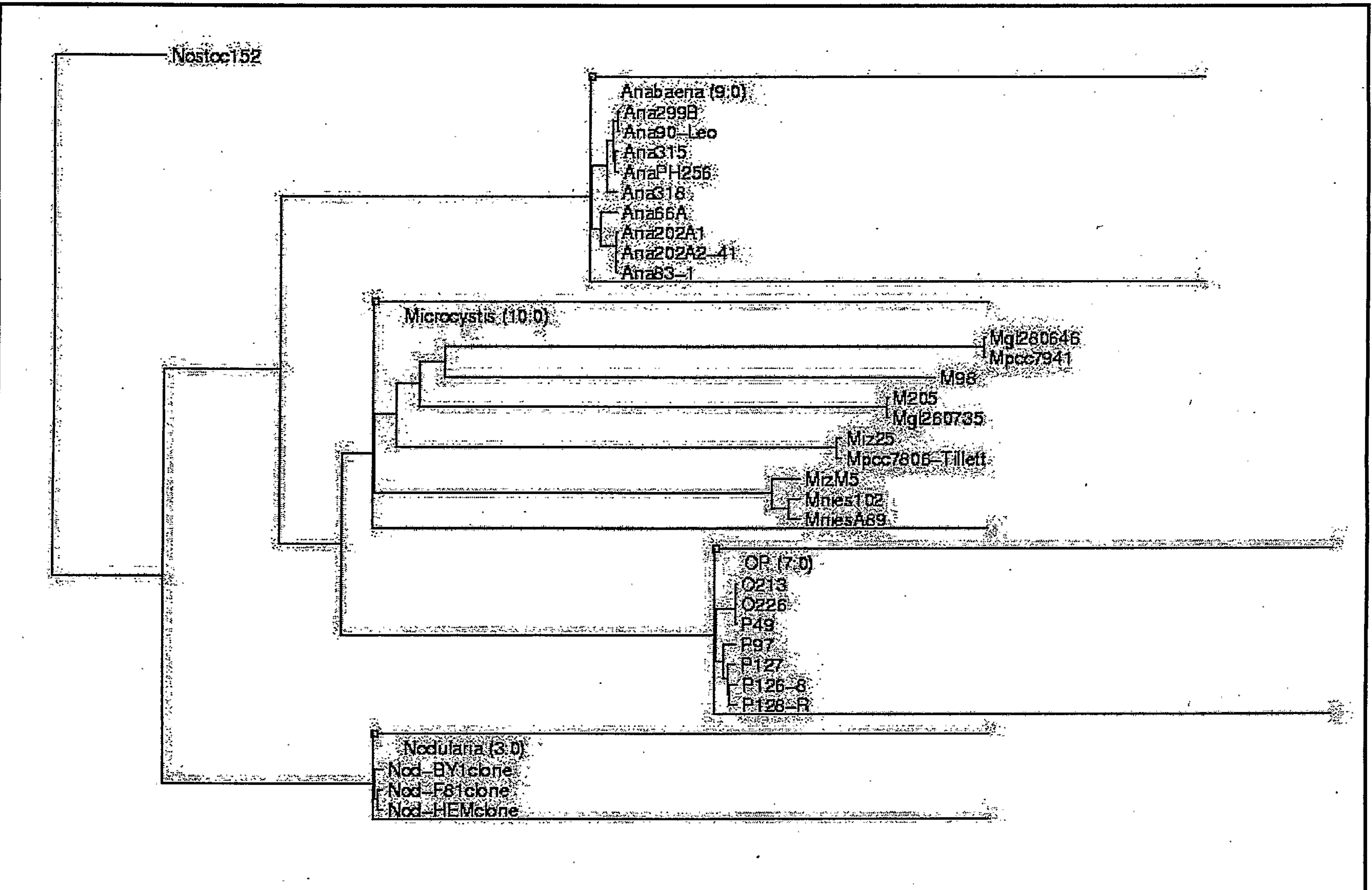


Fig. 14

```
                                            1                                                    50
Consensus_Anabaena_McyE                     AGCATTAGCA GTTGGTTATC GTAATCTTCC TGAAATTACC AGAGAAAAAT
Consensus_Microcystis_McyE                  CGCCCTAGCA TCGGGTTATC ATAACCAACC CGAAATGACT CAAGAAAAAT
Consensus_Nodularia_McyE                    AGCATTAGCA KCAGGTTATC ACAACCTCCC CCAAATCACC GCAGAAAAAT
Consensus_Nostoc_McyE                       AGCATTAGCC GCAGGTTATC ATAATCTTCC CGACATCACT ACAGAAAAAT
Consensus_Oscillatoria/Planktothrix_McyE    AGCCTTAGCA TCGGGTTATC ACAATCTTCC TCAAATCACA AAAGAAAAAT

                                            51                                                  100
Consensus_Anabaena_McyE                     TTCAACCCAA CTTTTTAAAT TCAGAAAAAA TTCTCTTTAG AACGGGAGAT
Consensus_Microcystis_McyE                  TTAAACCTAG CTTTCTTGAT GAGACAAAAA CTCTCTTTAG AACCGGCGAT
Consensus_Nodularia_McyE                    TTCAACCTAG CTYTATGACT GAGGGAAAAA CTATCTTTAG AACCGGAGAT
Consensus_Nostoc_McyE                       TTCAACCCAG CTTGATAAGT GAGGGAAAAA CTCTCTTTAG AACGGGAGAT
Consensus_Oscillatoria/Planktothrix_McyE    TTAAACCTGG CTTTTTTRAT CAGAAAACAA CGATGTTTAG AACCGGGGAT

                                            101                                                 150
Consensus_Anabaena_McyE                     TTAGGTAAAC AAATTGCTCC AGGTGTGATT GAATTTATAG GGCGAAAAGA
Consensus_Microcystis_McyE                  TTAGGCAAGC AAACTGCTCC SGGTATCATT GAGTTTATGG GACGAAAAGA
Consensus_Nodularia_McyE                    TTAGGTAAAC AAATTGCCCC AGGCGTGATT GAATTTCTTG GTCGTAAAGA
Consensus_Nostoc_McyE                       TTAGGTAAAC AAACTGCTCC AGGTGTCATT GAATTTATTG GGCGTAAAGA
Consensus_Oscillatoria/Planktothrix_McyE    TTAGGCAAAC AAACTGCTCC CGGTGTGATT GAATTTATGG GCAGAAAAGA

                                            151                                                 200
Consensus_Anabaena_McyE                     TAATCAAGTT AAGGTCAATG GCTATCGTGT AGATCCAGGA GAAMTTGAAT
Consensus_Microcystis_McyE                  TAATCAAGTT AAGGTCAATG GTTATCGAAT TGACCCCGGA GAAATTGAAT
Consensus_Nodularia_McyE                    TAATCAAGTT AAGGTGAATG GCTATCGYAT AGATCCAGGA GAAATTGAAT
Consensus_Nostoc_McyE                       TAATCAAGTA AAGGTGAATG GTTATCGTAT AGATCCAGGA GAAATTGAAT
Consensus_Oscillatoria/Planktothrix_McyE    CAATCAAGTT AAGGTAAATG GCTATCGTAT CGACCCCGAA GAAATTGAAT

                                            201                                                 250
Consensus_Anabaena_McyE                     ACCAAATTAG CCGCTATGCC GAGATTGAGA AAGCAATTGT CTTACCTATA
Consensus_Microcystis_McyE                  ATCAATTGAC TCGTTATGCT CCCATTGAAA GAGCGATTGT TTTACCCGTT
Consensus_Nodularia_McyE                    ACCAACTCAG CCGCCATTCT CAAATTGAGA GAGCAATCGT ATTGCCTACT
Consensus_Nostoc_McyE                       ATCAACTCAG CCGTCATGCT CAGATTGAAA GAGCGATTAT ATTGCCTATC
Consensus_Oscillatoria/Planktothrix_McyE    ATCAACTTAA TCGTTATCCT CAGATTGAGA GAGCTATTAT TCTACCGATA

                                            251                                                 300
Consensus_Anabaena_McyE                     GAGGTAAATA ACCAAATTCA ATTATCTGCT TATTGTCAAA CTGATAAAGA
Consensus_Microcystis_McyE                  CAAGTKAATA ATCAAACTCA ATTATCTGCT TACTGTCAAA CAGACAAAAC
Consensus_Nodularia_McyE                    AATGTAGATA ATCAAACCCA GTTATCAGCC TATTGTAAAA CTGAGTCAGA
Consensus_Nostoc_McyE                       AATGTAGATA ATCAAACTCA ATTATCTGCT TATTGTCAAA CTGATAAAGA
Consensus_Oscillatoria/Planktothrix_McyE    TCAGTCAATA ATCAAACTCA ATTATCAGCC TATTGTCAAA CMGATAAACA
```

