# Genetic Diversity and Microcystin Production by *Anabaena* in the Gulf of Finland, Baltic Sea

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**Academic Dissertation in Microbiology**

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Front cover Maria B. Raunio, *Boys at the seashore*
(2008; pencil, charcoal)

*To the pieces of my hope:*

*Aaro, Alvar, and Aatos,*
*Jolàn and Amrita,*
*& Miilo*

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## LIST OF ORIGINAL ARTICLES

**This thesis is based on the following original articles, which are referred to by their Roman numerals in the text.**

**I.** **Katrianna Halinen,** Jouni Jokela, David P. Fewer, Matti Wahlsten, and Kaarina Sivonen. 2007. Direct evidence for production of microcystins by *Anabaena* strains from the Baltic Sea. Appl. Environ. Microbiol. 73:6543-6550.

**II.** **Katrianna Halinen,** David P. Fewer, Leila M. Sihvonen, Christina Lyra, Eeva Eronen, and Kaarina Sivonen. 2008. Genetic diversity in strains of the genus *Anabaena* isolated from planktonic and benthic habitats of the Gulf of Finland (Baltic Sea). FEMS Microbiol. Ecol. 64:199-208.

**III.** David P. Fewer, Miikka Köykkä, **Katrianna Halinen**, Jouni Jokela, Christina Lyra, and Kaarina Sivonen. Culture-independent evidence for the persistent presence and genetic diversity of microcystin-producing *Anabaena* (cyanobacteria) in the Gulf of Finland. Environ.l Microbiol., doi: 10.1111/j.1462-2920.2008.01806.x

**IV.** **Katrianna Halinen,** David P. Fewer, and Kaarina Sivonen. Natural insertional inactivation of the microcystin synthetase gene cluster in the Baltic Sea *Anabaena*. Submitted manuscript.

## THE AUTHOR'S CONTRIBUTION

**I.** The author was responsible for the original idea of the study and experimental design. She isolated the strains, extracted DNA, and constructed phylogenetic trees based on the 16S rRNA gene sequences. The author extracted microcystins from the strains for toxin analyses. She did the PCA analysis based on environmental data. The author wrote the manuscript.

**II.** The author was responsible for the original idea and designed the study. She collected the majority of the planktonic strains, did the PCR work for the majority of the strains, and performed 16S rRNA, *rbcL*, and *rpoC1* phylogenetic analyses. The author wrote the manuscript.

**III.** The author participated in the inception of this study and experimental design. She conducted the statistical analyses of the environmental data together with Doc. Christina Lyra. The author contributed to the writing of the manuscript.

**IV.** The author designed this study together with Dr. David Fewer. She did the PCR work for *Anabaena* strains, and analyzed environmental water samples. She designed most of the primers used for screening. The author wrote the manuscript.

## ABBREVIATIONS AND DEFINITIONS

| | |
|---|---|
| Adda | (2S,3S,8S,9S)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6,-dienoic acid |
| Akinete | A specialised cell found in certain cyanobacteria that resists e.g. desiccation and cold |
| ANI | Average nucleotide identity of all conserved genes |
| Asp | Aspartic acid (aspartate) |
| Benthos (benthic) | The organisms living on or in the bottom of a body of water |
| Bloom | An abundant growth of algae (in this case cyanobacteria), often resulting from nutrient enrichment |
| bp | Base pair |
| CAP | Canonical analysis of principle coordinates |
| CTD | Conductivity-temperature-depth |
| Cytotoxic | A substance having a toxic effect on cells |
| DGGE | Denaturing gradient gel electrophoresis |
| Dha | Dehydroalanine |
| Diazotrophy (diazotrophic) | Ability to fix atmospheric nitrogen gas into a more usable form such as ammonia |
| DIN | Dissolved inorganic nitrogen |
| DIP | Dissolved inorganic phosphorus |
| DISTLM | Distance-based multivariate analysis for a linear model |
| DNA | Deoxyribonucleic acid |
| dNTP | A generic term referring to the deoxyribonucleotides: dATP, dCTP, dGTP and dTTP |
| dw | Dry weight |
| Ecotype | "*… is a population that has acquired a small number of extra genetic elements or mutations, enabling the population to exploit a slightly different ecological niche but preserving the genetic signature and the full ecological potential that characterizes its species*" (according to Konstantinidis and Tiedje, 2004) |
| ELISA | Enzyme-linked immunosorbent assay |
| Epilithon (epilithic) | The organisms that grow on stone |
| Epiphyte (epiphytic) | The organisms that grow attached to the plant without doing harm (no parasitic association) |
| ERIC-PCR | Enterobacterial repetitive intergenic consensus sequences –polymerase chain reaction |
| ESI | Electrospray ionisation |
| GC clamp | A stretch of guanine-cytosine -rich sequences used in denaturing gradient gel electrophoresis |
| GTR+I+G | A Generalised Time Reversible model of DNA substitution. |
| *gvpA* | Gene encoding the main structural gas vesicle protein |
| Hepatotoxin | A substance having an adverse effect on the liver |
| Heterocyte | A specialised cell responsible for nitrogen fixation in certain cyanobacteria |
| *hetR* | Gene responsible for heterocyst differentiation |

| | |
|---|---|
| HGT | Horizontal gene transfer |
| HPLC | High-performance liquid chromatography |
| ITS | Internal transcribed spacer region |
| LC-MS | Liquid chromatography – mass spectrometry |
| LD | The dose that kills 50 % of the test organisms |
| LGT | Lateral gene transfer |
| MALDI-TOF-MS | Matrix-assisted laser desorption ionisation time-of-flight mass spectrometry |
| *mcy* | Microcystin synthetase gene cluster |
| *mcyD* | Microcystin synthetase gene D |
| *mcyE* | Microcystin synthetase gene E |
| MCYST | Microcystin |
| MCYST-HtyR | Microcystin which has homotyrosine and arginine in positions X and Y, respectively (see **Fig. 4**) |
| MCYST-LR | Microcystin which has leucine and arginine in positions X and Y, respectively (see **Fig. 4**) |
| ML | Maximum likelihood |
| Morphospecies | A species distinguished solely on the basis of morphology |
| Morphotype | A specimen chosen to illustrate a morphological variation (physical characteristic) within a species population |
| MP | Maximum parsimony |
| MSN | Minimum spanning network |
| *m/z* | Ratio of molecular mass and ion charge |
| *ndaF* | Nodularin synthetase gene F |
| Neurotoxin | A substance having a toxic effect on the nervous system |
| *nif*H | Gene encoding the nitrogenase iron protein subunit |
| NJ | Neighbor-joining |
| nt | Nucleotide |
| ORF | Open reading frame; a portion of an organism's genome which contains a sequence that could encode a protein |
| PCA | Principal component analysis |
| PC-IGS | Phycocyanin operon intergenic spacer |
| PCR | Polymerase chain reaction |
| Phenotype | Any observable characteristic of an organism, such as e.g. its morphology, development or physiological properties |
| Phytoplankton | Photosynthetic microscopic aquatic organisms that float freely or have weak swimming abilities |
| Plankton | The organisms inhabiting the surface layer of a sea or lake |
| PSU | Practical salinity unit |
| Q-PCR | Quantitative polymerase chain reaction |
| *rbcL* | Gene encoding the D-ribulose 1,5-bisphosphate carboxylase-oxygenase |
| *rbcLX* | Gene encoding the D-ribulose 1,5-bisphosphate carboxylase-oxygenase and intergenic region |
| REP-PCR | Repetitive extragenic palindromic -polymerase chain reaction |

| | |
|---|---|
| *rpoB* | Gene encoding the DNA-dependent RNA-polymerase β-subunit |
| *rpoC1* | Gene encoding the DNA-dependent RNA-polymerase C-subunit |
| RNA | Ribonucleic acid |
| rRNA | Ribosomal ribonucleic acid |
| RT-PCR | Reverse-transcriptase polymerase chain reaction |
| sp. | Species |
| spp. | Species (plural) |
| WHO | World Health Organisation |
| $\pi$ | Nucleotide diversity; is used to measure the degree of polymorphism within a population |

## ABSTRACT

Cyanobacteria (blue-green algae) form blooms in the Baltic Sea during the warmest summer months. According to paleolimnological data, cyanobacteria have long history in the Baltic Sea, going back at least 7000 years. However, the intensity as well as the expanse of cyanobacterial blooms has increased during recent decades. Blooms attract regular attention in the media because of their visibility and the potential health risk they pose to humans and animals. The Gulf of Finland is the most eutrophied area of the Baltic Sea, and cyanobacterial blooms are widely believed to be the result of intense anthropogenic nutrient loading.

Cyanobacterial blooms are formed mainly by species of three genera in the Baltic Sea, *Nodularia*, *Anabaena* and *Aphanizomenon*. The focus of present-day research on Baltic Sea cyanobacteria has been on *Nodularia* and *Aphanizomenon*, while the genus *Anabaena* has been neglected. *Anabaena* is often considered to play a minor role in cyanobacterial blooms. However, *Anabaena* can form a significant part of the blooms, especially in the northern part of the Baltic Sea. Cyanobacterial blooms in the Baltic Sea are invariably toxic due to the production of hepatotoxic nodularin by *Nodularia spumigena*. According to systematic studies, *Aphanizomenon flos-aquae* was not found to produce hepatotoxins in the Baltic Sea. However, it has been speculated that Baltic Sea *Anabaena* spp. could produce microcystins. The genetic structure of the *Anabaena* populations in the Baltic Sea has not been systematically explored. The aim of this present study was to increase our understanding of the *Anabaena* - a component of the Baltic Sea phytoplankton.

Altogether, 49 planktonic *Anabaena* strains were isolated from the Gulf of Finland, five of which were microcystin-producing. This study provided unequivocal evidence that Baltic Sea *Anabaena* is able to produce microcystins. Each microcystin-producing *Anabaena* strain produced two to four dominant microcystin variants, including the highly toxic microcystin-LR. In this study, a culture-independent method was designed to detect putative microcystin and nodularin producers. By means of this DGGE method, microcystin-producing *Anabaena* populations were detected in cyanobacterial bloom samples from the summers of 2003 and 2004. Microcystin-producing *Anabaena* populations were detected throughout the Gulf of Finland. This excluded the possibility that the presence of microcystin-producing *Anabaena* was a chance phenomenon. Results suggest that salinity may limit the distribution of the microcystin-producing *Anabaena* although further studies are needed to confirm the interdependence of salinity and microcystin production.

Microcystin-producing *Anabaena* populations were found to be highly diverse on analyses of the 16S rRNA, *rbcL*, *rpoC1*, and *mcyE* gene sequences. In previous studies, freshwater microcystin-producing *Anabaena* strains were grouped together in phylogenetic analyses. All microcystin-producing Baltic Sea *Anabaena* strains belonged to this hepatotoxic cluster, with the exception of a single strain. Both planktonic and benthic *Anabaena* populations were genetically heterogeneous and closely related to freshwater *Anabaena* strains. However, genetic diversity in benthic *Anabaena* strains was higher than in planktonic strains. In phylogenetic analyses, novel *Anabaena* lineages, possibly specific to the Baltic Sea, were identified. This suggests ecotypic diversification within *Anabaena* populations.

We found two planktonic *Anabaena* strains which carried the entire *mcy* gene cluster, but were nonetheless incapable of producing microcystins. Natural genetic inactivation

of the *mcy* gene cluster was identified in *Anabaena* strain BIR259. This strain carried insertions which most likely caused the inactivation of the *mcy* genes. The insertions documented here were surprisingly common in the Baltic Sea bloom samples and they were present in samples from both studied summers, 2003 and 2004. However, these insertions were not identified in freshwater strains or in field samples from freshwater lakes.

The aim of this study was to establish a strain collection of Baltic Sea *Anabaena* and to shed light on the phylogeny, microcystin production, and genetic diversity of the Baltic Sea *Anabaena* populations. In addition to strain isolation, these research goals were approached by *in situ* molecular methods. Systematic toxin screening showed that *Anabaena* is able to produce microcystins, and this should be taken into account in future toxin monitoring programmes.

# TIIVISTELMÄ (ABSTRACT IN FINNISH)

Syanobakteerit (sinilevät) ovat olleet Itämeressä koko nykymuotoisen Itämeren ajan, sillä paleolimnologiset todisteet niiden olemassaolosta Itämeren alueella ovat noin 7000 vuoden takaa. Syanobakteerien massaesiintymät eli kukinnat ovat kuitenkin sekä levinneet laajemmille alueille että tulleet voimakkaimmiksi viimeisten vuosikymmenien aikana. Tähän on osasyynä ihmisten aiheuttama kuormitus, joka rehevöittää Itämerta. Suomenlahti, jota tämä tutkimus käsittelee, on kärsinyt tästä rehevöitymiskehityksestä muita Itämeren altaita enemmän.