Fig. 15A

```
                                           301                                                    350
Consensus_Anabaena_McyE                    TATAAAAWTT TCTGAAATCA GAGAATTTTT AGCTAAATAT TYGCCAGTTT
Consensus_Microcystis_McyE                 TCTAGAAATT GCTGAGATTC GAGAATTWCT TGCCAARTTT TTACCAGTTT
Consensus_Nodularia_McyE                   CATAGAAATT TCCGAAATTC GAGAATTTCT ATCGAACTTT TTGCCCGTTT
Consensus_Nostoc_McyE                      CATCGAAATT GCTGAAATTA GAGAATTTCT CTCTAAATTT TTGCCAGTTT
Consensus_Oscillatoria/Planktothrix_McyE   GATAGAAATT TCTGAAATYA GAGAATTTCT AGCTAATTTT CTGCCAGTTT

                                           351                                                    400
Consensus_Anabaena_McyE                    ACATGATTCC TAGTTCCTTT ATCTTCTTAA AGCAATTTCC CTTAACTAAA
Consensus_Microcystis_McyE                 ATATGATTCC SAGTTACTTT ATTTTTTTAA AGCAATTCCC CTTAACTCGA
Consensus_Nodularia_McyE                   AYATGATTCC TACTTTCTTT ATCTTCTTAA AGCAATTTCC CTTAACCAGA
Consensus_Nostoc_McyE                      ATATGATTCC TACTTCCTTT ATCTTCCTCA AGCAATTTCC TCTAACCAGA
Consensus_Oscillatoria/Planktothrix_McyE   ACATGATTCC TAGTTACTTT ATTTTCTTAA AGCAATTYCC CCTAACTAAA

                                           401                                                    450
Consensus_Anabaena_McyE                    CATGGCAAAY TTGACTTGCG ATCGCTCRTC GCTGTCAAGC CRACAGATCA
Consensus_Microcystis_McyE                 CATGGRAAAC TTGACCTGCA CTCCCTGAGA GAACTCAGAG AAACYGGTAA
Consensus_Nodularia_McyE                   CATGGGAAAA TTGATTTGCG ATCCCTGGCT GAATTCAAGG GAATAGGTAA
Consensus_Nostoc_McyE                      CATGGCAAAA TTGACTTGCG ATCGCTTGCT GAACTCCAAG GAATCGGTAA
Consensus_Oscillatoria/Planktothrix_McyE   CACGGCAAAC TTGACTTAAA CTCAMTGATT GCAGTCAATG AAACCGGGAA

                                           451                                                    500
Consensus_Anabaena_McyE                    AY...TAACA CAAGTCTCTT ATACTGCACC GCGTAATACT TTAGAATCAA
Consensus_Microcystis_McyE                 ATCTCTGGTK AATTCTAATT AYGTTGCACC CCGKAATYAT TTAGAATCCA
Consensus_Nodularia_McyE                   CT...TAACA CAGTTAGCGT ATACTGCACC GCGCAATAAT TTAGAGTCCA
Consensus_Nostoc_McyE                      GT...TAACA CAGGCAGATT ATACTGCACC GCGCAATGAT TTAGAGTCCA
Consensus_Oscillatoria/Planktothrix_McyE   AT...CTACC CARGTAAATT ATGTTGCACC GCGTAATAAT TTAGAGTCAA

                                           501                                                    550
Consensus_Anabaena_McyE                    AGCTAGTCCA TATTTGGGAA AAAATTCTCA CTAAACATCC CATTGGAATT
Consensus_Microcystis_McyE                 ATCTCGTTAG TATCTGGGAA AAAATTCTCT CTAAACATCC TATCGGTATT
Consensus_Nodularia_McyE                   AGCTCGTACA TATTTGGGAA AAAATTCTCA CCAAACAACC CATTGGCATT
Consensus_Nostoc_McyE                      AGCTAGTAAA GATTTGGGAA AAAATTCTCA CCACACATCC CATCGGCATT
Consensus_Oscillatoria/Planktothrix_McyE   ACCTAGTTAG AATCTGGGAA AAGATTCTRA CCAAACATCC CATCGGTATT

                                           551                                                    600
Consensus_Anabaena_McyE                    TTTGATAACT TTTTTGAAAT CGGCGGACAC TCTCTGCTCC TTTCTAGAGT
Consensus_Microcystis_McyE                 TTTGATAACT TCTTTGAAAT TGGCGGTCAT TCTCTACTCT TATCAAGGGT
Consensus_Nodularia_McyE                   TTTGATAACT TCTTTGAAAT TGGTGGACAC TCACTGCTGC TTTCCAGAGT
Consensus_Nostoc_McyE                      TTTGATAACT TCTTTGAAAT TGGTGGACAC TCCCTGCTGC TTTCGAGAGT
Consensus_Oscillatoria/Planktothrix_McyE   TTTGATAACT TCTTTGAAAT TGGCGGACAT TCTCTGATGC TTTCGAGAAT
```

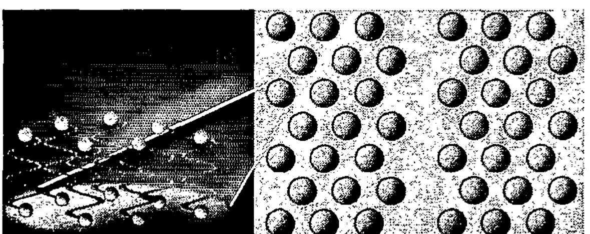
Fig. 15B

```
                                             601                                                   650
Consensus_Anabaena_McyE                      AGTCACTCAC GTCCATAAAG AATTAAATGT ATTAGTTAAA TTAGCTGATT
Consensus_Microcystis_McyE                   TGTAACCCGG GTTCATAAAG AACTAAATGT ATCCGTAAAA TTRGCTGACT
Consensus_Nodularia_McyE                     GGTAACTCAC GTTCATAAAG AATTAAATGT GTTGGTAAAA TTGGCTGAAT
Consensus_Nostoc_McyE                        GGTAACTTAC GTTCATAAAG AATTAAATGT GTTAGTCAAA TTGGCTGACT
Consensus_Oscillatoria/Planktothrix_McyE     CGTAACCCAC GTTCATAAAG AATTAAATGT ATCGGTAAAA TTGGCTGACT

                                             651                                                   700
Consensus_Anabaena_McyE                      TCTTCAAAGT TCCCACAATT CTTGGATTAG CAGCTTTAAT ATCTAAAGCT
Consensus_Microcystis_McyE                   TCTTTAAAGT TCCAACCRTT GCTGGATTGG CGACTTTAAT CTCCCAGACT
Consensus_Nodularia_McyE                     TCTTTAAAGT TCCCACAATC GCCGGATTAG CAGCTTTAGT ATCTAAAACC
Consensus_Nostoc_McyE                        TTTTTAAAGT TCCCACCATA GCCGGATTAG CAGCTTTAGT AGCTAAAACC
Consensus_Oscillatoria/Planktothrix_McyE     TCTTTAAAGT TCCTACCATT GCCGGATTAG CCGTTTTAGT CTCTAAAACT

                                             701                                                   750
Consensus_Anabaena_McyE                      CAATCTAACT ATCAAGAACC CATACCAGCA ATAACTCAAC AAGAATCTTA
Consensus_Microcystis_McyE                   CAATACAATT ATCAAGAACC CATTTCGGCA ATTCCCCCCC AAAAATCYTA
Consensus_Nodularia_McyE                     CAATATGACT ATCAAGAACC CATACCAGCA ATAACTCAGC AAACGTCTTA
Consensus_Nostoc_McyE                        CAATACGATT ATCAAGAACC CATACCTGCA ATAATTCAGC AAAAATCTTA
Consensus_Oscillatoria/Planktothrix_McyE     GAATATGATT ATCAAGAACC CATYCCCACA ATTCCTCTGC AAAAATCCTA

                                             751                 772
Consensus_Anabaena_McyE                      TCCCATGTCT CATGGACAAC GC  (SEQ ID NO:35)
Consensus_Microcystis_McyE                   TCCGATGTCT CATGGTCAGC GT  (SEQ ID NO:36)
Consensus_Nodularia_McyE                     TCCTATGTCT CATGGGCAAC GC  (SEQ ID NO:37)
Consensus_Nostoc_McyE                        TCCCATGTCT CATGGGCAAC GC  (SEQ ID NO:38)
Consensus_Oscillatoria/Planktothrix_McyE     TCCGATGTCC CATGGGCAAC GT  (SEQ ID NO:39)
```

Fig. 15C

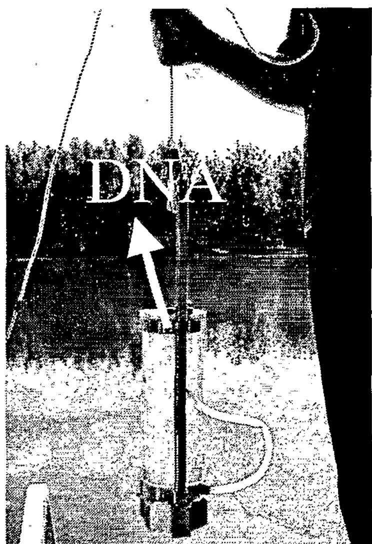
DNA-chip (Microarray)

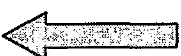
EU project ”MIDI-CHIP”

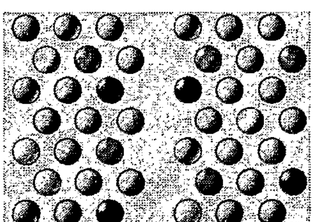


•Probes on a DNA-chip
•large amounts of sequence data is needed for probe design

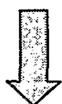
DNA extracted from environmental samples (PCR product) reacts with the probes on the chips

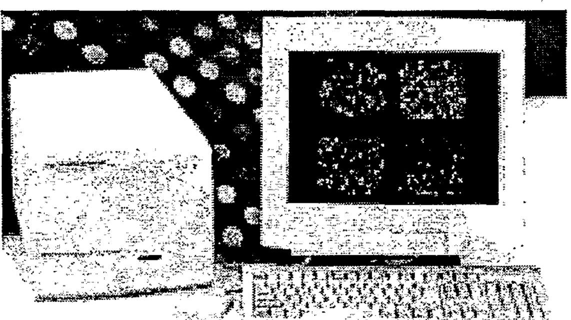






Chips designed by CNR-ITBA





<u>Chips are scanned with laser and results analyzed with computer</u>

- genetic identification of cyanobacteria present in the sample (16S rRNA)
- presence of cyanobacteria with microcystin synthetase genes (*mcy*E)

Fig. 16

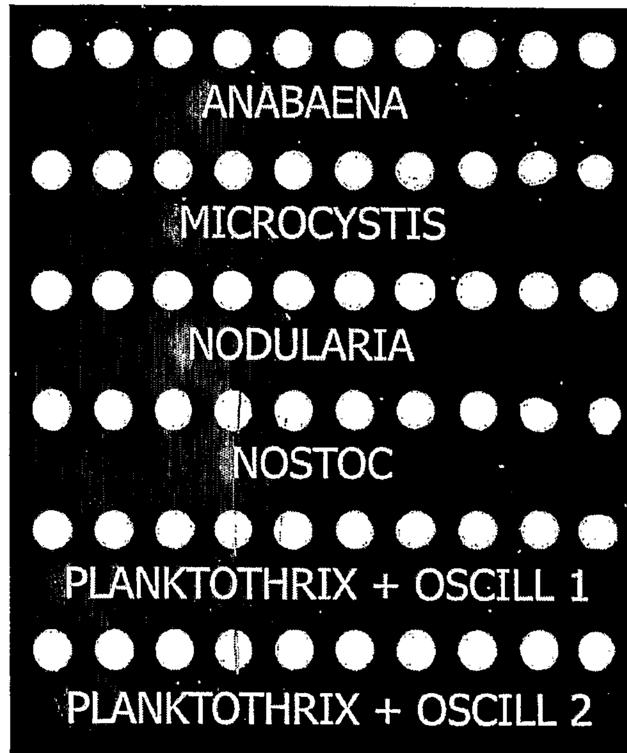
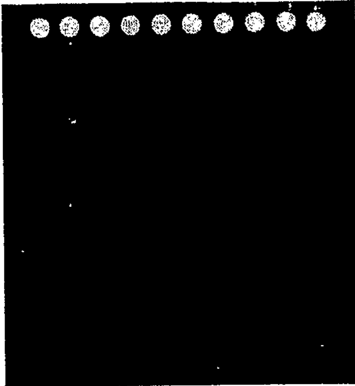