Syanobakteerit muodostavat jokakesäisiä kukintoja Suomenlahdella - niin sen avomerialueilla kuin rannoillakin. Yleisimmät kukintoja muodostavat syanobakteerisuvut ovat *Nodularia*, *Anabaena* ja *Aphanizomenon*. Kukinnat aiheuttavat paitsi esteettistä haittaa myös terveydellisen riskitekijän. Niiden myrkyllisyys liitetään usein *Nodularia*-suvun tuottamaan nodulariini-maksamyrkkyyn. Itämeren *Aphanizomenon*-suvun on todettu olevan myrkytön. Vaikka Itämeren kukintoja aiheuttavista *Nodularia*- ja *Aphanizomenon*-syanobakteereista tiedetään varsin paljon, on molekyylimenetelmiin pohjautuva syanobakteeritutkimus ohittanut Itämeren *Anabaena*-suvun monelta osin. Tämän työn tarkoituksena oli syventää käsitystämme Itämeren *Anabaena*-syanobakteerista, sen mahdollisesta myrkyllisyydestä, geneettisestä monimuotoisuudesta ja fylogeneettisista sukulaisuussuhteista.

Tässä työssä eristettiin 49 planktista *Anabaena*-kantaa, joista viisi tuottivat mikrokystiinejä. Tämä oli ensimmäinen yksiselitteinen todiste, että Itämeren *Anabaena* tuottaa maksamyrkyllisiä mikrokystiini-yhdisteitä. Jokainen eristetty myrkyllinen *Anabaena*-kanta tuotti useita mikrokystiini-variantteja. Lisäksi mikrokystiinejä löydettiin kukintanäytteistä, joissa oli myrkkyä syntetisoivia geenejä sisältäneitä *Anabaena*-syanobakteereita. Myrkkyjä löydettiin molempina tutkimusvuosina 2003 ja 2004. Myrkkyjen esiintyminen ei siten ollut vain yksittäinen ilmiö. Tässä työssä saimme viitteitä siitä, että maksamyrkyllinen *Anabaena*-syanobakteeri esiintyisi vähäsuolaisissa vesissä. Tämä riippuvuussuhde jää kuitenkin tulevien tutkimuksien selvitettäväksi.

Tässä työssä havaittiin mikrokystiinisyntetaasi-geenien inaktivoituminen Itämeren *Anabaena*-kannassa ja kukintanäytteissä. Kuvasimme *Anabaena*-kannan mikrokystiinisyntetaasigeenien sisältä insertioita, jotka hyvin todennäköisesti inaktivoivat myrkyntuoton. Insertion sisältäneeltä kannalta löysimme kuitenkin kaikki mikrokystiinisyntetaasigeenit osoittaen, että geenien olemassaolo ei välttämättä varmista kannan mikrokystiinintuottoa. Mielenkiintoista oli se, että inaktivaation aiheuttavia insertioita löytyi kukintanäytteistä molemmilta tutkimusvuosilta. Vastaavia insertioita ei kuitenkaan löydetty makean veden *Anabaena*-kannoista tai järvinäytteistä.

On yleistä, että syanobakteerikukinnoista löytyy usean syanobakteerisuvun edustajia. Myrkyllisiä sukuja tai lajeja ei voida kuitenkaan erottaa mikroskooppisesti myrkyttömistä. Käsillä olevassa tutkimuksessa kehitettiin molekyylimenetelmä, jolla on mahdollista määrittää kukinnan mahdollisesti maksamyrkylliset syanobakteerisuvut. Tätä menetelmää sovellettiin Itämeren kukintojen tutkimiseen.

Itämeren pintavesistä ja ranta-alueiden pohjasta eristetyt *Anabaena*-kannat osoittautuivat geneettisesti monimuotoisiksi. Tämä *Anabaena*-syanobakteerien geneettinen monimuotoisuus vahvistettiin monistamalla geenejä suoraan

kukintanäytteistä ilman kantojen eristystä. Makeiden vesien ja Itämeren *Anabaena*-kannat ovat geneettisesti hyvin samankaltaisia. Geneettisissä vertailuissa kävi kuitenkin ilmi, että pohjassa elävien *Anabaena*-kantojen geneettinen monimuotoisuus oli suurempaa kuin pintavesistä eristettyjen kantojen. Itämeren *Anabaena*-kantojen sekvenssit muodostivat omia ryhmiä sukupuun sisällä, jolloin on mahdollista, että nämä edustavat Itämeren omia *Anabaena*-ekotyyppejä.

Tämä tutkimus oli ensimmäinen, jossa uusin molekyylimenetelmin systemaattisesti selvitettiin Itämeren *Anabaena*-syanobakteerin geneettistä populaatiorakennetta, fylogeniaa ja myrkyntuottoa. Tulevaisuudessa monitorointitutkimuksissa on otettava huomioon myös Itämeren *Anabaena*-syanobakteerin mahdollinen maksamyrkyntuotto – erityisesti vähäsuolaisemmilla rannikkovesillä.

# PREFACE

*"But even with all our modern instruments for*
*probing and sampling ...*
*no one now can say that we shall ever resolve*
*the last, the ultimate mysteries of the sea."*

Rachel L. Carson, *The Sea Around Us* (1951)

Molecular ecology as a field of science has brought forward questions such as "*who is there*?", "*what are they like*?", or "*what do they do*?". The history of microbiology could be divided in two according to the methods used in answering these questions: The era of traditional microbiology using methods based on culturing and microscopy, and the era of molecular microbiology (Giovannoni and Rappé, 2000). Molecular methods have allowed a deeper understanding of phylogeny, genetic diversity, and biogeography of *Nodularia* and *Aphanizomenon* populations in the Baltic Sea.

However, little is known about Baltic Sea *Anabaena*, although it is one of the bloom-forming cyanobacteria in the Baltic Sea. The main task of this study was to address classical questions relevant to *Anabaena* in the Baltic Sea: *What is the genetic structure of* Anabaena *populations*? *Are they genetically diverse or homogeneous*? *Does the Baltic Sea* Anabaena *produce microcystins*? Following the history of microbial ecology, this study commenced with isolation of *Anabaena* strains from the Gulf of Finland and ended with *in situ* molecular studies of phytoplankton assemblages.

# 1. INTRODUCTION

## 1.1 The Baltic Sea

The Baltic Sea (**Fig. 1**) is one of the largest brackish water basins in the world, covering 392,978 km$^2$, and having a drainage area of 1,649,550 km$^2$ (Myrberg *et al*., 2006). It is a non-tidal and semi-enclosed basin with limited water exchange with the North Sea through the Kattegat. The hydrography of the Baltic Sea is strongly influenced by river inflow (annual runoff is 440 km$^3$, total volume being about 21,205 km$^3$; in the Gulf of Finland, annual runoff is 114 km$^3$ and total volume is 1,100 km$^3$) (Myrberg *et al*., 2006). This river inflow together with limited water exchange results in brackish nature of the Baltic Sea. The low salinity, ranging from 0 in the north to 32 PSU in the Kattegat (Myrberg *et al*., 2006), is due to the limited water input through the Danish sounds. For a number of reasons, the ecosystem of the Baltic Sea is particularly vulnerable. The Baltic Sea is approximately 9000 years old (Myrberg *et al*., 2006) and the low number of reported species (Johannesson and André, 2006) is thought to reflect its young age (Myrberg *et al*., 2006). The Baltic Sea is a marginal environment for both marine and freshwater species due to low winter temperatures as well as vertical and horizontal salinity gradients. In addition to postglacial flora and fauna, current species diversity is affected by the introduction of nonindigenous species from other fresh- and brackish water environments (Leppäkoski *et al*., 2002). Furthermore, it is under heavy anthropological use, with over 85 million inhabitants in the surrounding areas. The Baltic Sea is shallow; the mean depth is only 54 m, and the residence time of the water for the whole Baltic Sea is about 50 years (Myrberg *et al*., 2006). Thus, the Baltic Sea has more of a stagnant than a through-flow character (Ehlin, 1981).

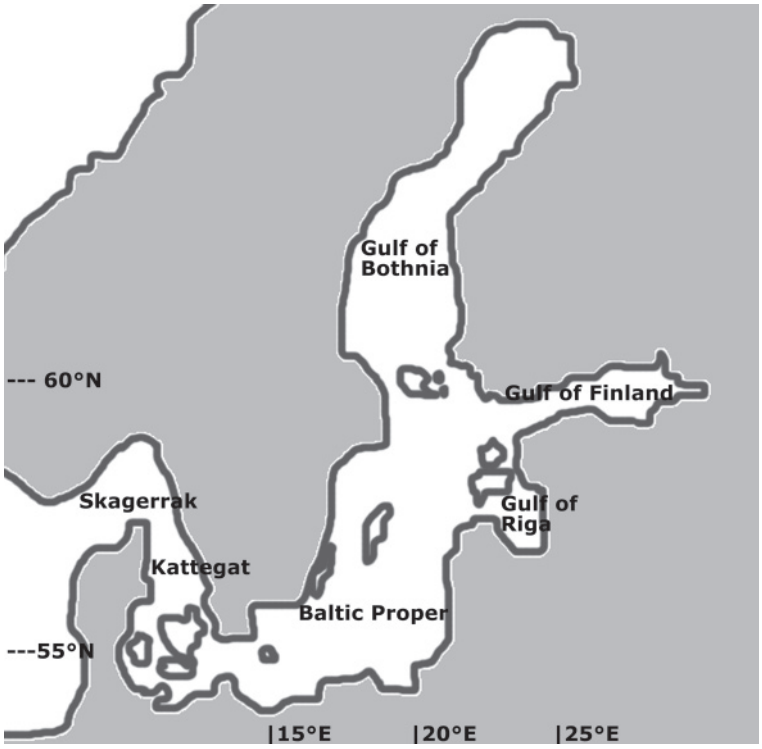



**Fig. 1.** The Baltic Sea and its basins.

The Gulf of Finland (**Fig. 1**) is a direct continuation of the Baltic Proper, without any sills, and has an area of 29,498 km$^2$ (Myrberg *et al*., 2006). The salinity of surface waters in the Gulf of Finland ranges from 0 to about 6.5 PSU (Kullenberg, 1981). The open waters of the Gulf of Finland are thus more saline in the west and more freshwater in the east due to freshwater inflow from the River Neva, which is the largest river in the catchment area of the Baltic Sea. Therefore, the hydrography of the Gulf of Finland resembles that of an estuary, with a salinity gradient and large land-derived nutrient inflows. The Gulf of Finland is considered to be the most eutrophied of the Baltic Sea basins, mainly due to this high nutrient inflow.

### 1.2 Cyanobacteria and their history in the Baltic Sea

Cyanobacteria (cyanoprokaryota, cyanophyta, blue-green algae) can be broadly classified as oxygenic phototrophs containing chlorophyll-*a* and are among the oldest life forms on earth. Cyanobacteria are estimated to have appeared on Earth at least 2700-3000 Mya (Knoll, 2008). *Anabaena* is one of the cyanobacteria whose morphotype has been recorded from 1500-2000 Mya old fossil assemblages (Knoll, 2008). Cyanobacteria are primary producers and have the ability to use water as the electron source in photosynthesis. They are thought to be responsible for oxygen production early in Earth's history.

Cyanobacteria are a morphologically diverse group of organisms ranging from single-celled to filamentous. Cyanobacteria may inhabit diverse environments because of their autotrophy (as phototrophs they require light), and, furthermore, they possess a number of adaptation mechanisms such as nitrogen fixation (occurs in specialised cells called *heterocytes*), chromatic adaptation, the ability to regulate buoyancy as well as differentiation of resting cells (*akinetes*). Thus, they are common in all kinds of habitats, such as planktonic and benthic habitats in freshwater, brackish and marine waters (Castenholz, 2001), as well as e.g. in stromatolites (Stal, 2000), salt lakes and sulfur springs (Oren, 2000). Cyanobacteria also form symbiotic associations with diverse eukaryotes (Bergman *et al*., 2008). The occurrence of cyanobacteria in a diversity of environments can be attributed to their old evolutionary history (Whitton and Potts, 2000). In phylogenetic trees, cyanobacteria form a coherent group well-separated from other bacterial groups (Woese, 1987) (**Fig. 2**). However, the cyanobacterial cluster contains also the plastids of eukaryotes (Turner, 1997).

Cyanobacteria have a long history in the Baltic Sea extending back at least 7 000 years, according to paleolimnological reconstructions (Bianchi *et al*., 2000). Marine plankton, including cyanobacteria, interested Baltic Sea scientists as far back as the late 19$^{th}$ century (Elmgren, 2001; Finni *et al*., 2001a and 2001b). However, cyanobacterial blooms were seldom reported before the Second World War (Finni *et al*., 2001a; Poutanen and Nikkilä, 2001). Eutrophication was identified as a problem, although it was popularly considered harmful mainly in coastal waters in the 1960s (Elmgren, 2001). The Water Conservation Laboratory of the City of Helsinki started annual monitoring of phytoplankton samples in 1964, and the study area also included open sea waters (Finni *et al*., 2001b). The intensity as well as the extent of cyanobacterial blooms have increased since the 1960s, probably because of increased nutrient loads, although the patchiness of blooms has complicated the documentation of such an increase (Kahru *et al*., 1994; Kahru *et al*., 2000; Finni *et al*., 2001a; Kahru *et al*., 2007; Suikkanen *et al.*, 2007). Cyanobacterial blooms pose a health hazard, especially in lakeshore waters and coastal waters of the Baltic Sea, since they produce toxins, e.g. hepatotoxins (Sivonen and Jones, 1999). Animal poisoning outbreaks caused by cyanobacterial blooms were reported from the Baltic Sea in Denmark in 1975

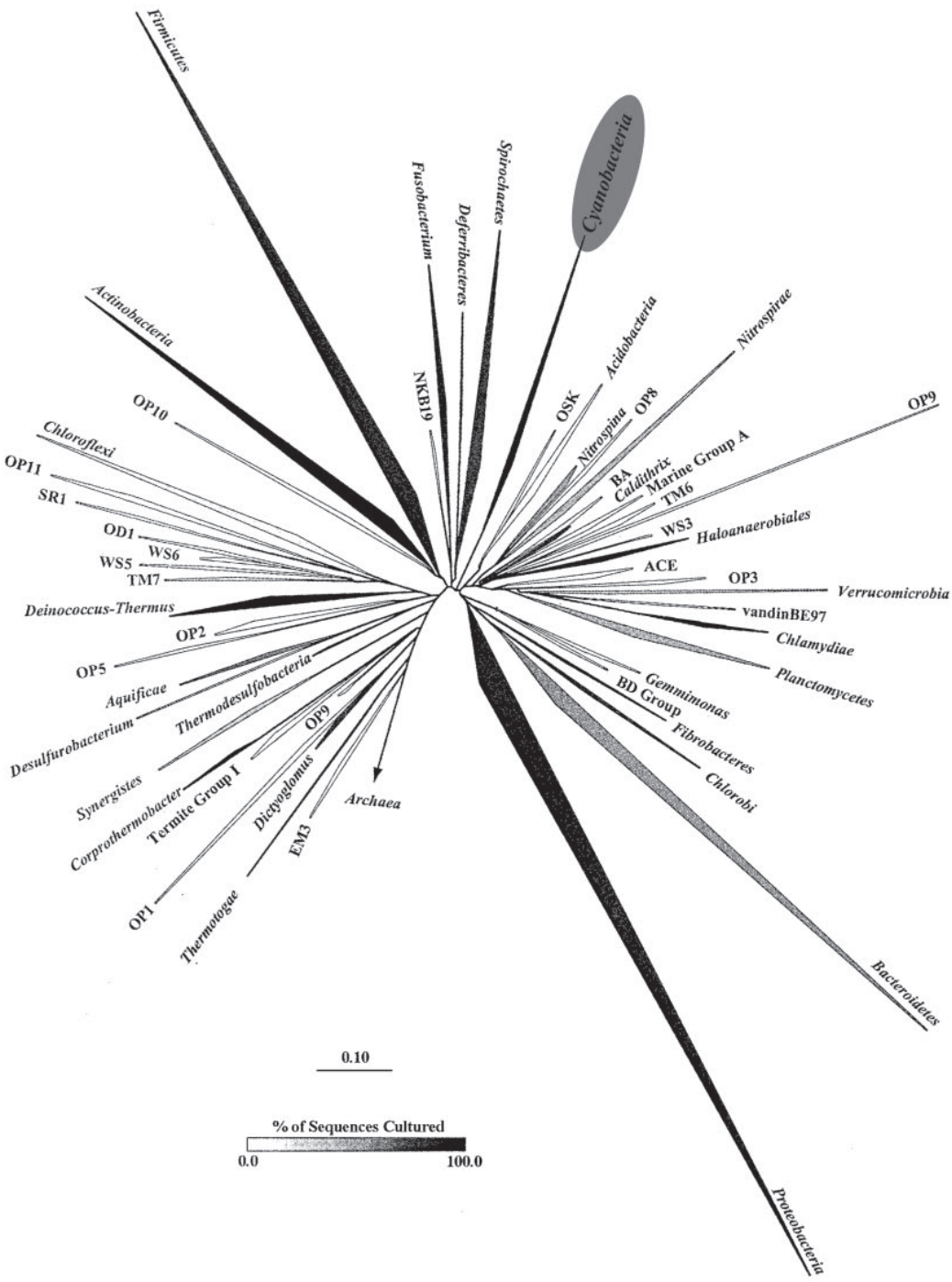



**Fig. 2.** The phylogenetic tree of the Bacteria according to Schloss and Handelsman (2004) with 16,964 16S rRNA gene sequences (length over 1,000 bp). Cyanobacteria form a monophyletic cluster within the bacterial domain, illustrated as gray circle. The vertex angle of each wedge shows the abundance of sequences in a phylum. The shading of each wedge corresponds to the proportion of sequences in that phylum obtained from cultured organisms. The tree has been reproduced with the kind permission from the publisher American Society for Microbiology (license number 2057760498743).

(Lindström, 1976), in Sweden in 1982 (Lundberg *et al*., 1983), and in Finland in 1984 (Persson *et al*., 1984).

### 1.3 Cyanobacterial blooms in the Baltic Sea

Baltic Sea late summer blooms, which can cover areas of up to 100,000 km$^2$ (Kahru, 1997), are often formed by several cyanobacterial genera (Kanoshina *et al*., 2003; Stal *et al*., 2003; Seppälä *et al*., 2007). The genera *Nodularia*, *Aphanizomenon*, and *Anabaena* are present in these blooms in the pelagic Baltic Proper as well as in the Gulf of Finland (e.g. Poutanen and Nikkilä, 2001; Kanoshina *et al*., 2003; Stal *et al.*, 2003; Seppälä *et al*., 2007; Suikkanen *et al*., 2007) (**Fig. 3**). They are all diazotrophic organisms and the low availability of inorganic nitrogen favors such nitrogen-fixing cyanobacteria (Niemi, 1979). Baltic Sea blooms usually occur when the water temperature exceeds about 15 ºC (Kanoshina *et al*., 2003). The spatial prevalence of cyanobacterial blooms is also said to be determined by wind force, with calm weather favoring *Nodularia* blooms (Kanoshina *et al*., 2003). On the other hand, wind forces transport open sea blooms towards the coastal sea regions causing harmful event (Laanemets *et al*., 2006). Stal *et al*. (2003) considered the amount of light energy to be important for bloom formation.

#### 1.3.1 *Anabaena*/*Aphanizomenon*

*Anabaena* and *Aphanizomenon* can occur as the dominant bloom formers in the Baltic Sea, especially in its northern part (Kanoshina *et al*., 2003; Karlsson *et al*., 2005; Seppälä *et al*., 2007; Suikkanen *et al*., 2007). *Aphanizomenon* is thought not to produce microcystins in the Baltic Sea (Sivonen *et al.*, 1990; Repka *et al.*, 2004). However, in freshwater environments *Aphanizomenon* produces neurotoxic anatoxin-a, saxitoxins and the hepatotoxic cylindrospermopsin (Sivonen and Jones, 1999). *Anabaena* produces a number of microcystin variants, anatoxin-a, anatoxin-a(S), cylindrospermopsin, and saxitoxins in freshwater environments (Sivonen and Jones, 1999; Spoof *et al*., 2006), but the production of microcystin (or other toxins) by Baltic Sea *Anabaena* has remained a matter of speculation (e.g. in Stal *et al*., 2003).

Heterocyte-forming cyanobacteria, including *Anabaena*/*Aphanizomenon*, form a monophyletic group (Turner, 1997) and thus have an apparently single evolutionary origin (Swingley *et al.*, 2008). *Anabaena* and *Aphanizomenon* are closely related and intermixed in phylogenetic trees based on the 16S rRNA gene (Lyra *et al*., 2001; Gugger *et al*.,

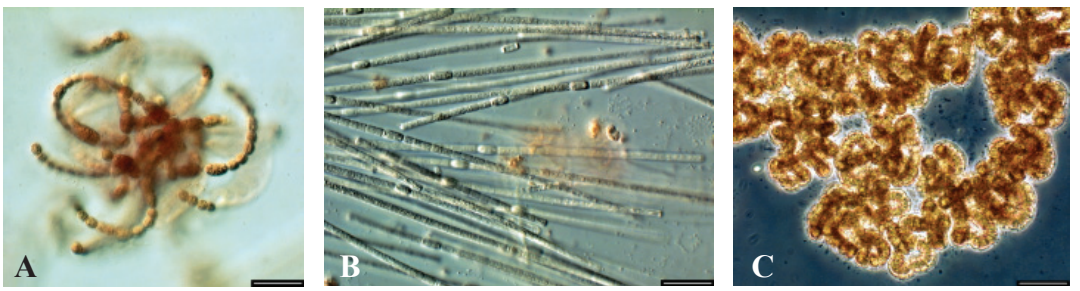



**Fig. 3.** Photomicrographs of representatives of the main bloom forming cyanobacterial genera in the Baltic Sea: a.) *Anabaena lemmermannii* b.) *Aphanizomenon flos-aquae*, and c.) *Nodularia spumigena*. The scale bar in all figures corresponds to 30 µm. Photomicrographs by Seija Hällfors/ Finnish Institute of Marine Research, Finland.

2002a; Iteman *et al*., 2002; Rajaniemi *et al*., 2005) as well as ITS1 (Gugger *et al*., 2002a) and *rbcLX* regions (Gugger *et al*., 2002a). Furthermore, cellular fatty acids have been employed to resolve their classification (Gugger *et al*., 2002b). *Anabaena*/*Aphanizomenon* are an example of the conflict between morphological classification and phylogenetic reconstruction. Morphological characters, such as the coiling of trichomes or the presence of gas vesicles, are not in concordance with genetic grouping (Rajaniemi *et al*., 2005). The close genetic relationship throws doubt on the taxonomic validity of these two genera (Gugger *et al*., 2002a; Rajaniemi *et al*., 2005). It has been suggested that *Anabaena* and *Aphanizomenon* form a single genus (Gugger *et al.* 2002a).

However, there are inconsistencies in comparisons of physiological properties of *Anabaena* and *Aphanizomenon*. On one hand, some of their physiological properties such as salinity tolerance as well as optimal and maximal temperatures for growth seem to be parallel (Iteman *et al.*, 2002), whereas, on the other hand, some properties, such as growth under conditions of nutrient and light excesses, have shown that they have different ecophysiological characters (De Nobel *et al.*, 1997). Temperature does not play a critical role for the presence of *Aphanizomenon* in the water column (Kanoshina *et al*., 2003) and it is present in the Baltic Sea all year round (Laamanen and Kuosa, 2005). In contrast, *Anabaena* is present only in the warmest summer period (Laamanen and Kuosa, 2005). Another difference is their depth distribution, since *Anabaena* populations have been observed mainly above depths of 10 m, whereas *Aphanizomenon* populations have been found in deeper waters as well (Hajdu *et al*., 2007).