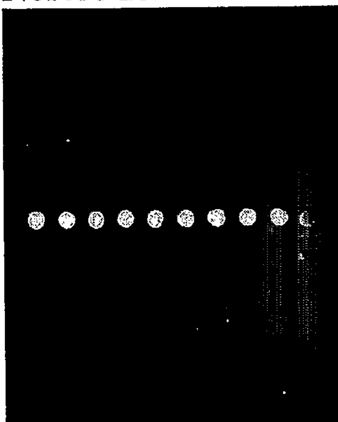
Fig. 17

Anabaena 202A1:

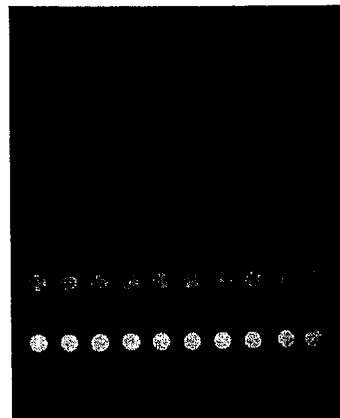


Microcystis 205:

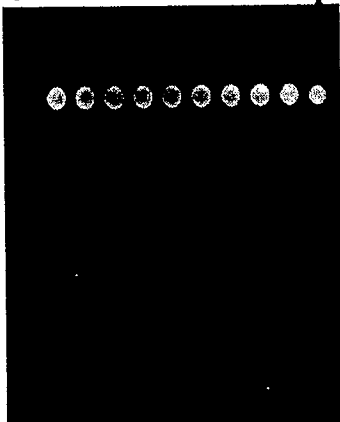
Nostoc 152:



Planktothrix 49:



OTU 33 bloom sample:



OTU 35 >10 um fraction:

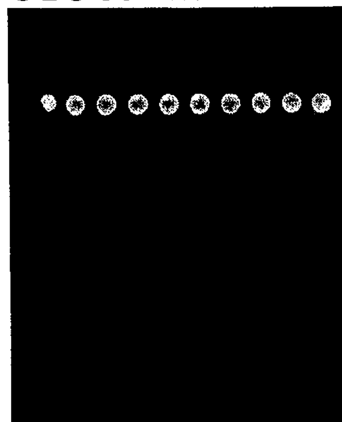


Fig. 18

Fig. 19A

Alignment

	5	15	25	35	45	55
Ana90-Leo	AGCATTAGCA	GTTGGTTATC	GTAATCTTCC	TGAAATTACC	AGAGAAAAAT	TTCAACCCAA
Ana299B	AGCATTAGCA	GTTGGTTATC	GTAATCTTCC	TGAAATTACC	AGAGAAAAAT	TTCAACCCAA
Ana315	AGCATTAGCA	GTTGGTTATC	GTAATCTTCC	TGAAATTACC	AGAGAAAAAT	TTCAACCCAA
AnaPH256	AGCATTAGCA	GTTGGTTATC	GTAATCTTCC	TGAAATTACC	AGAGAAAAAT	TTCAACCCAA
Ana318	AGCATTAGCA	GTTGGTTATC	GTAATCTTCC	TGAAATTACC	AGAGAAAAAT	TTCAACCCAA
Ana202A1	AGCATTAGCA	GTTGGTTATC	GTAATCTTCC	TGAAATTACC	AGAGAAAAAT	TTCAACCCAA
Ana202A2-41	AGCATTAGCA	GTTGGTTATC	GTAATCTTCC	TGAAATTACC	AGAGAAAAAT	TTCAACCCAA
Ana66A	AGcATTAGCA	GTTGGTTATC	GTAATCTTCC	TGAAATTACC	AGAGAAAAAT	TTCAACCCAA
Ana83-1	AGCATTAGCA	GTTGGTTATC	GTAATCTTCC	TGAAATTACC	AGAGAAAAAT	TTCAACCCAA
Cons-Ana98%	AGCATTAGCA	GTTGGTTATC	GTAATCTTCC	TGAAATTACC	AGAGAAAAAT	TTCAACCCAA
Mpcc7806-Tillet	CGCCCTAGCA	TCGGGTTATC	ATAACCAACC	CGAAATGACT	CAAGAAAAAT	TTAAACCTAG
M205	CGCCCTAGCA	TCGGGTTATC	ATAACCAACC	CGAAATGACT	CAAGAAAAAT	TTAAACCTAG
M98	CGCCCTAGCA	TCGGGTTATC	ATAACCAACC	CGAAATGACT	CAAGAAAAAT	TTAAACCTAG
Mgl260735	CGCCCTAGCA	TCGGGTTATC	ATAACCAACC	CGAAATGACT	CAAGAAAAAT	TTAAACCTAG
Mgl280646	CGCCCTAGCA	TCGGGTTATC	ATAACCAACC	CGAAATGACT	CAAGAAAAAT	TTAAACCTAG
Mpcc7941	CGCCCTAGCA	TCGGGTTATC	ATAACCAACC	CGAAATGACT	CAAGAAAAAT	TTAAACCTAG
Miz25	CGCCCTAGCA	TCGGGTTATC	ATAACCAACC	CGAAATGACT	CAAGAAAAAT	TTAAACCTAG
MizM5	CGCCCTAGCA	TCGGGTTATC	ATAACCAACC	CGAAATGACT	CAAGAAAAAT	TTAAACCTAG
Mnies102	CGCCCTAGCA	TCGGGTTATC	ATAACCAACC	CGAAATGACT	CAAGAAAAAT	TTAAACCTAG
MniesA89	CGCCCTAGCA	TCGGGTTATC	ATAACCAACC	CGAAATGACT	CAAGAAAAAT	TTAAACCTAG
Cons-Mic97,4%	CGCCCTAGCA	TCGGGTTATC	ATAACCAACC	CGAAATGACT	CAAGAAAAAT	TTAAACCTAG
P49	AGCCTTAGCA	TCGGGTTATC	ACAATCTTCC	TCAAATCACA	AAAGAAAAAT	TTAAACCTGG
P97	AGCCTTAGCA	TCGGGTTATC	ACAATCTTCC	TCAAATCACA	AAAGAAAAAT	TTAAACCTGG
P126-8	AGCCTTAGCA	TCGGGTTATC	ACAATCTTCC	TCAAATCACA	AAAGAAAAAT	TTAAACCTGG
P127	AGCCTTAGCA	TCggGTTATC	ACAATCTTCC	TCAAATCACA	AAAGAAAAAT	TTAAACCTGG
P128-R	AGCCTTAGCA	TCGGGTTATC	ACAATCTTCC	TCAAATCACA	AAAGAAAAAT	TTAAACCTGG
O213	AGCCTTAGCA	TCGGGTTATC	ACAATCTTCC	TCAAATCACA	AAAGAAAAAT	TTAAACCTGG
O226	AGCCTTAGCA	TCGGGTTATC	ACAATCTTCC	TCAAATCACA	AAAGAAAAAT	TTAAACCTGG
Cons-Plank98,7%	AGCCTTAGCA	TCGGGTTATC	ACAATCTTCC	TCAAATCACA	AAAGAAAAAT	TTAAACCTGG
Nod-HEMclone	AGCATTAGCA	TCAGGTTATC	ACAACCTCCC	CCAAATCACC	GCAGAAAAAT	TTCAACCTAG
Nod-BY1clone	AGCATTAGCA	GCAGGTTATC	ACAACCTCCC	CCAAATCACC	GCAGAAAAAT	TTCAACCTAG
Nod-F81clone	AGCATTAGCA	TCAGGTTATC	ACAACCTCCC	CCAAATCACC	GCAGAAAAAT	TTCAACCTAG
Cons-Nod99,4%	AGCATTAGCA	TCAGGTTATC	ACAACCTCCC	CCAAATCACC	GCAGAAAAAT	TTCAACCTAG
Nostoc152	AGCATTAGCC	GCAGGTTATC	ATAATCTTCC	CGACATCACT	ACAGAAAAAT	TTCAACCCAG

	65	75	85	95	105	115
Ana90-Leo	CTTTTTAAAT	TCAGAAAAAA	TTCTCTTTAG	AACGGGAGAT	TTAGGTAAAC	AAATTGCTCC
Ana299B	CTTTTTAAAT	TCAGAAAAAA	TTCTCTTTAG	AACGGGAGAT	TTAGGTAAAC	AAATTGCTCC
Ana315	CTTTTTAAAT	TCAGAAAAAA	TTCTCTTTAG	AACGGGAGAT	TTAGGTAAAC	AAATTGCTCC
AnaPH256	CTTTTTAAAT	TCAGAAAAAA	TTCTCTTTAG	AACGGGAGAT	TTAGGTAAAC	AAATTGCTCC
Ana318	CTTTTTAAAT	TCAGAAAAAA	TTCTCTTTAG	AACGGGAGAT	TTAGGTAAAC	AAATTGCTCC
Ana202A1	CTTTTTAAAT	TCAGAAAAAA	TTCTCTTTAG	AACGGGAGAT	TTAGGTAAAC	AAATTGCTCC
Ana202A2-41	CTTTTTAAAT	TCAGAAAAAA	TTCTCTTTAG	AACGGGAGAT	TTAGGTAAAC	AAATTGCTCC
Ana66A	CTTTTTAAAT	TCAGGAAAAA	TTCTCTTTAG	AACGGGAGAT	TTAGGTAAAC	AAATTGCTCC
Ana83-1	CTTTTTAAAT	TCAGAAAAAA	TTCTCTTTAG	AACGGGAGAT	TTAGGTAAAC	AAATTGCTCC
Cons-Ana98%	CTTTTTAAAT	TCAGAAAAAA	TTCTCTTTAG	AACGGGAGAT	TTAGGTAAAC	AAATTGCTCC
Mpcc7806-Tillet	CTTTCTTGAT	GAGACAAAAA	CTCTCTTTAG	AACCGGCGAT	TTAGGCAAGC	AAACTGCTCC
M205	CTTTCTTGAT	GAGACAAAAA	CTCTCTTTAG	AACCGGCGAT	TTAGGCAAAC	AAACTGCTCC
M98	CTTTCTTGAT	GAGACAAAAA	CTCTCTTTAG	AACCGGCGAT	TTAGGCAAGC	AAACTGCTCC
Mgl260735	CTTTCTTGAT	GAGACAAAAA	CTCTCTTTAG	AACCGGCGAT	TTAGGCAAAC	AAACTGCTCC
Mgl280646	CTTTCTTGAT	GAGACAAAAA	CTCTCTTTAG	AACCGGCGAT	TTAGGCAAGC	AAACTGCTCC
Mpcc7941	CTTTCTTGAT	GAGACAAAAA	CTCTCTTTAG	AACCGGCGAT	TTAGGCAAGC	AAACTGCTCC
Miz25	CTTTCTTGAT	GAGACAAAAA	CTCTCTTTAG	AACCGGCGAT	TTAGGCAAGC	AAACTGCTCC