Previously it was thought that *Aphanizomenon* is represented by a single genotype in the Baltic Sea (Barker *et al*., 2000b; Laamanen *et al*., 2002), however, recent results suggest that more than one *Aphanizomenon* genotype exists. Boström *et al*. (2007) found evidence for genetic diversity in *Aphanizomenon* populations from the Baltic Proper by cloning *nif*H genes. However, it must be taken into account that the *nifH* gene is not as well conserved as the 16S rRNA gene (Zehr *et al*., 2003). Rantasärkkä (2008) detected three different 16S rRNA *Aphanizomenon* gene sequences in the Gulf of Finland, and Halinen *et al*. (unpublished) found four different *Aphanizomenon* 16S rRNA gene sequences in the Baltic Proper. However, multiple *rrn* operons have been found within *Aphanizomenon* strains (Iteman *et al*., 2002), and therefore diversity in 16S rRNA gene sequences may due to intragenomic variation. The only *Aphanizomenon* strain isolated from the Baltic Sea (TR183) does not represent the dominant *Aphanizomenon* genotype there (Janson and Granéli, 2002; Laamanen *et al*., 2002).

*Anabaena* populations are morphologically diverse in the Baltic Sea, and 18 *Anabaena* morphospecies have been described from planktonic and benthic environments (Hällfors, 2004). Seven planktonic species (*A. baltica*, *A. circinalis*, *A. flos-aquae*, *A. lemmermannii*, *A. macrospora*, *A. solitaria*, and *A. spiroides*) and 3 morphospecies from benthic/littoral habitats (*A. cylidrica*, *A. inaequalis*, *A. torulosa*) have been described from the Gulf of Finland (Hällfors, 2004). Only a few planktonic *Anabaena* strains have been isolated from the Baltic Sea. Janson and Granéli (2002) studied four *Anabaena* strains, each of which had a unique *hetR* genotype, suggesting genetically diverse *Anabaena* populations in the Baltic Sea. Three benthic (from sediment, epilithic and epiphytic) *Anabaena* strains from the Gulf of Finland have been studied phylogenetically (Rajaniemi *et al.*, 2005). These three strains were intermixed with freshwater *Anabaena* and *Aphanizomenon* strain sequences in phylogenetic analyses (Rajaniemi *et al.*, 2005). None of these strains were microcystin-producers as determined by *mcyE*-specific PCR (Rajaniemi *et al*., 2005). However, five

benthic *Anabaena* strains, isolated from the coastal Gulf of Finland, were reported to contain cytotoxic compounds, but did not produce microcystins or nodularins (Surakka *et al.*, 2005).

### 1.3.2 *Nodularia*

*Nodularia spumigena* is the dominant bloom former in the Baltic Sea (Kononen *et al*., 1998; Stal *et al*., 2003; Seppälä *et al*., 2007), but is also present in various saline and brackish waters and soil environments worldwide (Sivonen and Jones, 1999; Lehtimäki, 2000). *Nodularia* is considered to account for the toxicity of the cyanobacterial blooms in the Baltic Sea, since it produces the hepatotoxic pentapeptide nodularin (Sivonen *et al*., 1989). Unlike *Aphanizomenon*, *Nodularia* is present in the water column only during summer periods (Laamanen and Kuosa, 2005), perhaps because of its sensitivity to water temperature (Lehtimäki *et al*., 1994 and 1997; Kanoshina *et al*., 2003; Repka *et al*., 2004). *Nodularia* filaments are usually concentrated in the surface layer of the water column (Kononen *et al*., 1998; Koskenniemi *et al*., 2007), in association with genetically rich assemblages of filament-associated bacteria (Salomon *et al*., 2003; Tuomainen *et al*., 2006).

According to molecular analysis of planktonic *Nodularia* strains or filaments using 16S rRNA (Lehtimäki *et al*., 2000; Lyra *et al*., 2005), *hetR* (Janson and Granéli, 2002), *rpoB* (Lyra *et al*., 2005), and *rbcLX* (Lyra *et al*., 2005) genes or regions *gvpA*-IGS (Barker *et al*., 1999), 16S-23S rRNA-ITS (Barker *et al*., 1999; Laamanen *et al*., 2001), and PC-IGS (Barker *et al*., 1999; Laamanen *et al*., 2001), only one planktonic *Nodularia* species is valid (Barker *et al*., 1999; Laamanen *et al*., 2001; Lyra *et al*., 2005). There is intraspecies genetic diversity in the Baltic Sea *Nodularia* populations (Barker *et al*., 1999; Barker *et al.*, 2000a; Lehtimäki *et al*., 2000; Laamanen *et al.*, 2001; Lyra *et al*., 2005; Boström *et al*., 2007).

Benthic *Nodularia* strains are morphologically and genetically more diverse than strains isolated from planktonic habitats, and they can be divided into two species, *N. sphaerocarpa* and *N. harveyana* (Lyra *et al*., 2005). To date, all isolated benthic *Nodularia* strains are non-nodularin producing (Lyra *et al*., 2005; Surakka *et al*., 2005). However, benthic *Nodularia* isolated from the Baltic Sea are able to produce other cytotoxic compounds (Surakka *et al*., 2005).

## 1.4 Other cyanobacteria present in the Baltic Sea

Cyanobacteria often form diverse populations during the bloom period (Seppälä *et al*., 2007), as well as outside the summer months. The number of cyanobacterial taxa is generally higher in coastal waters than in pelagic environments (Sivonen *et al.*, 1989). According to the Checklist of Baltic Sea Phytoplankton Species (Hällfors, 2004), the number of cyanobacterial morphospecies recorded from the Baltic Sea is large: Orders Chroococcales, Oscillatoriales, and Nostocales are all present and represented by a number of genera and species. *Synechococcus* are abundant in the Baltic Sea, especially during the warmest months (Kuosa, 1991; Stal and Walsby 2000; Hajdu *et al.* 2007). *Planktothrix* and *Microcystis* may form blooms in coastal areas as well as in the eastern Gulf of Finland (Sivonen *et al.*, 1989; Pitkänen *et al*., 1993; Kauppila, 2007). Cyanobacteria are constitutive members of benthic habitats of marine coasts (Hoffmann, 1999; Stal, 2000). The cyanobacterial genera *Calothrix* and *Rivularia* (Sihvonen *et al*., 2007) as well as *Nodularia* (Lyra *et al*., 2005) display a high genetic diversity in Baltic Sea benthic habitats.

### 1.5 How to classify cyanobacteria? – challenges therein and complexities of 'species' definitions

Biologists, especially since the time of Carl von Linné (1707 – 1778) and his magnum opus *Systema Naturae*, have classified organisms. The 'species' is considered the basic unit of classification of organisms. However, as Ernst Mayr writes: "… *the species problem is the oldest and most frustrating problem in biology*" (Mayr, 1992). Within the cyanobacteria, the case of the morphologically different but phylogenetically closely related and inseparable *Anabaena* and *Aphanizomenon* strains is an excellent illustration of the difficulties that can be encountered in species demarcation and classification. Furthermore, the classification of *Anabaena* and other cyanobacteria is confused by the several parallel taxonomic systems used.

Historically, cyanobacterial taxonomy followed the botanical approach (Code of Botanical Nomenclature) as for algae, which cyanobacteria resemble in containing chlorophyll *a* and in being capable of oxygenic photosynthesis. These traditional classification criteria, which have changed over time [reviewed by Hoffmann (1994), Wilmotte (1994), and Turner (1997)], were based on morphological features such as cell-shape, cell-size, or shape of filaments. Morphological identification of natural samples is still widely used (Komárek, 2003). The weak point of the traditional morphological classification system is the morphological transformation of cells exposed to different environmental conditions.

Since the 1970´s cyanobacteria have been classified as bacteria because of e.g. their lack of membrane-bound nucleus or other organelles, and since then cyanobacteria have been integrated into the Bacterial Code (Oren, 2004). However, the bacterial species concept is still under debate [e.g. Rosselló-Mora and Amann (2001)]. Mayr's classical 'biological species concept', which defines species "*as groups of actually or potentially interbreeding natural populations which are reproductively isolated from other such groups*" [E. Mayr (1942) from Rosselló-Mora and Amann (2001)], included the idea of evolutionary relationships into the concept of species but emphasised sexual interbreeding. Thus, it is difficult to apply this to (usually) asexual bacteria (Cohan, 2002). Rippka *et al.* (1979) created a bacteriological classification system which was based on morphology and development. This system categorised cyanobacteria into five groups: I. Chroococcales, II. Pleurocapsales, III. Oscillatoriales, IV. Nostocales, and V. Stigonematales (Rippka *et al*., 1979). In this classification system *Anabaena* belonged to Section IV, containing "filamentous heterocystous cyanobacteria that divide in only one plane". The classification of Rippka *et al.* (1979) was modified and adopted into Bergey's Manual of Systematic Bacteriology (Castenholz, 2001). In Bergey's Manual, *Anabaena* is classified in subsection IV.I (Rippka *et al*., 2001).

Two genetic methods play a dominant role in bacterial species demarcation: DNA-DNA hybridization and 16S rRNA gene sequence comparison (Rosselló-Mora and Amann, 2001). Stackebrandt and Goebel (1994) recommended a 16S rRNA gene sequence similarity cut-off value of at least 97.5 % to describe a species. However, this view was recently revised, and Stackebrandt and Ebers (2006) recommended a 16S rRNA similarity value of (above) "*98.7-99% as the point at which DNA-DNA association experiments should be mandatory…*" (Stackebrandt and Ebers, 2006). The problem is that the phylogeny based on 16S rRNA gene sequences of *Anabaena* follows neither the morphological classification

nor the classification in Bergey's Manual of Systematic Bacteriology (Rajaniemi *et al*., 2005). Furthermore, the genus *Anabaena* has no standing in bacteriological nomenclature according the Bacteriological Code, because no single representative has been validly published (Oren, 2004)**.** This discussion of classification of cyanobacteria will continue until a theory-based concept of bacterial species is accepted - by both bacteriologists and by botanists.

*Anabaena* and *Aphanizomenon*, which are genetically closely related (Lyra *et al*., 2001; Gugger *et al*., 2002a; Rajaniemi *et al*., 2005; **Fig. 12**), are morphologically (**Fig. 3**) and ecologically (De Nobel *et al*., 1997; Laamanen and Kuosa, 2005) different. Currently a 'polyphasic approach' has been used to define cyanobacterial species based on their morphology, ecology, physiology, and phylogeny. According to Komárek (2003), this approach defines 'species' as follows: "*a group of populations (+strains) which belongs to one genotype (genus), is characterized by stabilized phenotypic features (definable and recognizable, with distinct limits of variation), and by identical ecological demands. These characters should occur repeatedly (in time) in various localities with the same ecological conditions*". The polyphasic approach has been carried out for example in a study by Rajaniemi *et al.* (2005) to resolve the relationship between heterocystous cyanobacteria, including *Anabaena* and *Aphanizomenon*. However, *Anabaena* was not confirmed as a species (Rajaniemi *et al*., 2005). To compensate for the lack of resolving power of highly conserved genes such as 16S rRNA within closely related cyanobacteria, many studies have employed more variable gene regions. The internal transcribed spacer region between 16S and 23S rRNA genes has been widely used (Ward, 1998; Otsuka *et al*., 1999; Boyer *et al*., 2001; Laamanen *et al*., 2001; Gugger *et al*., 2002a; Laamanen *et al*., 2002; Rocap *et al*., 2002; Ernst *et al*., 2003; Janse *et al*., 2004b; Gugger *et al*., 2005; Humbert *et al*., 2005; Taton *et al*., 2006; Haverkamp *et al*., 2008). Protein coding genes have also been used to study the phylogeny of cyanobacteria. Such protein genes used to resolve the relationships of *Anabaena* and *Aphanizomenon* are e.g. *rbcLX* genes (encoding the RubisCO large subunit and intergenic spacer region) (Gugger *et al*., 2002a; Rajaniemi *et al*., 2005), *nif* genes (nitrogenase) (Henson *et al*., 2002 and 2004), *rpo* genes (DNA-dependent RNA polymerase) (Rajaniemi *et al*., 2005), and the *hetR* gene (heterocyst differentiation) (Janson and Granéli, 2002).

Whole genome sequencing has created novel insights into the concept of a bacterial species, since high-throughput sequencing is adding to our understanding of the evolutionary history of cyanobacteria (Swingley *et al*., 2008). The genes of a species can be divided into the *core genome* and *dispensable genome* and together these two form the *pan-genome* (Medini *et al*., 2005). The core genome includes all genes defining major phenotypic traits, the maintenance and survival of a species (i.e. *house-keeping genes*) (Lan and Reeves, 2000). Dispensable genome brings advantages e.g. in adaptation into the new environment (Lan and Reeves, 2000). At present, 35 cyanobacterial genomes are either completed or nearly completed (Hess, 2008; Swingley *et al*., 2008). Molecular phylogenies are based on the assumption of vertical inheritance of genes, and the species definition is made more complicated by phenomena such as gene loss, gene duplication, conversion, and horizontal (or lateral) gene transfer (HGT/LGT) (Swingley *et al*., 2008). The phylogenetic trees of 340 protein families from 24 cyanobacterial genomes were largely consistent with findings based on single genes (Swingley *et al*., 2008). Zhaxybayeva *et al*. (2008) found that cyanobacterial genes from all functional categories were subjected to HGT. Furthermore, around 50 % of cyanobacterial gene families had a history of HGT (Zhaxybayeva *et al*.,

2008). The danger of gene exchange exists especially when closely related organisms are studied (Rudi *et al*., 1998; Hess, 2008). Gene flow has been suggested to occur within the *Nodularia* populations in the Baltic Sea (Barker *et al*., 1999; Barker *et al*., 2000a). It is proposed that marine *Synechococcus* and *Prochlorococcus* have shared RubisCO genes with proteobacteria through HGT (Swingley *et al*., 2008). In *Nostoc* and *Microcystis* lineages, 16S rDNA and *rbcLX* are proposed to have had different evolutionary histories (Rudi *et al*., 1998). To reconstruct cyanobacterial evolution (and species classification as well), Swingley *et al*. (2008, p. 22) suggests two goals: " … *to understand how and why the phylogenies of individual genes differ from one another, and to determine whether some core of genes common to a group of organisms are predominantly inherited vertically*".

**1.6 Cyanobacterial toxins**

The first documentation of diseases or deaths of animals or humans associated with cyanobacteria comes from the mid 19th century (Kuiper-Goodman *et al*., 1999). Cyanobacteria are a prolific source of intracellular secondary metabolites, including peptides, polyketides, alkaloids, and lipopolysaccharides, some of which are toxic (Neilan *et al*., 1999; Sivonen and Jones, 1999; Smith and Doan, 1999; Burja *et al*., 2001; Sivonen and Börner, 2008). The most common cyanobacterial toxins are microcystins and nodularins, which are cyclic peptide hepatotoxins (Sivonen and Jones, 1999). Hepatotoxic blooms of cyanobacteria have been reported worldwide (Sivonen and Jones, 1999). These toxins cause death by liver hemorrhage within a few hours in mouse bioassays (Sivonen and Jones, 1999). Both microcystins and nodularins also have non-toxic variants (Sivonen and Börner, 2008). There are several chemically and toxicologically distinct cyanobacterial alkaloid neurotoxins: anatoxin-a, anatoxin-a(s), and saxitoxin. Neurotoxins cause rapid death by respiratory arrest in mouse bioassays (Sivonen and Jones, 1999). The cytotoxic and hepatotoxic alkaloid cylindrospermopsin occurs in cyanobacteria growing in tropical and subtropical environments (Sivonen and Börner, 2008). Other alkaloids include the dermatotoxic aplysiatoxins and lyngbyatoxin (Sivonen and Jones, 1999). Lipopolysaccharides, as constituents of the gram-negative cell wall, are common to all cyanobacteria (Sivonen and Jones, 1999). They are also called endotoxins, and may cause pyrogenic or allergenic responses in humans and animals (Sivonen and Jones, 1999).

A number of analytical, biochemical, and immunological methods have been developed to detect cyanobacterial toxins (Sivonen and Börner, 2008). During the 1980s, the mouse bioassay was a common method to detect toxins in bloom samples. However, in addition to ethical questions, mouse bioassays produced detection problems, with the primary reaction being to the more toxic compounds, while less toxic compounds remained unrecognised. Later on, structural characterisation of the toxic compounds enabled the development of sensitive and fast screening methods. The enzyme-linked immuno-sorbent assay (ELISA) is an immunological detection method that allows sensitive detection of microcystin, nodularin and saxitoxin (An and Carmichael 1994; Carmichael and An, 2000; Tillmanns *et al*., 2006; Sivonen and Börner, 2008). However, ELISA does not differentiate between structurally related compounds (An and Carmichael, 1994; Carmichael and An, 2000; Sivonen and Börner, 2008). High performance liquid chromatography (HPLC) together with UV detection is widely used in toxin detection (Sivonen and Jones, 1999). Microcystins can be identified quite precisely using matrix-assisted laser desorption ionisation time-of-flight mass spectrometry (MALDI-TOF-MS) or liquid chromatography-mass spectrometry (LC-MS), the latter also providing quantitative analysis (Sivonen and Börner, 2008).

### 1.6.1 The presence of hepatotoxins in the Baltic Sea

*Nodularia spumigena* produces the hepatotoxic pentapeptide nodularin, and its concentration measured in the Baltic Sea (19 mg $g^{-1}$ dw) seems to be one of the highest cyanobacterial toxin concentration measured in the environment (Sivonen and Jones, 1999). Microcystins have been detected in coastal waters of the southern Baltic Sea (Mazur and Pliński, 2003; Luckas *et al*., 2005) and at the entrance of the Gulf of Finland (Karlsson *et al*., 2005). However, the microcystin producer was not identified in any of these studies.

### 1.6.2 Microcystins - the most prolific and predominant of the known cyanobacterial toxins

Microcystins are the most prevalent cyanobacterial toxins, and in contrast to the restricted production of nodularin by *Nodularia spumigena* (Sivonen and Jones, 1999), microcystins are produced by several evolutionary clades of cyanobacterial species representing the genera *Anabaena*, *Anabaenopsis*, *Haphalosiphon*, *Microcystis*, *Nostoc*, *Phormidium*, *Planktothrix*, *Radiocystis*, and *Synechococcus* (Sivonen and Jones, 1999; Carmichael and Li, 2006; Lompardo *et al.*, 2006; Izaguirre *et al*., 2007). Microcystins are predominantly produced by the genera *Anabaena*, *Microcystis*, and *Planktothrix* (Sivonen and Jones, 1999), although all these genera also contain non-microcystin producing strains. *Anabaena* is one of the most important microcystin-producing genera in temperate lakes (Sivonen and Jones, 1999). Microcystins are also produced in benthic and terrestrial environments (Mez *et al*., 1997; Oksanen *et al.*, 2004; Jungblut *et al*., 2006; Mohamed *et al*., 2006; Izaguirre *et al*., 2007).

To date, over 80 microcystin variants have been described (Sivonen and Börner, 2008), and this list grows continually. Microcystins share a common structure, cyclo(-Adda-D-Glu-Mdha-D-Ala-L-X-D-MeAsp-L-Z), where X and Z are variable L-amino acids (**Fig. 4**). Adda is 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyl-4,6-decadienoic acid and is one

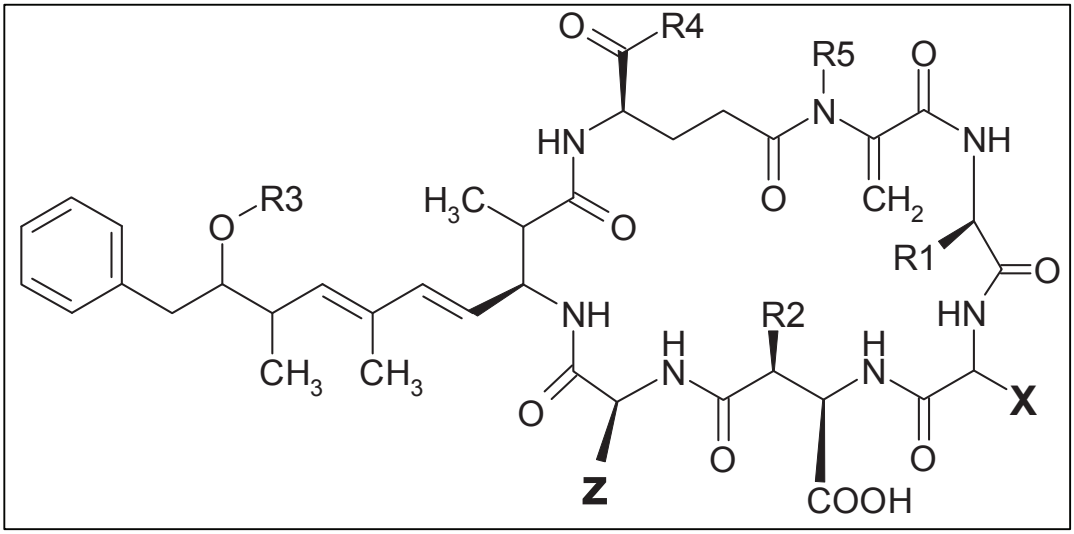



**Fig. 4.** The general structure of microcystin, cyanobacterial heptapeptide hepatotoxin. X and Z are variable L-amino acids. R1 to R5 mark positions in which the structure varies. The cyclic microcystin structure is shown; linear microcystins are less toxic than cyclic compounds (Sivonen and Jones, 1999). The illustration was constructed by J. Jokela, University of Helsinki.

of the critical amino acids involved in inhibition of protein phosphatases (Rinehart *et al.*, 1994). Microcystin-LR is the most toxic and the most frequently reported variant (Sivonen and Jones, 1999).

Microcystins pose a serious health risk for wild and domestic animals as well as for humans (Mez *et al*., 1997; Kuiper-Goodman *et al*., 1999; Carmichael *et al.*, 2001; Malbrouck and Kestemont, 2006; Soares *et al.*, 2006). Microcystins are water soluble and concentrate in liver cells, due to active transport by the bile acid carrier transport system, where they lead to cytoskeletal disorganization, possible lipid peroxidation, DNA damage, apoptosis, necrosis, intrahepatic bleeding, and death by hemorrhagic shock (Wiegand and Pflugmacher, 2005; Malbrouck and Kestemont, 2006). Furthermore, chronic exposure to microcystins may promote liver cancer (Kuiper-Goodman *et al*., 1999). Microcystins are potent inhibitors of protein phosphatases 1 and 2A (Kuiper-Goodman *et al*., 1999). The $LD_{50}$ toxicity value of the microcystin variants ranges from non-toxic to 50 μg $kg^{-1}$ (Sivonen and Jones, 1999). The World Health Organization has set a drinking water guideline value for MCYST-LR of no more than 1 μg $L^{-1}$ (WHO, 2006).

Although the effects of microcystins have been studied intensively in various organisms (Sivonen and Börner, 2008), their natural function is unknown and it is unclear why they are produced in cyanobacteria. It is widely believed that microcystins are protective agents against other photoautotrophic organisms (Babica *et al*., 2006) or act as grazing deterrents against herbivores such as zooplankton (Sivonen and Börner, 2008). However, their old evolutionary history, dating back to the time when herbivores or eukaryotic photoautotrophs had not evolved, suggests that protection against grazers or allelopathy was not the primary role of microcystins (Rantala *et al*., 2004). Another explanation pertains to the role of microcystins in basic cyanobacterial metabolism e.g. as iron chelators or intraspecific signaling molecules (Sivonen and Börner, 2008). However, such central role in cyanobacterial metabolism is arguable, since microcystins are not universally produced by all cyanobacteria. In addition to microcystins, cyanobacteria are able to produce other bioactive compounds, which possible play similar roles in non-microcystin-producing cyanobacteria.