Fig. 19B

```
MizM5              CTTTCTTGAT GAGACAAAAA CTCTCTTTAG AACCGGCGAT TTAGGCAAGC AAACTGCTCC
Mnies102           CTTTCTTGAT GAGACAAAAA CTCTCTTTAG AACCGGCGAT TTAGGCAAGC AAACTGCTCC
MniesA89           CTTTCTTGAT GAGACAAAAA CTCTCTTTAG AACCGGCGAT TTAGGCAAGC AAACTGCTCC
Cons-Mic97,4%      CTTTCTTGAT GAGACAAAAA CTCTCTTTAG AACCGGCGAT TTAGGCAAGC AAACTGCTCC
P49                CTTTTTTAAT CAGAAAACAA CGATGTTTAG AACCGGGGAT TTAGGGAAAC AAACTGCTCC
P97                CTTTTTTGAT CAGAAAACAA CGATGTTTAG AACCGGGGAT TTAGGGAAAC AAACTGCTCC
P126-8             CTTTTTTGAT CAGAAAACAA CGATGTTTAG AACCGGGGAT TTAGGGAAAC AAACTGCTCC
P127               CTTTTTTGAT CAGAAAACAA CGATGTTTAG AACCGGGGAT TTAGGGAAAC AAACTGCTCC
P128-R             CTTTTTTGAT CAGAAAACAA CGATGTTTAG AACCGGGGAT TTAGGGAAAC AAACTGCTCC
O213               CTTTTTTAAT CAGAAAACAA CGATGTTTAG AACCGGGGAT TTAGGGAAAC AAACTGCTCC
O226               CTTTTTTAAT CAGAAAACAA CGATGTTTAG AACCGGGGAT TTAGGGAAAC AAACTGCTCC
Cons-Plank98,7%    CTTTTTTGAT CAGAAAACAA CGATGTTTAG AACCGGGGAT TTAGGGAAAC AAACTGCTCC
Nod-HEMclone       CTTTATGACT GAGGGAAAAA CTATCTTTAG AACCGGAGAT TTAGGTAAAC AAATTGCCCC
Nod-BY1clone       CTTTATGACT GAGGGAAAAA CTATCTTTAG AACCGGAGAT TTAGGTAAAC AAATTGCCCC
Nod-F81clone       CTCTATGACT GAGGGAAAAA CTATCTTTAG AACCGGAGAT TTAGGTAAAC AAATTGCCCC
Cons-Nod99,4%      CTTTATGACT GAGGGAAAAA CTATCTTTAG AACCGGAGAT TTAGGTAAAC AAATTGCCCC
Nostoc152          CTTGATAAGT GAGGGAAAAA CTCTCTTTAG AACGGGAGAT TTAGGTAAAC AAACTGCTCC

                   ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                     125        135        145        155        165        175
Ana90-Leo          AGGTGTGATT GAATTTATAG GGCGAAAAGA TAATCAAGTT AAGGTCAATG GCTATCGTGT
Ana299B            AGGTGTGATT GAATTTATAG GGCGAAAAGA TAATCAAGTT AAGGTCAATG GCTATCGTGT
Ana315             AGGTGTGATT GAATTTATAG GGCGAAAAGA TAATCAAGTT AAGGTCAATG GCTATCGTGT
AnaPH256           AGGTGTGATT GAATTTATAG GGCGAAAAGA TAATCAAGTT AAGGTCAATG GCTATCGTGT
Ana318             AGGTGTGATT GAATTTATAG GGCGAAAAGA TAATCAAGTT AAGGTCAATG GCTATCGTGT
Ana202A1           AGGTGTGATT GAATTTATAG GGCGAAAAGA TAATCAAGTT AAGGTCAATG GCTATCGTGT
Ana202A2-41        AGGTGTGATT GAATTTATAG GGCGAAAAGA TAATCAAGTT AAGGTCAATG GCTATCGTGT
Ana66A             AGGTGTGATT GAATTTATAG GGCGAAAAGA TAATCAAGTT AAGGTCAATG GCTATCGTGT
Ana83-1            AGGTGTGATT GAATTTATAG GGCGAAAAGA TAATCAAGTT AAGGTCAATG GCTATCGTGT
Cons-Ana98%        AGGTGTGATT GAATTTATAG GGCGAAAAGA TAATCAAGTT AAGGTCAATG GCTATCGTGT
Mpcc7806-Tillet    CGGTATCATT GAGTTTATGG GACGAAAAGA TAATCAAGTT AAGGTCAATG GTTATCGAAT
M205               AGGTATCATT GAGTTTATGG GACGAAAAGA TAATCAAGTT AAGGTCAATG GTTATCGAAT
M98                GGGTATCATT GAGTTTATGG GACGAAAAGA TAATCAAGTT AAGGTCAATG GTTATCGAAT
Mgl260735          AGGTATCATT GAGTTTATGG GACGAAAAGA TAATCAAGTT AAGGTCAATG GTTATCGAAT
Mgl280646          GGGTATCATT GAGTTTATGG GACGAAAAGA TAATCAAGTT AAGGTCAATG GTTATCGAAT
Mpcc7941           GGGTATCATT GAGTTTATGG GACGAAAAGA TAATCAAGTT AAGGTCAATG GTTATCGAAT
Miz25              CGGTATCATT GAGTTTATGG GACGAAAAGA TAATCAAGTT AAGGTCAATG GTTATCGAAT
MizM5              GGGTATCATT GAGTTTATGG GACGAAAAGA TAATCAAGTT AAGGTCAATG GTTATCGAAT
Mnies102           CGGTATCATT GAGTTTATGG GACGAAAAGA TAATCAAGTT AAGGTCAATG GTTATCGAAT
MniesA89           CGGTATCATT GAGTTTATGG GACGAAAAGA TAATCAAGTT AAGGTCAATG GTTATCGAAT
Cons-Mic97,4%      CGGTATCATT GAGTTTATGG GACGAAAAGA TAATCAAGTT AAGGTCAATG GTTATCGAAT
P49                CGGTGTGATT GAATTTATGG GCAGAAAAGA CAATCAAGTT AAGGTAAATG GCTATCGTAT
P97                CGGTGTAATT GAATTTATGG GCAGAAAAGA CAATCAAGTT AAGGTAAATG GCTATCGTAT
P126-8             CGGTGTGATT GAATTTATGG GCAGAAAAGA CAATCAAGTT AAGGTAAATG GCTATCGTAT
P127               CGGTGTGATT GAATTTATGG GCAGAAAAGA CAATCAAGTT AAGGTAAATG GCTATCGTAT
P128-R             CGGTGTGATT GAATTTATGG GCAGAAAAGA CAATCAAGTT AAGGTAAATG GCTATCGTAT
O213               CGGTGTGATT GAATTTATGG GCAGAAAAGA CAATCAAGTT AAGGTAAATG GCTATCGTAT
O226               CGGTGTGATT GAATTTATGG GCAGAAAAGA CAATCAAGTT AAGGTAAATG GCTATCGTAT
Cons-Plank98,7%    CGGTGTGATT GAATTTATGG GCAGAAAAGA CAATCAAGTT AAGGTAAATG GCTATCGTAT
Nod-HEMclone       AGGCGTGATT GAATTTCTTG GTCGTAAAGA TAATCAAGTT AAGGTGAATG GCTATCGTAT
Nod-BY1clone       AGGCGTGATT GAATTTCTTG GTCGTAAAGA TAATCAAGTT AAGGTGAATG GCTATCGCAT
Nod-F81clone       AGGCGTGATT GAATTTCTTG GTCGTAAAGA TAATCAAGTT AAGGTGAATG GCTATCGTAT
Cons-Nod99,4%      AGGCGTGATT GAATTTCTTG GTCGTAAAGA TAATCAAGTT AAGGTGAATG GCTATCGTAT
Nostoc152          AGGTGTCATT GAATTTATTG GGCGTAAAGA TAATCAAGTA AAGGTGAATG GTTATCGTAT

                   ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                     185        195        205        215        225        235
Ana90-Leo          AGATCCAGGA GAAATTGAAT ACCAAATTAG CCGCTATGCC GAGATTGAGA AAGCAATTGT
Ana299B            AGATCCAGGA GAAATTGAAT ACCAAATTAG CCGCTATGCC GAGATTGAGA AAGCAATTGT
Ana315             AGATCCAGGA GAAATTGAAT ACCAAATTAG CCGCTATGCC GAGATTGAGA AAGCAATTGT
AnaPH256           AGATCCAGGA GAAATTGAAT ACCAAATTAG CCGCTATGCC GAGATTGAGA AAGCAATTGT
```