### 1.6.3 Nodularins

The pentapeptide nodularin is thought to be unique to *Nodularia*, since it has not been detected in any other cyanobacterial genera (Sivonen and Jones, 1999). Its structure is cyclo-(D-MeAsp-L-arginine-Adda-D-glutamate-Mdhb) (Rinehart *et al*., 1988). D-MeAsp is D-erythro-β-methylaspartic acid, Mdhb is 2-(methylamino)-2-dehydrobutyric acid, and Adda is (2S,3S,8S,9S)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid. Only a small number of nodularin variants have been reported in the literature (Sivonen and Jones, 1999). Toxicological actions of nodularins are similar to microcystins, because of their parallel chemical structures. (**Fig. 5**)

### 1.6.4 Microcystin synthetase genes in *Anabaena*

Microcystins and nodularins are produced nonribosomally by the microcystin/nodularin synthetase enzyme complex via a thiotemplate mechanism (Sivonen and Börner, 2008). This large enzyme complex, encoded by the *mcy* gene cluster, is composed of peptide synthetases and polyketide synthases, as well as tailoring enzymes (Tillett *et al.*, 2000; Christiansen *et al.*, 2003; Rouhiainen *et al.*, 2004). *Anabaena* strain 90, isolated from the Finnish Lake Vesijärvi in 1986, is the only *Anabaena* strain thus far for which the microcystin

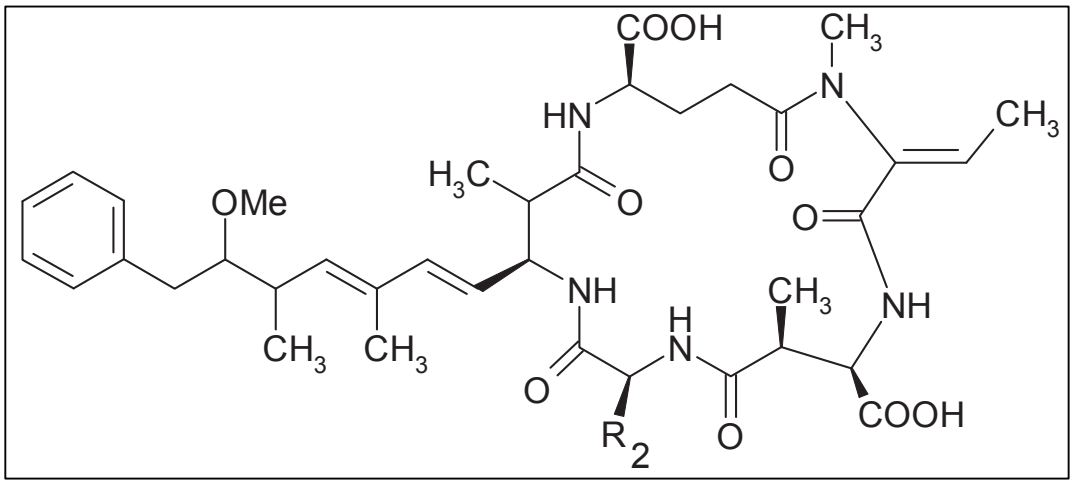



**Fig. 5.** The general structure of nodularin, cyanobacterial pentapeptide hepatotoxin. R refers to the location of alternative structural component. Microcystins and nodularins have similar toxicologic functions, since both have Adda-component ("tail" on the left, seen also in Fig. 4 in microcystin structure) responsible for toxic effects. The illustration was constructed by J. Jokela, University of Helsinki.

synthetase gene cluster has been characterized (Rouhiainen *et al.*, 2004). The total size of the cluster is 55.4 kb and the cluster is comprised of ten genes (*mcyA-J*) and three operons: (*mcyA-mcyB-mcyC*), (*mcyG-mcyD-mcyJ-mcyE-mcyF-mcyI*), and (*mcyH*) (Rouhiainen *et al.*, 2004). In addition to *Anabaena*, the *mcy* cluster has been sequenced from *Microcystis* (Nishizawa *et al.*, 1999 and 2000; Tillett *et al*., 2000), *Planktothrix* (Christiansen *et al*., 2003), and *Nostoc* (D.P. Fewer *et al*., unpublished results) strains as has the *nda* cluster from *Nodularia* (Moffitt and Neilan, 2004). The organization of the genes in each of these

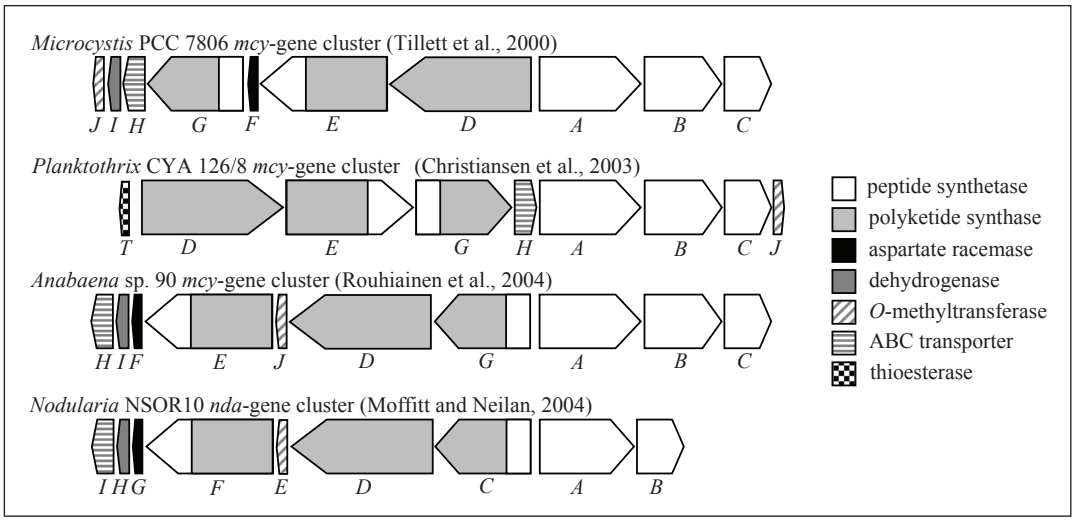



**Fig. 6.** Microcystin/nodularin synthetase gene (*mcy/nda*) cluster structures of *Microcystis, Planktothrix, Anabaena*, and *Nodularia*. The illustration was produced by A. Rantala-Ylinen, University of Helsinki.

genera is different (**Fig. 6**). The *mcy/nda* genes are transcribed from a bidirectional promoter region in *Anabaena*, *Microcystis*, and *Nodularia*. In *Planktothrix* all *mcy* genes except *mcyT* are transcribed unidirectionally from a promoter located upstream of the *mcyD* gene. The horizontal transfer of toxin biosynthesis genes was proposed almost 30 years ago because of the scattered distribution of toxin production in cyanobacterial genera (Dittmann and Börner, 2005). However, this organizational disparity of *mcy* genes challenges the idea of a recent and ongoing horizontal gene transfer of *mcy* gene clusters between cyanobacterial genera (Dittmann and Börner, 2005). Further evidence for a vertical evolutionary history for *mcy* genes is found in the congruence between the phylogeny of house-keeping genes and *mcy* genes (Rantala *et al*., 2004). Although the *mcy* gene cluster is ancient (Rantala *et al*., 2004), recent recombination events leading to replacement or modification of individual domains or genes have occurred (Christiansen *et al*., 2003; Mikalsen *et al.*, 2003; Tanabe *et al.*, 2004; Kurmayer and Gumpenberger, 2006; Fewer *et al*., 2007). Recombination between *mcy* genes may in part explain how new structural variants of microcystins are produced.

### 1.6.5 PCR-based methods to detect potentially toxic cyanobacteria

Many cyanobacteria produce identical toxin variants (Sivonen and Jones, 1999), so that the detection of the toxin does not necessarily allow the identification of the producer organism. *Nodularia spumigena*, the only producer of nodularin, is an exception (Sivonen and Jones, 1999). Toxic and non-toxic cyanobacterial strains are morphologically indistinguishable (e.g. Laamanen *et al*., 2001; Bittencourt-Oliveira, 2003; Yoshida *et al*., 2008). However, morphology seems to show correlation with toxicity in some instances, and the presence of *mcy* genes was found most frequently with the *M. aeruginosa* and *M. botrys* morphospecies, while *mcy* genes were not detected in samples of the *M. wesenbergii* morphotype (Via-Ordorika *et al.*, 2004). Previously, toxicity was tested by strain isolation and measurement of toxin production from a mass culture. Prior to the discovery of *mcy*/*nda* gene clusters, alternative DNA–based detection methods were employed to differentiate toxic from non-toxic strains. However, *Microcystis* strains show no grouping according to microcystin production in trees based on different house-keeping genes (e.g. Neilan *et al.*, 1997; Lyra *et al*., 2001; Tillett *et al.*, 2001; Yoshida *et al*., 2008). Similarly, microcystin-producing *Planktothrix* strains are indistinguishable from non-microcystin-producing strains in phylogenetic analyses (Lyra *et al.*, 2001). Toxic and non-toxic strains of the genus *Nodularia* are an exception, since they form distinct clusters in phylogenetic trees based on various genes and intergenic regions (Lehtimäki *et al.*, 2000; Laamanen *et al*., 2001; Moffitt *et al.*, 2001; Lyra *et al.*, 2005). This grouping has been used to design diagnostic PCR to identify nodularin-producing *Nodularia* (Laamanen *et al*., 2001). In *Anabaena* strains, all microcystin-producing strain sequences group together based on different house-keeping genes (Lyra *et al*., 2001; Gugger *et al*., 2002a; Rajaniemi *et al*., 2005).

Microcystin/nodularin synthetase genes are useful in the detection of putative toxin producers, since they provide the possibility to relate the microcystin/nodularin producing phenotype to the genotype independently of the taxonomic characterization of the organisms (Dittmann and Börner, 2005; Rantala, 2007). Microcystin/nodularin biosynthesis genes have been used widely to predict toxicity in laboratory strains of e.g. *Anabaena*, *Microcystis*, and *Planktothrix* (Rantala, 2007). Furthermore, microcystin synthetase genes have been used for identification of the toxin producer in field samples (Rantala, 2007). Microarrays with subgroup-specific primers have been successfully used for the detection of hepatotoxin-producing cyanobacteria (Rantala *et al*., 2008). Specific primers were designed to

simultaneously detect *mcy* and *nda* genes of *Anabaena*, *Microcystis*, *Nostoc*, *Planktothrix*, and *Nodularia* from Finnish lake and Baltic Sea water samples (Rantala *et al*., 2008). The array showed very good detection sensitivity and, in the future, may be applied to routine high-throughput analysis in environmental monitoring. In quantitative PCR (Q-PCR) the amount of PCR products can be measured online by a fluorometer, which detects fluorescent probes attached to PCR amplification products. Thus, the original number of specific genes can be measured. Q-PCR has been used to detect the dominant toxin producer in Finnish lakes (Vaitomaa *et al*., 2003). Specific primers were designed to detect the *mcyE* gene in *Anabaena* and *Microcystis* (Vaitomaa *et al*., 2003). Kurmayer and Kutzenberger (2003) employed Q-PCR to monitor *Microcystis mcyB* genes in Lake Wannsee. Furukawa *et al*. (2006) used Q-PCR to quantify *mcyA* genes in lake water samples. Only 8.8 *Microcystis* cells per reaction were enough for detection (Furukawa *et al*., 2006). Koskenniemi *et al*. (2007) measured the presence of nodularin-producing *Nodularia* in the Baltic Sea. With the aid of gene expression methods (targeting mRNA transcripts of *mcy*/*nda* genes) we can gain an understanding of *mcy* gene regulation and inactivation mechanisms (Kaebernick *et al.*, 2000 and 2002).

PCR-based community fingerprinting methods, such as denaturing gradient gel electrophoresis (DGGE), have been progressively introduced to the study of toxin-producing cyanobacterial communities following the discovery and characterization of *mcy* and *nda* gene clusters. Toxic blooms are often formed by several cyanobacterial taxa (Rantala *et al*., 2006), and the DGGE method is a means to identify and characterise the genetic diversity of the putative toxin-producers. In DGGE, sequence fragments of equal lengths are separated based on their melting domains as determined by nucleotide composition (Muyzer *et al*., 1993). These fingerprints may distinguish sequences differing by even a single base pair. Parameters such as diversity and richness, and shifts in bacterial communities, can be measured from DGGE fingerprints. Separated sequence fragments can be identified via direct sequencing. However, DGGE has not been previously used to detect *mcy* or *nda* genes. Janse *et al*. (2004b) applied DGGE for recognition of microcystin-producing *Microcystis* colonies but they used the rRNA ITS as a marker, which does not work robustly as a genetic marker for microcystin production (Otsuka *et al*., 1999; Yoshida *et al*., 2008).

Mbedi *et al.* (2005) studied the co-occurrence of eight *mcy* genes in non-microcystin- and microcystin-producing *Planktothrix* strains. According to their study, the *mcyE* gene was discovered to be the most robust predictor of toxicity in *Planktothrix* strains (Mbedi *et al.*, 2005). *mcyE* has been intensively used for screening cyanobacterial strains and/or field samples (Vaitomaa *et al.*, 2003; Rajaniemi *et al*., 2005; Rantala *et al.*, 2006). Ouahid *et al*. (2005) suggested that the simultaneous PCR detection of a number of *mcy* genes should be used as a criterion in specifying potential toxicities in field samples. However, PCR-based methods to detect *mcy* genes are not without problems. Firstly, *mcy* genes are not found exclusively in microcystin-producing cyanobacteria. False positives can be produced in cases where a strain carries an inactivated *mcy* gene cluster, as has been observed in *Microcystis* (Nishizawa *et al.*, 2000; Kaebernick *et al*., 2001; Tillett *et al.*, 2001; Mikalsen *et al.*, 2003; Via-Ordorika *et al.*, 2004) and *Planktothrix* strains (Kurmayer *et al.*, 2004; Mbedi *et al.*, 2005). Secondly, DNA polymorphisms at priming sites create variability in *mcy* genes and may yield false negative results (Via-Ordorika *et al.*, 2004; Mbedi *et al.*, 2005). Although the *mcy* gene cluster is ancient and its evolution has been mainly vertical, recombination events have occurred in *mcy* genes (Mikalsen *et al*., 2003; Tanabe *et al*.,

2004; Kurmayer and Gumpenberger, 2006; Fewer *et al*., 2007). This polymorphism is reflected in the diversity of microcystin isoforms produced by cyanobacteria (Mikalsen *et al.*, 2003; Kurmayer *et al*., 2005; Kurmayer and Gumpenberger, 2006; Fewer *et al*., 2007 and 2008). Recombination events may also lead to both false positive as well as false negative results in *mcy*-detecting PCR reactions.

## 2. AIMS OF THE STUDY

Research on Baltic Sea cyanobacteria has focused on *Nodularia* and *Aphanizomenon*, and little is known about the genus *Anabaena,* presumably due to the perception that it plays a minor role in cyanobacterial blooms (e.g. Stal *et al*., 2003). However, at times *Anabaena* may form a significant part of blooms, especially in the Gulf of Finland and in the northern part of the Baltic Sea (Kanoshina *et al*., 2003; Karlsson *et al*., 2005; Seppälä *et al*., 2007). The range of *Anabaena* morphospecies found in the Baltic Sea plankton and benthos are listed in Hällfors (2004). Molecular biological methods enable biogeographical, ecological and evolutionary approaches to questions about cyanobacterial populations, but nevertheless these methods have not been previously applied to Baltic Sea *Anabaena*. The current deficiency in understanding of the Baltic Sea *Anabaena* may due to the lack of isolates in culture collections. In this study, the focus was to advance our understanding of Baltic Sea *Anabaena*, and specific objectives are listed in **Table 1**.

**Table 1.** Table of the specific aims of this study. The Roman numerals refer to the papers in which each problem was approached.

| **Gaps in current knowledge of Baltic Sea *Anabaena*** | **Objectives and approaches of this study** |
|---|---|
| What is the genetic structure of *Anabaena* populations in the Gulf of Finland, Baltic Sea?<br><br>Few *Anabaena* strains have been isolated from the Baltic Sea. | To isolate planktonic and benthic *Anabaena* strains from the Gulf of Finland of the Baltic Sea (**I, II**), and to study the genetic structure of Baltic Sea planktonic and benthic *Anabaena* populations using several molecular marker genes (**I**, **II**, **III**, **IV**). |
| It is uncertain if Baltic Sea *Anabaena* produces toxins.<br><br>Why do some *Anabaena* strains contain *mcy* genes but do not produce microcystins. | To screen isolated planktonic *Anabaena* strains for microcystin production and to characterise dominant microcystin variants (**I**). To characterize microcystin-synthetase gene inactivation in the Baltic Sea *Anabaena* strains (**IV**). |
| How frequent are microcystin-producing *Anabaena* in the Gulf of Finland?<br><br>Hepatotoxic blooms in the Baltic Sea are formed by several cyanobacterial genera. How can the toxin producer be identified? | To develop a culture-independent detection method specific for microcystin-producing *Anabaena* and nodularin-producing *Nodularia* (**III**) and apply this method to study the distribution of microcystin-producing *Anabaena* in the Gulf of Finland by DGGE (**III**). |

# 3. MATERIALS AND METHODS

The research methods used in this study are listed in **Table 2** and are described in more detail in the respective papers **I-IV**. The *Anabaena* strains used in this study are listed in **Table 3**. The sampling locations for *Anabaena* isolates (papers **I**, **II**, and **IV**) are illustrated in **Fig. 7**, and the locations of water sampling for paper **III** are illustrated in **Fig. 8**.

**Table 2.** The methods used in this study. The Roman numerals refer to the papers in which each method was applied and described in details.

| Method | Paper |
|---|---|
| Sampling | I, II, III |
| Determination of physicochemical parameters from water samples | I, III |
| PCA analysis | I |
| Canonical analysis of principle coordinates | III |
| Distance-based multivariate analysis for a linear model by forward selection | III |
| Strain isolation | I, II |
| Strain identification by microscopy | I, II |
| Microcystin/nodularin extraction from strains and filtered water samples | I, III |
| LC-MS analysis | I, III |
| DNA extraction and purification | I, II, III, IV |
| PCR amplification (16S rRNA, *rbcL*, *rpoC1*, *mcyE*, and *ndaF* partial genes, and the *mcy* gene cluster) | I, II, III, IV |
| Primer design | II, III, IV |
| 16S rRNA gene sequencing | I, II |
| *rpoC1* sequencing | II |
| *rbcL* sequencing | II |
| *mcy* genes / *ndaF* sequencing | III, IV |
| BLAST searching | I, II, III, IV |
| Denaturing gradient gel electrophoresis (DGGE) optimization | III |
| Denaturing gradient gel electrophoresis (DGGE) | III |
| Phylogenetic sequence analyses (NJ, ML, MP) | I, II |
| Minimum spanning network analysis | III |

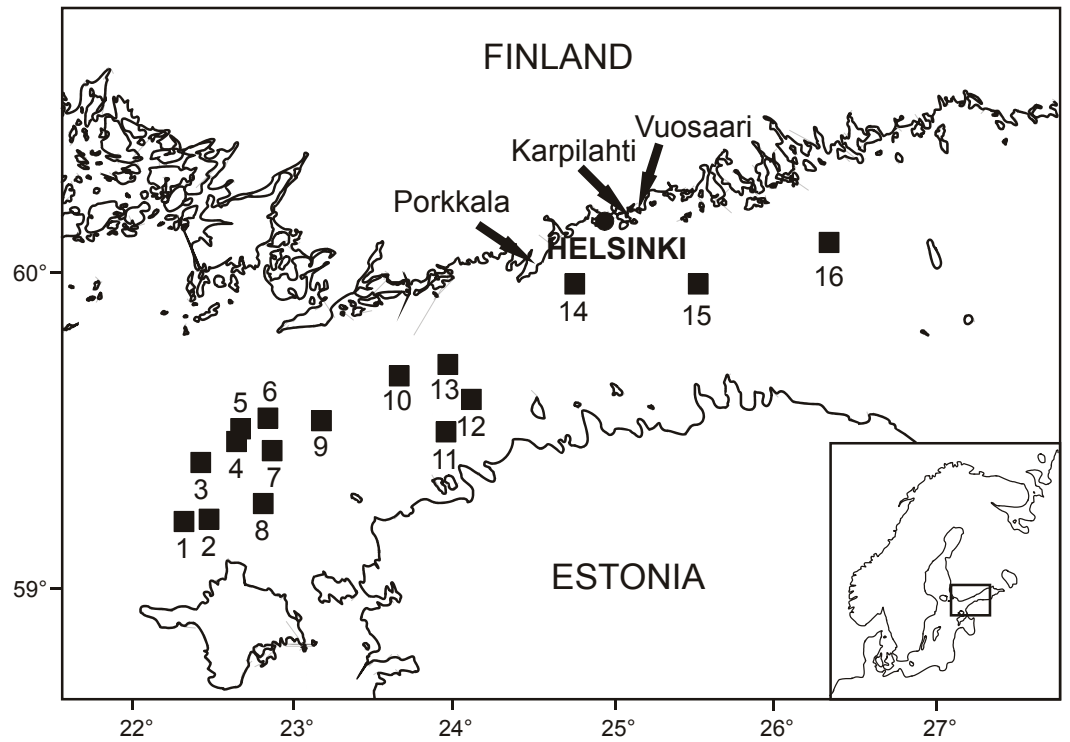



**Fig. 7.** Altogether, 49 planktonic *Anabaena* strains were isolated from the sampling stations shown as squares and numbered 1 to 16 (see the reference in Table 2). Benthic strains were isolated from the littoral zone of Karpilahti, Porkkala, and Vuosaari, indicated with arrows. The map was produced by K. Koskenniemi, University of Helsinki.

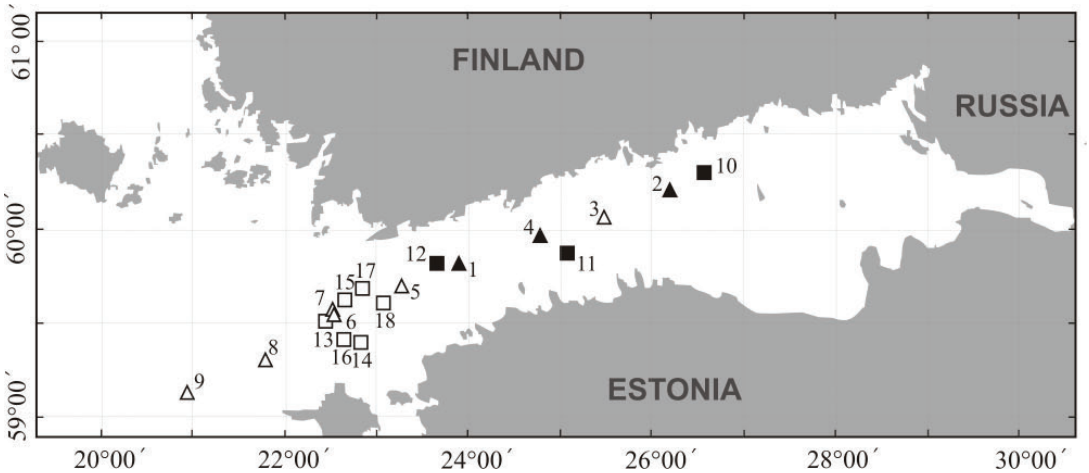



**Fig. 8.** Sampling locations of seawater for paper III. To study the distribution of microcystin-producing *Anabaena* populations and their genetic diversity by *mcyE*-specific DGGE, water samples were taken over a two-year time period, 2003 (illustrated as triangles) and 2004 (illustrated as squares). The study area covered the northern Baltic proper and the Gulf of Finland. Filled triangles and squares indicate the stations where *mcyE* genes were detected (see sections 4.3 and 4.6).

**Table 3.** *Anabaena* strains used in this study including their original habitat, geographical origin, year of isolation and accession numbers of sequences obtained in this study. The listed *Anabaena* strains were used in papers I, II, III, and IV. [1] GF = Gulf of Finland; [2] Station numbers correspond to the station numbers in Fig. 7; [3] MC = microcystin.

| Strain | Habitat | Geographical origin and year of isolation [1] | Station No. [2] | Reference | MC-prod. [3] | 16S rRNA | *mcyD* | *mcyE* | *rbcL* | *rpoC1* |
|---|---|---|---|---|---|---|---|---|---|---|
| **BECID8** | epilithic | Karpilahti, GF 2001 | - | this study | - | EF583856 | - | - | - | - |
| **BECID20** | epiphytic | Vuosaari, GF 2001 | - | Surakka *et al.*; 2005 | - | EF583858 | - | - | - | - |
| **BECID23** | epiphytic | Vuosaari, GF 2001 | - | Surakka *et al.*; 2005 | - | EF583859 | - | - | - | - |
| **BECID31** | epilithic | Vuosaari, GF 2001 | - | this study | - | EF583862 | - | - | - | - |
| **BIR2** | plankton | GF 2003 | 15 | Rantasärkkä, 2008 | - | EF568920 | - | - | - | - |
| **BIR3** | plankton | GF 2003 | 15 | Rantasärkkä, 2008 | - | EF568918 | - | - | - | - |
| **BIR4** | plankton | GF 2003 | 15 | Rantasärkkä, 2008 | - | EF568919 | - | - | - | - |
| **BIR5** | plankton | GF 2003 | 15 | Rantasärkkä, 2008 | - | EF568921 | - | - | - | - |
| **BIR19** | plankton | GF 2004 | 11 | this study | - | EF568909 | - | - | EF565296 | EF568822 |
| **BIR25** | plankton | GF 2004 | 11 | this study | - | EF568914 | - | - | EF565297 | EF568823 |
| **BIR28** | plankton | GF 2004 | 11 | this study | - | EF568910 | - | - | EF565298 | EF568824 |
| **BIR30** | plankton | GF 2004 | 11 | this study | - | EF568911 | - | - | EF565299 | EF568825 |
| **BIR31** | plankton | GF 2004 | 11 | this study | - | EF568868 | - | - | EF565300 | EF568826 |
| **BIR41** | plankton | GF 2004 | 14 | this study | - | EF568869 | - | - | EF565301 | EF568827 |
| **BIR49** | plankton | GF 2004 | 14 | this study | - | EF568915 | - | - | EF565302 | EF568828 |
| **BIR50** | plankton | GF 2004 | 12 | this study | - | EF568870 | - | - | EF565303 | EF568829 |
| **BIR52** | plankton | GF 2004 | 12 | this study | - | EF568871 | - | - | EF565304 | EF568830 |
| **BIR53** | plankton | GF 2004 | 12 | this study | - | EF568872 | - | - | EF565305 | EF568831 |
| **BIR54** | plankton | GF 2004 | 12 | this study | - | EF568873 | - | - | EF565306 | EF568832 |
| **BIR56** | plankton | GF 2004 | 12 | this study | - | EF568874 | - | - | EF565307 | EF568833 |
| **BIR66** | plankton | GF 2004 | 7 | this study | - | EF568875 | - | - | - | EF568834 |
| **BIR67** | plankton | GF 2004 | 7 | this study | - | EF568876 | - | - | EF565308 | EF568835 |
| **BIR73** | plankton | GF 2004 | 14 | this study | - | EF568916 | - | - | EF565309 | EF568836 |
| **BIR76** | plankton | GF 2004 | 14 | this study | - | EF568877 | - | - | EF565310 | EF568837 |
| **BIR78** | plankton | GF 2004 | 14 | this study | - | EF568878 | - | - | EF565311 | EF568838 |
| **BIR84** | plankton | GF 2004 | 7 | this study | - | EF568879 | - | - | EF565312 | EF568839 |