Fig. 19C


```
Ana318              AGATCCAGGA GAAATTGAAT ACCAAATTAG CCGCTATGCC GAGATTGAGA AAGCAATTGT
Ana202A1            AGATCCAGGA GAACTTGAAT ACCAAATTAG CCGCTATGCC GAGATTGAGA AAGCAATTGT
Ana202A2-41         AGATCCAGGA GAACTTGAAT ACCAAATTAG CCGCTATGCC GAGATTGAGA AAGCAATTGT
Ana66A              AGATCCAGGA GAACTTGAAT ACCAAATTAG CCGCTATGCC GAGATTGAGA AAGCAATTGT
Ana83-1             AGATCCAGGA GAACTTGAAT ACCAAATTAG CCGCTATGCC GAGATTGAGA AAGCAATTGT
Cons-Ana98%         AGATCCAGGA GAAATTGAAT ACCAAATTAG CCGCTATGCC GAGATTGAGA AAGCAATTGT
Mpcc7806-Tillet     TGACCCCGGA GAAATTGAAT ATCAATTGAC TCGTTATGCT CCCATTGAAA GAGCGATTGT
M205                TGACCCCGGA GAAATTGAAT ATCAATTGAC TCGTTATGCT CCCATTGAAA GAGCGATTGT
M98                 TGACCCCGGA GAAATTGAAT ATCAATTGAC TCGTTATGCT CCCATTGAAA GAGCGATTGT
Mgl260735           TGACCCCGGA GAAATTGAAT ATCAATTGAC TCGTTATGCT CCCATTGAAA GAGCGATTGT
Mgl280646           TGACCCCGGA GAAATTGAAT ATCAATTGAC TCGTTATGCT CCCATTGAAA GAGCGATTGT
Mpcc7941            TGACCCCGGA GAAATTGAAT ATCAATTGAC TCGTTATGCT CCCATTGAAA GAGCGATTGT
Miz25               TGACCCCGGA GAAATTGAAT ATCAATTGAC TCGCTATGCT CCCATTGAAA GAGCGATTGT
MizM5               TGATCCCGGA GAAATTGAAT ATCAATTGAC TCGTTATGCT CCCATTGAAA GAGCGATTGT
Mnies102            TGACCCCGGA GAAATTGAAT ATCAATTGAC TCGTTATGCT CCCATTGAAA GAGCGATTGT
MniesA89            TGACCCCGGA GAAATTGAAT ATCAATTGAC TCGTTATGCT CCCATTGAAA GAGCGATTGT
Cons-Mic97,4%       TGACCCCGGA GAAATTGAAT ATCAATTGAC TCGTTATGCT CCCATTGAAA GAGCGATTGT
P49                 CGACCCCGAA GAAATTGAAT ATCAACTTAA TCGTTATCCT CAGATTGAGA GAGCTATTAT
P97                 CGACCCCGAA GAAATTGAAT ATCAACTTAA TCGTTATCCT CAGATTGAGA GAGCTATTAT
P126-8              CGACCCCGAA GAAATTGAAT ATCAACTTAA TCGTTATCCT CAGATTGAGA GAGCTATTAT
P127                CGACCCCGAA GAAATTGAAT ATCAACTTAA TCGTTATCCT CAGATTGAGA GAGCTATTAT
P128-R              CGACCCCGAA GAAATTGAAT ATCAACTTAA TCGTTATCCT CAGATTGAGA GAGCTATTAT
O213                CGACCCCGAA GAAATTGAAT ATCAACTTAA TCGTTATCCT CAGATTGAGA GAGCTATTAT
O226                CGACCCCGAA GAAATTGAAT ATCAACTTAA TCGTTATCCT CAGATTGAGA GAGCTATTAT
Cons-Plank98,7%     CGACCCCGAA GAAATTGAAT ATCAACTTAA TCGTTATCCT CAGATTGAGA GAGCTATTAT
Nod-HEMclone        AGATCCAGGA GAAATTGAAT ACCAACTCAG CCGCCATTCT CAAATTGAGA GAGCAATCGT
Nod-BY1clone        AGATCCAGGA GAAATTGAAT ACCAACTCAG CCGCCATTCT CAAATTGAGA GAGCAATCGT
Nod-F81clone        AGATCCAGGA GAAATTGAAT ACCAACTCAG CCGCCATTCT CAAATTGAGA GAGCAATCGT
Cons-Nod99,4%       AGATCCAGGA GAAATTGAAT ACCAACTCAG CCGCCATTCT CAAATTGAGA GAGCAATCGT
Nostoc152           AGATCCAGGA GAAATTGAAT ATCAACTCAG CCGTCATGCT CAGATTGAAA GAGCGATTAT

                    ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                        245        255        265        275        285        295
Ana90-Leo           CTTACCTATA GAGGTAAATA ACCAAATTCA ATTATCTGCT TATTGTCAAA CTGATAAAGA
Ana299B             CTTACCTATA GAGGTAAATA ACCAAATTCA ATTATCTGCT TATTGTCAAA CTGATAAAGA
Ana315              CTTACCTATA GAGGTAAATA ACCAAATTCA ATTATCTGCT TATTGTCAAA CTGATAAAGA
AnaPH256            CTTACCTATA GAGGTAAATA ACCAAATTCA ATTATCTGCT TATTGTCAAA CTGATAAAGA
Ana318              CTTACCTATA GAGGTAAATA ACCAAATTCA ATTATCTGCT TATTGTCAAA CTGATAAAGA
Ana202A1            CTTACCTATA GAGGTAAATA ACCAAATTCA ATTATCTGCT TATTGTCAAA CTGATAAAGA
Ana202A2-41         CTTACCTATA GAGGTAAATA ACCAAATTCA ATTATCTGCT TATTGTCAAA CTGATAAAGA
Ana66A              CTTACCTATA GAGGTAAATA ACCAAATTCA ATTATCTGCT TATTGTCAAA CTGATAAAGA
Ana83-1             CTTACCTATA GAGGTAAATA ACCAAATTCA ATTATCTGCT TATTGTCAAA CTGATAAAGA
Cons-Ana98%         CTTACCTATA GAGGTAAATA ACCAAATTCA ATTATCTGCT TATTGTCAAA CTGATAAAGA
Mpcc7806-Tillet     TTTACCCGTT CAAGTTAATA ATCAAACTCA ATTATCTGCT TACTGTCAAA CAGACAAAAC
M205                TTTACCCGTT CAAGTTAATA ATCAAACTCA ATTATCTGCT TACTGTCAAA CAGACAAAAC
M98                 TTTACCCGTT CAAGTTAATA ATCAAACTCA ATTATCTGCT TACTGTCAAA CAGACAAAAC
Mgl260735           TTTACCCGTT CAAGTTAATA ATCAAACTCA ATTATCTGCT TACTGTCAAA CAGACAAAAC
Mgl280646           TTTACCCGTT CAAGTTAATA ATCAAACTCA ATTATCTGCT TACTGTCAAA CAGACAAAAC
Mpcc7941            TTTACCCGTT CAAGTTAATA ATCAAACTCA ATTATCTGCT TACTGTCAAA CAGACAAAAC
Miz25               TTTACCCGTT CAAGTTAATA ATCAAACTCA ATTATCTGCT TACTGTCAAA CAGACAAAAC
MizM5               TTTACCCATT CAAGTGAATA ATCAAACTCA ATTATCTGCT TACTGTCAAA CAGAAAAAAC
Mnies102            TTTACCCGTT CAAGTGAATA ATCAAACTCA ATTATCTGCT TACTGTCAAA CAGACAAAAC
MniesA89            TTTACCCGTT CAAGTGAATA ATCAAACTCA ATTATCTGCT TACTGTCAAA CAGACAAAAC
Cons-Mic97,4%       TTTACCCGTT CAAGTTAATA ATCAAACTCA ATTATCTGCT TACTGTCAAA CAGACAAAAC
P49                 TCTACCGATA TCAGTCAATA ATCAAACTCA ATTATCAGCC TATTGTCAAA CCGATAAACA
P97                 TCTACCGATA TCAGTCAATA ATCAAACTCA ATTATCAGCC TATTGTCAAA CAGATAAACA
P126-8              TCTACCGATA TCAGTCAATA ATCAAACTCA ATTATCAGCC TATTGTCAAA CAGATAAACA
P127                TCTACCGATA TCAGTCAATA ATCAAACTCA ATTATCAGCC TATTGTCAAA CAGATAAACA
P128-R              TCTACCGATA TCAGTCAATA ATCAAACTCA ATTATCAGCC TATTGTCAAA CAGATAAACA
O213                TCTACCGATA TCAGTCAATA ATCAAACTCA ATTATCAGCC TATTGTCAAA CCGATAAACA
O226                TCTACCGATA TCAGTCAATA ATCAAACTCA ATTATCAGCC TATTGTCAAA CCGATAAACA
```

Fig. 19D


```
Cons-Plank98,7%      TCTACCGATA TCAGTCAATA ATCAAACTCA ATTATCAGCC TATTGTCAAA CAGATAAACA
Nod-HEMclone         ATTGCCTACT AATGTAGATA ATCAAACCCA GTTATCAGCC TATTGTAAAA CTGAGTCAGA
Nod-BY1clone         ATTGCCTACT AATGTAGATA ATCAAACCCA GTTATCAGCC TATTGTAAAA CTGAGTCAGA
Nod-F81clone         ATTGCCTACT AATGTAGATA ATCAAACCCA GTTATCAGCC TATTGTAAAA CTGAGTCAGA
Cons-Nod99,4%        ATTGCCTACT AATGTAGATA ATCAAACCCA GTTATCAGCC TATTGTAAAA CTGAGTCAGA
Nostoc152            ATTGCCTATC AATGTAGATA ATCAAACTCA ATTATCTGCT TATTGTCAAA CTGATAAAGA

                     ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                         305        315        325        335        345        355
Ana90-Leo            TATAAAATTT TCTGAAATCA GAGAATTTTT AGCTAAATAT TTGCCAGTTT ACATGATTCC
Ana299B              TATAAAATTT TCTGAAATCA GAGAATTTTT AGCTAAATAT TTGCCAGTTT ACATGATTCC
Ana315               TATAAAATTT TCTGAAATCA GAGAATTTTT AGCTAAATAT TTGCCAGTTT ACATGATTCC
AnaPH256             TATAAAATTT TCTGAAATCA GAGAATTTTT AGCTAAATAT TTGCCAGTTT ACATGATTCC
Ana318               TATAAAATTT TCTGAAATCA GAGAATTTTT AGCTAAATAT TTGCCAGTTT ACATGATTCC
Ana202A1             TATAAAAATT TCTGAAATCA GAGAATTTTT AGCTAAATAT TCGCCAGTTT ACATGATTCC
Ana202A2-41          TATAAAAATT TCTGAAATCA GAGAATTTTT AGCTAAATAT TCGCCAGTTT ACATGATTCC
Ana66A               TATAAAAATT TCTGAAATCA GAGAATTTTT AGCTAAATAT TCGCCAGTTT ACATGATTCC
Ana83-1              TATAAAAATT TCTGAAATCA GAGAATTTTT AGCTAAATAT TCGCCAGTTT ACATGATTCC
Cons-Ana98%          TATAAAATTT TCTGAAATCA GAGAATTTTT AGCTAAATAT TTGCCAGTTT ACATGATTCC
Mpcc7806-Tillet      TCTAGAAATT GCTGAGATTC GAGAATTACT TGCCAAATTT TTACCAGTTT ATATGATTCC
M205                 TCTAGAAATT GCTGAGATTC GAGAATTACT TGCCAAATTT TTACCAGTTT ATATGATTCC
M98                  TCTAGAAATT GCTGAGATTC GAGAATTACT TGCCAAATTT TTACCAGTTT ATATGATTCC
Mgl260735            TCTAGAAATT GCTGAGATTC GAGAATTACT TGCCAAATTT TTACCAGTTT ATATGATTCC
Mgl280646            TCTAGAAATT GCTGAGATTC GAGAATTACT TGCCAAATTT TTACCAGTTT ATATGATTCC
Mpcc7941             TCTAGAAATT GCTGAGATTC GAGAATTACT TGCCAAATTT TTACCAGTTT ATATGATTCC
Miz25                TCTAGAAATT GCTGAGATTC GAGAATTACT TGCCAAATTT TTACCAGTTT ATATGATTCC
MizM5                TCTAGAAATT GCTGAGATTC GAGAATTTCT TGCCAAGTTT TTACCAGTTT ATATGATTCC
Mnies102             TCTAGAAATT GCTGAGATTC GAGAATTTCT TGCCAAGTTT TTGCCAGTTT ATATGATTCC
MniesA89             TCTAGAAATT GCTGAGATTC GAGAATTTCT TGCCAAGTTT TTGCCAGTTT ATATGATTCC
Cons-Mic97,4%        TCTAGAAATT GCTGAGATTC GAGAATTACT TGCCAAATTT TTACCAGTTT ATATGATTCC
P49                  GATAGAAATT TCTGAAATCA GAGAATTTCT AGCTAATTTT CTGCCAGTTT ACATGATTCC
P97                  GATAGAAATT TCTGAAATTA GAGAATTTCT AGCTAATTTT CTGCCAGTTT ACATGATTCC
P126-8               GATAGAAATT TCTGAAATTA GAGAATTTCT AGCTAATTTT TTGCCAGTTT ACATGATTCC
P127                 GATAGAAATT TCTGAAATTA GAGAATTTCT AGCTAATTTT TTGCCAGTTT ACATGATTCC
P128-R               GATAGAAATT TCCGAAATTA GAGAATTTTT AGCTAATTTT CTGCCAGTTT ACATGATTCC
O213                 GATAGAAATT TCTGAAATCA GAGAATTTCT AGCTAATTTT CTGCCAGTTT ACATGATTCC
O226                 GATAGAAATT TCTGAAATCA GAGAATTTCT AGCTAATTTT CTGCCAGTTT ACATGATTCC
Cons-Plank98,7%      GATAGAAATT TCTGAAATTA GAGAATTTCT AGCTAATTTT CTGCCAGTTT ACATGATTCC
Nod-HEMclone         CATAGAAATT TCCGAAATTC GAGAATTTCT ATCGAACTTT TTGCCCGTTT ACATGATTCC
Nod-BY1clone         CATAGAAATT TCCGAAATTC GAGAATTTCT ATCGAACTTT TTGCCCGTTT ACATGATTCC
Nod-F81clone         CATAGAAATT TCCGAAATTC GAGAATTTCT ATCGAACTTT TTGCCCGTTT ATATGATTCC
Cons-Nod99,4%        CATAGAAATT TCCGAAATTC GAGAATTTCT ATCGAACTTT TTGCCCGTTT ACATGATTCC
Nostoc152            CATCGAAATT GCTGAAATTA GAGAATTTCT CTCTAAATTT TTGCCAGTTT ATATGATTCC

                     ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                         365        375        385        395        405        415
Ana90-Leo            TAGTTCCTTT ATCTTCTTAA AGCAATTTCC CTTAACTAAA CATGGCAAAT TTGACTTGCG
Ana299B              TAGTTCCTTT ATCTTCTTAA AGCAATTTCC CTTAACTAAA CATGGCAAAT TTGACTTGCG
Ana315               TAGTTCCTTT ATCTTCTTAA AGCAATTTCC CTTAACTAAA CATGGCAAAT TTGACTTCCG
AnaPH256             TAGTTCCTTT ATCTTCTTAA AGCAATTTCC CTTAACTAAA CATGGCAAAT TTGACTTCCG
Ana318               TAGTTCCTTT ATCTTCTTAA AGCAATTTCC CTTAACTAAA CATGGCAAAT TTGACTTGCG
Ana202A1             TAGTTCCTTT ATCTTCTTAA AGCAATTTCC CTTAACTAAA CATGGCAAAC TTGACTTGCG
Ana202A2-41          TAGTTCCTTT ATCTTCTTAA AGCAATTTCC CTTAACTAAA CATGGCAAAC TTGACTTGCG
Ana66A               TAGTTCCTTT ATCTTCTTAA AGCAATTTCC CTTAACTAAA CATGGCAAAC TTGACTTGCG
Ana83-1              TAGTTCCTTT ATCTTCTTAA AGCAATTTCC CTTAACTAAA CATGGCAAAC TTGACTTGCG
Cons-Ana98%          TAGTTCCTTT ATCTTCTTAA AGCAATTTCC CTTAACTAAA CATGGCAAAT TTGACTTGCG
Mpcc7806-Tillet      GAGTTACTTT ATTTTTTTAA AGCAATTCCC CTTAACTCGA CATGGGAAAC TTGACCTGCA
M205                 GAGTTACTTT ATTTTTTTAA AGCAATTCCC CTTAACTCGA CATGGAAAAC TTGACCTGCA
M98                  GAGTTACTTT ATTTTTTTAA AGCAATTCCC CTTAACTCGA CATGGAAAAC TTGACCTGCA
Mgl260735            GAGTTACTTT ATTTTTTTAA AGCAATTCCC CTTAACTCGA CATGGAAAAC TTGACCTGCA
Mgl280646            GAGTTACTTT ATTTTTTTAA AGCAATTCCC CTTAACTCGA CATGGAAAAC TTGACCTGCA
```

Fig. 19E


```
Mpcc7941            GAGTTACTTT ATTTTTTTAA AGCAATTCCC CTTAACTCGA CATGGAAAAC TTGACCTGCA
Miz25               GAGTTACTTT ATTTTTTTAA AGCAATTCCC CTTAACTCGA CATGGGAAAC TTGACCTGCA
MizM5               CAGTTACTTT ATTTTTTTAA AGCAATTCCC TTTAACTCGA CATGGGAAAC TTGACCTGCA
Mnies102            CAGTTACTTT ATTTTTTTAA AGCAATTCCC CTTAACTCGA CATGGGAAAC TTGACCTGCA
MniesA89            CAGTTACTTT ATTTTTTTAA AGCAATTCCC CTTAACTCGA CATGGGAAAC TTGACCTGCA
Cons-Mic97,4%       GAGTTACTTT ATTTTTTTAA AGCAATTCCC CTTAACTCGA CATGGAAAAC TTGACCTGCA
P49                 TAGTTACTTT ATTTTCTTAA AGCAATTCCC CCTAACTAAA CACGGCAAAC TTGACTTAAA
P97                 TAGTTACTTT ATTTTCTTAA AGCAATTTCC CCTAACTAAA CACGGCAAAC TTGACTTAAA
P126-8              TAGTTACTTT ATTTTCTTAA AGCAATTTCC CCTAACTAAA CACGGCAAAC TTGACTTAAA
P127                TAGTTACTTT ATTTTCTTAA AGCAATTTCC CCTAACTAAA CACGGCAAAC TTGACTTAAA
P128-R              TAGTTACTTT ATTTTCTTAA AGCAATTTCC CCTAACTAAA CACGGCAAAC TTGACTTAAA
O213                TAGTTACTTT ATTTTCTTAA AGCAATTCCC CCTAACTAAA CACGGCAAAC TTGACTTAAA
O226                TAGTTACTTT ATTTTCTTAA AGCAATTCCC CCTAACTAAA CACGGCAAAC TTGACTTAAA
Cons-Plank98,7%     TAGTTACTTT ATTTTCTTAA AGCAATTTCC CCTAACTAAA CACGGCAAAC TTGACTTAAA
Nod-HEMclone        TACTTTCTTT ATCTTCTTAA AGCAATTTCC CTTAACCAGA CATGGGAAAA TTGATTTGCG
Nod-BY1clone        TACTTTCTTT ATCTTCTTAA AGCAATTTCC CTTAACCAGA CATGGGAAAA TTGATTTGCG
Nod-F81clone        TACTTTCTTT ATCTTCTTAA AGCAATTTCC CTTAACCAGA CATGGGAAAA TTGATTTGCG
Cons-Nod99,4%       TACTTTCTTT ATCTTCTTAA AGCAATTTCC CTTAACCAGA CATGGGAAAA TTGATTTGCG
Nostoc152           TACTTCCTTT ATCTTCCTCA AGCAATTTCC TCTAACCAGA CATGGCAAAA TTGACTTGCG

                    ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                        425        435        445        455        465        475
Ana90-Leo           ATCGCTCATC GCTCTCAAGC CAACAGATCA ATTAACACAA GTCTC~~~TT ATACTGCACC
Ana299B             ATCGCTCATC GCTCTCAAGC CAACAGATCA ATTAACACAA GTCTC~~~TT ATACTGCACC
Ana315              ATCGCTCATC GCTCTCAAGC CAACAGATCA ATTAACACAA GTCTC~~~TT ATACTGCACC
AnaPH256            ATCGCTCATC GCTCTCAAGC CAACAGATCA ATTAACACAA GTCTC~~~TT ATACTGCACC
Ana318              ATCGCTCATC GCTCTCAAGC CAACAGATCA ATTAACACAA GTCTC~~~TT ATACTGCACC
Ana202A1            ATCGCTCGTC GCTCTCAAGC CGACAGATCA ACTAACACAA GTCTC~~~TT ATACTGCACC
Ana202A2-41         ATCGCTCGTC GCTCTCAAGC CGACAGATCA ACTAACACAA GTCTC~~~TT ATACTGCACC
Ana66A              ATCGCTCGTC GCTCTCAAGC CGACAGATCA ACTAACACAA GTCTC~~~TT ATACTGCACC
Ana83-1             ATCGCTCGTC GCTCTCAAGC CGACAGATCA ACTAACACAA GTCTC~~~TT ATACTGCACC
Cons-Ana98%         ATCGCTCATC GCTCTCAAGC CAACAGATCA ATTAACACAA GTCTC~~~TT ATACTGCACC
Mpcc7806-Tillet     CTCCCTGAGA GAACTCAGAG AAACTGGTAA ATCTCTGGTG AATTCTAATT ATGTTGCACC
M205                CTCCCTGAGA GAACTCAGAG AAACTGGTAA ATCTCTGGTG AATTCTAATT ACGTTGCACC
M98                 CTCCCTGAGA GAACTCAGAG AAACTGGTAA ATCTCTGGTG AATTCTAATT ACGTTGCACC
Mgl260735           CTCCCTGAGA GAACTCAGAG AAACTGGTAA ATCTCTGGTG AATTCTAATT ACGTTGCACC
Mgl280646           CTCCCTGAGA GAACTCAGAG AAACTGGTAA ATCTCTGGTG AATTCTAATT ACGTTGCACC
Mpcc7941            CTCCCTGAGA GAACTCAGAG AAACTGGTAA ATCTCTGGTG AATTCTAATT ACGTTGCACC
Miz25               CTCCCTGAGA GAACTCAGAG AAACTGGTAA ATCTCTGGTG AATTCTAATT ATGTTGCACC
MizM5               CTCCCTGAGA CAACTCAGAG AAACCGGTAA ATATCTGGTT AATTCTAATT ATGTTGCACC
Mnies102            CTCCCTGAGA GAACTCAAAG AAACCAGTAA ATCTCTGGTT AATTCTAATT ATGTTGCACC
MniesA89            CTCCCTGAGA GAACTCAAAG AAACCAGTAA ATCTCTGGTT AATTCTAATT ATGTTGCACC
Cons-Mic97,4%       CTCCCTGAGA GAACTCAGAG AAACTGGTAA ATCTCTGGTG AATTCTAATT ACGTTGCACC
P49                 CTCACTGATT GCACTCAATG AAACCGGGAA ATCTACCCAG GTA~~~AATT ATGTTGCACC
P97                 CTCAATGATT GCACTCAATG AAACCGGGAA ATCTACCCAA GTA~~~AATT ATGTTGCACC
P126-8              CTCAATGATT GCACTCAATG AAACCGGGAA ATCTACCCAA GTA~~~AATT ATGTTGCACC
P127                CTCAATGATT GCACTCAATG AAACCGGGAA ATCTACCCAA GTA~~~AATT ATGTTGCACC
P128-R              CTCAATGATT GCACTCAATG AAACCGGGAA ATCTACCCAA GTA~~~AATT ATGTTGCACC
O213                CTCACTGATT GCACTCAATG AAACCGGGAA ATCTACCCAG GTA~~~AATT ATGTTGCACC
O226                CTCACTGATT GCACTCAATG AAACCGGGAA ATCTACCCAG GTA~~~AATT ATGTTGCACC
Cons-Plank98,7%     CTCAATGATT GCACTCAATG AAACCGGGAA ATCTACCCAA GTA~~~AATT ATGTTGCACC
Nod-HEMclone        ATCCCTGGCT GAATTCAAGG GAATAGGTAA CTTAACACAG TTAGC~~~GT ATACTGCACC
Nod-BY1clone        ATCCCTGGCT GAATTCAAGG GAATAGGTAA CTTAACACAG TTAGC~~~GT ATACTGCACC
Nod-F81clone        ATCCCTGGCT GAATTCAAGG GAATAGGTAA CTTAACACAG TTAGC~~~GT ATACTGCACC
Cons-Nod99,4%       ATCCCTGGCT GAATTCAAGG GAATAGGTAA CTTAACACAG TTAGC~~~GT ATACTGCACC
Nostoc152           ATCGCTTGCT GAACTCCAAG GAATCGGTAA GTTAACACAG GCAGA~~~TT ATACTGCACC

                    ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                        485        495        505        515        525        535
Ana90-Leo           GCGTAATACT TTAGAATCAA AGCTAGTCCA TATTTGGGAA AAAATTCTCA CTAAACATCC
Ana299B             GCGTAATACT TTAGAATCAA AGCTAGTCCA TATTTGGGAA AAAATTCTCA CTAAACATCC
```

Fig. 19F


```
Ana315              GCGTAATACT TTAGAATCAA AGCTAGTCCA TATTTGGGAA AAAATTCTCA CTAAACATCC
AnaPH256            GCGTAATACT TTAGAATCAA AGCTAGTCCA TATTTGGGAA AAAATTCTCA CTAAACATCC
Ana318              GCGTAATACT TTAGAATCAA AGCTAGTCCA TATTTGGGAA AAAATTCTCA CTAAACATCC
Ana202A1            GCGTAATACT TTAGAATCAA AGCTAGTCCA TATTTGGGAA AAAATTCTCA CTAAACATCC
Ana202A2-41         GCGTAATACT TTAGAATCAA AGCTAGTCCA TATTTGGGAA AAAATTCTCA CTAAACATCC
Ana66A              GCGTAATACT TTAGAATCAA AGCTAGTCCA TATTTGGGAA AAAATTCTCA CTAAACATCC
Ana83-1             GCGTAATACT TTAGAATCAA AGCTAGTCCA TATTTGGGAA AAAATTCTCA CTAAACATCC
Cons-Ana98%         GCGTAATACT TTAGAATCAA AGCTAGTCCA TATTTGGGAA AAAATTCTCA CTAAACATCC
Mpcc7806-Tillet     CCGGAATTAT TTAGAATCCA ATCTCGTTAG TATCTGGGAA AAAATTCTCT CTAAACATCC
M205                CCGGAATTAT TTAGAATCCA ATCTCGTTAG TATCTGGGAA AAAATTCTCT CTAAACATCC
M98                 CCGGAATTAT TTAGAATCCA ATCTCGTTAG TATCTGGGAA AAAATTCTCT CTAAACATCC
Mgl260735           CCGGAATTAT TTAGAATCCA ATCTCGTTAG TATCTGGGAA AAAATTCTCT CTAAACATCC
Mgl280646           CCGGAATTAT TTAGAATCCA ATCTCGTTAG TATCTGGGAA AAAATTCTCT CTAAACATCC
Mpcc7941            CCGGAATTAT TTAGAATCCA ATCTCGTTAG TATCTGGGAA AAAATTCTCT CTAAACATCC
Miz25               CCGGAATTAT TTAGAATCCA ATCTCGTTAG TATCTGGGAA AAAATTCTCT CTAAACATCC
MizM5               CCGTAATCAT TTAGAATCCA ATCTCGTTAG TATCTGGGAA AAAATTCTCT CTAAACATCC
Mnies102            CCGTAATCAT TTAGAATCCA ATCTCGTTAG TATCTGGGAA AAAATTCTCT CTAAACATCC
MniesA89            CCGTAATCAT TTAGAATCCA ATCTCGTTAG TATCTGGGAA AAAATTCTCT CTAAACATCC
Cons-Mic97,4%       CCGGAATTAT TTAGAATCCA ATCTCGTTAG TATCTGGGAA AAAATTCTCT CTAAACATCC
P49                 GCGTAATAAT TTAGAGTCAA ACCTAGTTAG AATCTGGGAA AAGATTCTGA CCAAACATCC
P97                 GCGTAATAAT TTAGAGTCAA ACCTAGTTAG AATCTGGGAA AAGATTCTGA CCAAACATCC
P126-8              GCGTAATAAT TTAGAGTCAA ACCTAGTTAG AATCTGGGAA AAGATTCTAA CCAAACATCC
P127                GCGTAATAAT TTAGAGTCAA ACCTAGTTAG AATCTGGGAA AAGATTCTAA CCAAACATCC
P128-R              GCGTAATAAT TTAGAGTCAA ACCTAGTTAG AATCTGGGAA AAGATTCTAA CCAAACATCC
O213                GCGTAATAAT TTAGAGTCAA ACCTAGTTAG AATCTGGGAA AAGATTCTGA CCAAACATCC
O226                GCGTAATAAT TTAGAGTCAA ACCTAGTTAG AATCTGGGAA AAGATTCTGA CCAAACATCC
Cons-Plank98,7%     GCGTAATAAT TTAGAGTCAA ACCTAGTTAG AATCTGGGAA AAGATTCTGA CCAAACATCC
Nod-HEMclone        GCGCAATAAT TTAGAGTCCA AGCTCGTACA TATTTGGGAA AAAATTCTCA CCAAACAACC
Nod-BY1clone        GCGCAATAAT TTAGAGTCCA AGCTCGTACA TATTTGGGAA AAAATTCTCA CCAAACAACC
Nod-F81clone        GCGCAATAAT TTAGAGTCCA AGCTCGTACA TATTTGGGAA AAAATTCTCA CCAAACAACC
Cons-Nod99,4%       GCGCAATAAT TTAGAGTCCA AGCTCGTACA TATTTGGGAA AAAATTCTCA CCAAACAACC
Nostoc152           GCGCAATGAT TTAGAGTCCA AGCTAGTAAA GATTTGGGAA AAAATTCTCA CCACACATCC

                    ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                        545        555        565        575        585        595
Ana90-Leo           CATTGGAATT TTTGATAACT TTTTTGAAAT CGGCGGACAC TCTCTGCTCC TTTCTAGAGT
Ana299B             CATTGGAATT TTTGATAACT TTTTTGAAAT CGGCGGACAC TCTCTGCTCC TTTCTAGAGT
Ana315              CATTGGAATT TTTGATAACT TTTTTGAAAT CGGCGGACAC TCTCTGCTCC TTTCTAGAGT
AnaPH256            CATTGGAATT TTTGATAACT TTTTTGAAAT CGGCGGACAC TCTCTGCTCC TTTCTAGAGT
Ana318              CATTGGAATT TTTGATAACT TTTTTGAAAT CGGCGGACAC TCTCTGCTCC TTTCTAGAGT
Ana202A1            CATTGGAATT TTTGATAACT TTTTTGAAAT CGGCGGACAC TCTCTGCTCC TTTCTAGAGT
Ana202A2-41         CATTGGAATT TTTGATAACT TTTTTGAAAT CGGCGGACAC TCTCTGCTCC TTTCTAGAGT
Ana66A              CATTGGAATT TTTGATAACT TTTTTGAAAT CGGCGGACAC TCTCTGCTCC TTTCTAGAGT
Ana83-1             CATTGGAATT TTTGATAACT TTTTTGAAAT CGGCGGACAC TCTCTGCTCC TTTCTAGAGT
Cons-Ana98%         CATTGGAATT TTTGATAACT TTTTTGAAAT CGGCGGACAC TCTCTGCTCC TTTCTAGAGT
Mpcc7806-Tillet     TATCGGTATT TTTGATAACT TCTTTGAAAT TGGCGGTCAT TCTCTACTCT TATCAAGGGT
M205                TATCGGTATT TTTGATAACT TCTTTGAAAT TGGCGGTCAT TCTCTACTCT TATCAAGGGT
M98                 TATCGGTATT TTTGATAACT TCTTTGAAAT TGGCGGTCAT TCTCTACTCT TATCAAGGGT
Mgl260735           TATCGGTATT TTTGATAACT TCTTTGAAAT TGGCGGTCAT TCTCTACTCT TATCAAGGGT
Mgl280646           TATCGGTATT TTTGATAACT TCTTTGAAAT TGGCGGTCAT TCTCTACTCT TATCAAGGGT
Mpcc7941            TATCGGTATT TTTGATAACT TCTTTGAAAT TGGCGGTCAT TCTCTACTCT TATCAAGGGT
Miz25               TATCGGTATT TTTGATAACT TCTTTGAAAT TGGCGGTCAT TCTCTACTCT TATCAAGGGT
MizM5               TATCGGTATT TTTGATAACT TCTTTGAAAT TGGCGGTCAT TCTCTACTCT TATCAAGGGT
Mnies102            TATCGGTATT TTTGATAACT TTTTTGAAAT TGGTGGTCAT TCTCTACTCT TATCAAGGGT
MniesA89            TATCGGTATT TTTGATAACT TCTTTGAAAT TGGCGGTCAT TCTCTACTCT TATCAAGGGT
Cons-Mic97,4%       TATCGGTATT TTTGATAACT TCTTTGAAAT TGGCGGTCAT TCTCTACTCT TATCAAGGGT
P49                 CATCGGTATT TTTGATAACT TCTTTGAAAT TGGCGGACAT TCTCTGATGC TTTCGAGAAT
P97                 CATCGGTATT TTTGATAACT TCTTTGAAAT TGGCGGACAT TCTCTGATGC TTTCGAGAAT
P126-8              CATCGGTATT TTTGATAACT TCTTTGAAAT TGGCGGACAT TCTCTGATGC TTTCGAGAAT
P127                CATCGGTATT TTTGATAACT TCTTTGAAAT TGGCGGACAT TCTCTGATGC TTTCGAGAAT
P128-R              CATCGGTATT TTTGATAACT TCTTTGAAAT TGGCGGACAT TCTCTGATGC TTTCGAGAAT
```

Fig. 19G

```
O213                 CATCGGTATT TTTGATAACT TCTTTGAAAT TGGCGGACAT TCTCTGATGC TTTCGAGAAT
O226                 CATCGGTATT TTTGATAACT TCTTTGAAAT TGGCGGACAT TCTCTGATGC TTTCGAGAAT
Cons-Plank98,7%      CATCGGTATT TTTGATAACT TCTTTGAAAT TGGCGGACAT TCTCTGATGC TTTCGAGAAT
Nod-HEMclone         CATTGGCATT TTTGATAACT TCTTTGAAAT TGGTGGACAC TCACTGCTGC TTTCCAGAGT
Nod-BY1clone         CATTGGCATT TTTGATAACT TCTTTGAAAT TGGTGGACAC TCACTGCTGC TTTCCAGAGT
Nod-F81clone         CATTGGCATT TTTGATAACT TCTTTGAAAT TGGTGGACAC TCACTGCTGC TTTCCAGAGT
Cons-Nod99,4%        CATTGGCATT TTTGATAACT TCTTTGAAAT TGGTGGACAC TCACTGCTGC TTTCCAGAGT
Nostoc152            CATCGGCATT TTTGATAACT TCTTTGAAAT TGGTGGACAC TCGCTGCTGC TTTCGAGAGT

                     ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                           605        615        625        635        645        655
Ana90-Leo            AGTCACTCAC GTCCATAAAG AATTAAATGT ATTAGTTAAA TTAGCTGATT TCTTCAAAGT
Ana299B              AGTCACTCAC GTCCATAAAG AATTAAATGT ATTAGTTAAA TTAGCTGATT TCTTCAAAGT
Ana315               AGTCACTCAC GTCCATAAAG AATTAAATGT ATTAGTTAAA TTAGCTGATT TCTTCAAAGT
AnaPH256             AGTCACTCAC GTCCATAAAG AATTAAATGT ATTAGTTAAA TTAGCTGATT TCTTCAAAGT
Ana318               AGTCACTCAC GTCCATAAAG AATTAAATGT ATTAGTTAAA TTAGCTGATT TCTTCAAAGT
Ana202A1             AGTCACTCAC GTCCATAAAG AATTAAATGT ATTAGTTAAA TTAGCTGATT TCTTCAAAGT
Ana202A2-41          AGTCACTCAC GTCCATAAAG AATTAAATGT ATTAGTTAAA TTAGCTGATT TCTTCAAAGT
Ana66A               AGTCACTCAC GTCCATAAAG AATTAAATGT ATTAGTTAAA TTAGCTGATT TCTTCAAAGT
Ana83-1              AGTCACTCAC GTCCATAAAG AATTAAATGT ATTAGTTAAA TTAGCTGATT TCTTCAAAGT
Cons-Ana98%          AGTCACTCAC GTCCATAAAG AATTAAATGT ATTAGTTAAA TTAGCTGATT TCTTCAAAGT
Mpcc7806-Tillet      TGTAACCCGG GTTCATAAAG AACTAAATGT ATCCGTAAAA TTGGCTGACT TCTTTAAAGT
M205                 TGTAACCCGG GTTCATAAAG AACTAAATGT ATCCGTAAAA TTAGCTGACT TCTTTAAAGT
M98                  TGTAACCCGG GTTCATAAAG AACTAAATGT ATCCGTAAAA TTAGCTGACT TCTTTAAAGT
Mgl260735            TGTAACCCGG GTTCATAAAG AACTAAATGT ATCCGTAAAA TTAGCTGACT TCTTTAAAGT
Mgl280646            TGTAACCCGG GTTCATAAAG AACTAAATGT ATCCGTAAAA TTAGCTGACT TCTTTAAAGT
Mpcc7941             TGTAACCCGG GTTCATAAAG AACTAAATGT ATCCGTAAAA TTAGCTGACT TCTTTAAAGT
Miz25                TGTAACCCGG GTTCATAAAG AACTAAATGT ATCCGTAAAA TTGGCTGACT TCTTTAAAGT
MizM5                TGTAACCCGG GTTCATAAAG AACTAAATGT ATCCGTAAAA TTGGCTGACT TCTTTAAAGT
Mnies102             TGTAACCCGG GTTCATAAAG AACTAAATGT ATCCGTAAAA TTAGCTGACT TCTTTAAAGT
MniesA89             TGTAACCCGG GTTCATAAAG AACTAAATGT ATCCGTAAAA TTGGCTGACT TCTTTAAAGT
Cons-Mic97,4%        TGTAACCCGG GTTCATAAAG AACTAAATGT ATCCGTAAAA TTAGCTGACT TCTTTAAAGT
P49                  CGTAACCCAC GTTCATAAAG AATTAAATGT ATCGGTAAAA TTGGCTGACT TCTTTAAAGT
P97                  CGTAACCCAC GTTCATAAAG AATTAAATGT ATCGGTAAAA TTGGCTGACT TCTTTAAAGT
P126-8               CGTAACCCAC GTTCATAAAG AATTAAATGT ATCGGTAAAA TTGGCTGACT TCTTTAAAGT
P127                 CGTAACCCAC GTTCATAAAG AATTAAATGT ATCGGTAAAA TTGGCTGACT TCTTTAAAGT
P128-R               CGTAACCCAC GTTCATAAAG AATTAAATGT ATCGGTAAAA TTGGCTGACT TCTTTAAAGT
O213                 CGTAACCCAC GTTCATAAAG AATTAAATGT ATCGGTAAAA TTGGCTGACT TCTTTAAAGT
O226                 CGTAACCCAC GTTCATAAAG AATTAAATGT ATCGGTAAAA TTGGCTGACT TCTTTAAAGT
Cons-Plank98,7%      CGTAACCCAC GTTCATAAAG AATTAAATGT ATCGGTAAAA TTGGCTGACT TCTTTAAAGT
Nod-HEMclone         GGTAACTCAC GTTCATAAAG AATTAAATGT GTTGGTAAAA TTGGCTGAAT TCTTTAAAGT
Nod-BY1clone         GGTAACTCAC GTTCATAAAG AATTAAATGT GTTGGTAAAA TTGGCTGAAT TCTTTAAAGT
Nod-F81clone         GGTAACTCAC GTTCATAAAG AATTAAATGT GTTGGTAAAA TTGGCTGAAT TCTTTAAAGT
Cons-Nod99,4%        GGTAACTCAC GTTCATAAAG AATTAAATGT GTTGGTAAAA TTGGCTGAAT TCTTTAAAGT
Nostoc152            GGTAACTTAC GTTCATAAAG AATTAAATGT GTTAGTCAAA TTGGCTGACT TTTTTAAAGT

                     ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                           665        675        685        695        705        715
Ana90-Leo            TCCCACAATT CTTGGATTAG CAGCTTTAAT ATCTAAAGCT CAATCTAACT ATCAAGAACC
Ana299B              TCCCACAATT CTTGGATTAG CAGCTTTAAT ATCTAAAGCT CAATCTAACT ATCAAGAACC
Ana315               TCCCACAATT CTTGGATTAG CAGCTTTAAT ATCTAAAGCT CAATCTAACT ATCAAGAACC
AnaPH256             TCCCACAATT CTTGGATTAG CAGCTTTAAT ATCTAAAGCT CAATCTAACT ATCAAGAACC
Ana318               TCCCACAATT CTTGGATTAG CAGCTTTAAT ATCTAAAGCT CAATCTAACT ATCAAGAACC
Ana202A1             TCCCACAATT CTTGGATTAG CAGCTTTAAT ATCTAAAGCT CAATCTAACT ATCAAGAACC
Ana202A2-41          TCCCACAATT CTTGGATTAG CAGCTTTAAT ATCTAAAGCT CAATCTAACT ATCAAGAACC
Ana66A               TCCCACAATT GTTGGATTAG CAGCTTTAAT ATCTAAAGCT CAATCTAACT ATCAAGAACC
Ana83-1              TCCCACAATT CTTGGATTAG CAGCTTTAAT ATCTAAAGCT CAATCTAACT ATCAAGAACC
Cons-Ana98%          TCCCACAATT CTTGGATTAG CAGCTTTAAT ATCTAAAGCT CAATCTAACT ATCAAGAACC
Mpcc7806-Tillet      TCCAACCATT GCTGGATTGG CTACTTTAAT CTCCCAGACT CAATACAATT ATCAAGAACC
M205                 TCCAACCGTT GCTGGATTGG CGACTTTAAT CTCCCAGACT CAATACAATT ATCAAGAACC
M98                  TCCAACCGTT GCTGGATTGG CGACTTTAAT CTCCCAGACT CAATACAATT ATCAAGAACC
```