**Table 3 continuing**

| Strain | Habitat | Geographical origin and year of isolation [1] | Station No. [2] | Reference | MC-prod. [3] | 16S rRNA | *mcyD* | *mcyE* | *rbcL* | *rpoC1* |
|---|---|---|---|---|---|---|---|---|---|---|
| **BIR94** | plankton | GF 2004 | 10 | this study | - | EF568880 | - | - | EF565313 | EF568840 |
| **BIR96** | plankton | GF 2004 | 10 | this study | - | EF568881 | - | - | EF565314 | EF568841 |
| **BIR130** | plankton | GF 2004 | 5 | this study | - | EF568882 | - | - | EF565315 | EF568842 |
| **BIR132** | plankton | GF 2004 | 5 | this study | - | EF568883 | - | - | EF565316 | EF568843 |
| **BIR162** | plankton | GF 2004 | 1 | this study | - | EF568884 | - | - | EF565317 | EF568844 |
| **BIR169** | plankton | GF 2004 | 1 | this study | - | EF568885 | - | - | EF565318 | EF568845 |
| **BIR180** | plankton | GF 2004 | 1 | this study | - | EF568886 | - | - | EF565319 | EF568846 |
| **BIR202** | plankton | GF 2004 | 9 | this study | - | EF568917 | - | - | EF565320 | EF568847 |
| **BIR208** | plankton | GF 2004 | 9 | this study | - | EF568887 | - | - | EF565321 | EF568848 |
| **BIR219** | plankton | GF 2004 | 6 | this study | - | EF568888 | - | - | EF565322 | EF568849 |
| **BIR232** | plankton | GF 2004 | 4 | this study | - | EF568889 | - | - | EF565323 | EF568850 |
| **BIR241** | plankton | GF 2004 | - | this study | - | EF568890 | - | - | EF565324 | EF568851 |
| **BIR246** | plankton | GF 2004 | 16 | this study | + | EF547190 | EF565271 | EF565279 | EF565286 | EF568812 |
| **BIR250A** | plankton | GF 2004 | 16 | this study | + | EF547191 | EF565272 | EF565280 | EF565287 | EF568813 |
| **BIR256** | plankton | GF 2004 | 16 | this study | - | EF568912 | EF565273 | EF565281 | EF565288 | EF568814 |
| **BIR257** | plankton | GF 2004 | 16 | this study | + | EF547192 | EF565274 | EF565282 | EF565289 | EF568815 |
| **BIR258** | plankton | GF 2004 | 16 | this study | + | EF547193 | EF565275 | EF565283 | EF565290 | EF568816 |
| **BIR259** | plankton | GF 2004 | 16 | this study | - | EF568913 | - | EF565284 | EF565291 | EF568817 |
| **BIR260** | plankton | GF 2004 | 16 | this study | + | EF547194 | EF565276 | EF565285 | EF565292 | EF568818 |
| **BIR272** | plankton | GF 2004 | 6 | this study | - | EF568891 | - | - | EF565325 | EF568852 |
| **BIR274** | plankton | GF 2004 | 6 | this study | - | EF568892 | - | - | EF565326 | EF568853 |
| **BIR300** | plankton | GF 2004 | 10 | this study | - | EF568908 | - | - | EF565295 | EF568821 |
| **BIR348** | plankton | GF 2004 | 3 | this study | - | EF568893 | - | - | EF565327 | EF568854 |
| **BIR358** | plankton | GF 2004 | 3 | this study | - | EF568894 | - | - | EF565328 | EF568855 |
| **BIR361** | plankton | GF 2004 | 1 | this study | - | EF568895 | - | - | EF565329 | EF568856 |
| **BIR370A** | plankton | GF 2004 | 2 | this study | - | EF568896 | - | - | EF565330 | EF568857 |
| **BIR370B** | plankton | GF 2004 | 2 | this study | - | EF568897 | - | - | EF565331 | EF568858 |
| **BIR374** | plankton | GF 2004 | 8 | this study | - | EF568898 | - | - | EF565332 | EF568859 |
| **BIR406** | plankton | GF 2004 | 13 | this study | - | EF568899 | - | - | EF565333 | EF568860 |

**Table 3 continuing**

| Strain | Habitat | Geographical origin and year of isolation [1] | Station No. [2] | Reference | MC-prod. [3] | 16S rRNA | *mcyD* | *mcyE* | *rbcL* | *rpoC1* |
|---|---|---|---|---|---|---|---|---|---|---|
| **BIR440** | plankton | GF 2004 | 12 | this study | - | EF568900 | - | - | EF565334 | - |
| **BIR441** | plankton | GF 2004 | 12 | this study | - | EF568901 | - | - | EF565335 | EF568861 |
| **XP15D** | epilithic | Porkkala, GF 1999 | - | Surakka *et al.* 2005 | - | EF583860 | - | - | - | - |
| **XP34A** | epilithic | Porkkala, GF 1999 | - | Surakka *et al.*; 2005, Herfindal *et al.*; 2005 | - | EF583863 | - | - | - | - |
| **XP35A** | periphytic | Porkkala, GF 1999 | - | Surakka *et al.*; 2005 | - | EF583855 | - | - | - | - |
| **XP36D** | sediment | Porkkala, GF 1999 | - | Surakka *et al.*; 2005 | - | EF583853 | - | - | - | - |
| **XP6A** | sediment | Porkkala, GF 1999 | - | Surakka *et al.*; 2005 | - | EF568902 | - | - | EF565336 | EF568863 |
| **XP6B** | sediment | Porkkala, GF 1999 | - | Rajaniemi *et al.*;2005 | | EF568903 | | | EF565337 | EF568862 |
| **XP6C** | sediment | Porkkala, GF 1999 | - | Surakka *et al.*; 2005 | - | EF568904 | - | - | EF565338 | EF568864 |
| **XPORK27C** | epiphytic | Porkkala, GF 1999 | - | Surakka *et al.*; 2005 | - | EF568906 | - | - | EF565340 | EF568866 |
| **XPORK36C** | sediment | Porkkala, GF 1999 | - | Surakka *et al.*; 2005 | - | EF568907 | - | - | EF565341 | EF568867 |
| **XSP2A** | epiphytic | Porkkala, GF 1999 | - | Surakka *et al.*; 2005 | - | EF583854 | - | - | - | - |
| **XSP36B** | sediment | Porkkala, GF 1999 | - | Surakka *et al.*; 2005 | - | EF583861 | - | - | - | - |
| **315** | plankton | Coastal waters of Helsinki, GF 1997 | - | Vaitomaa *et al.*; 2003, Herfindal *et al.*; 2005 | + | EF547195 | EF565277 | AY382548 | EF565293 | EF568819 |
| **318** | plankton | Coastal waters of Helsinki, GF 1998 | - | Vaitomaa *et al.*; 2003, Herfindal *et al.*; 2005 | + | EF547196 | EF565278 | AY382549 | EF565294 | EF568820 |

# 4. RESULTS AND DISCUSSION

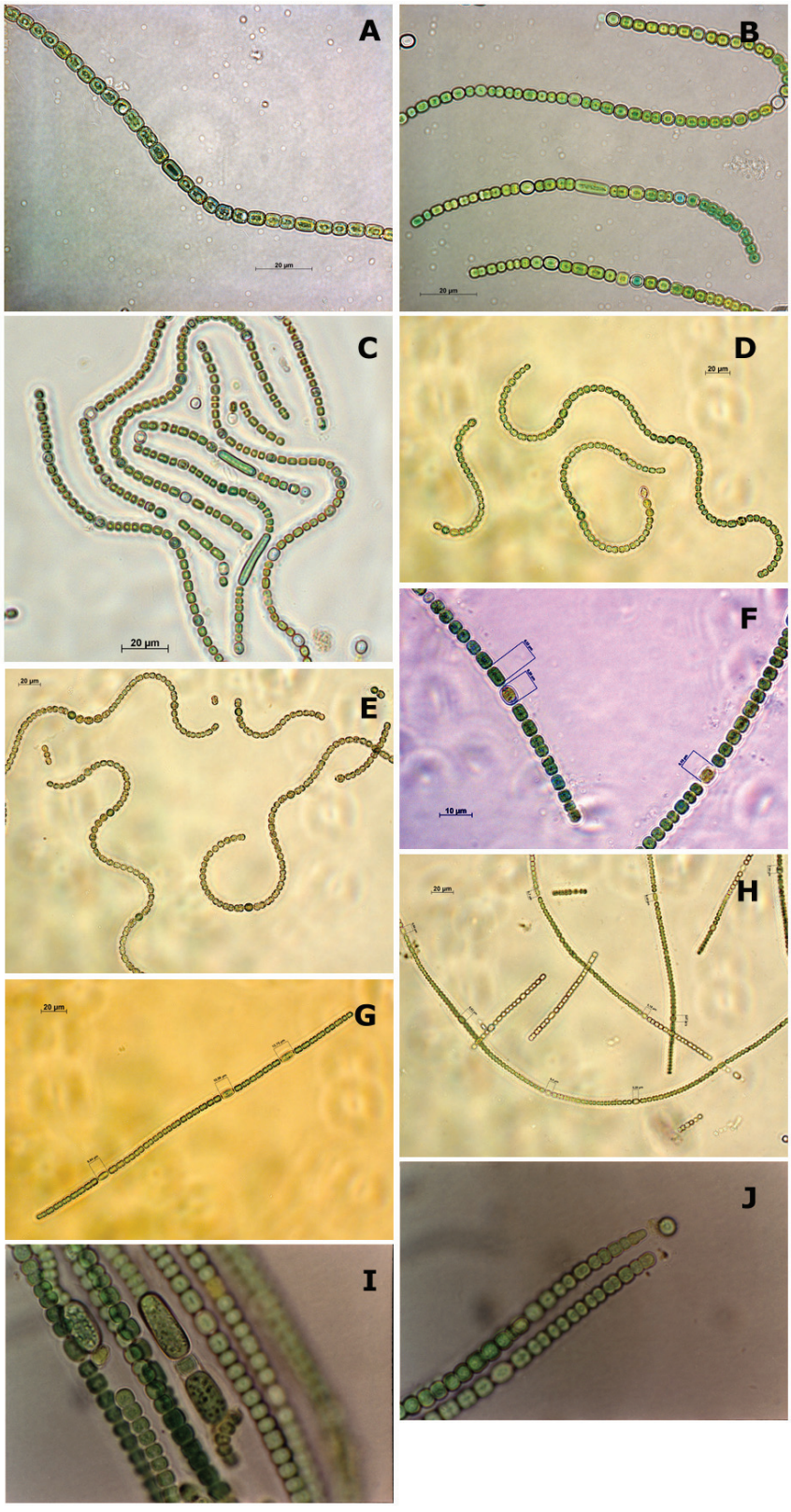
### 4.1 Isolation of planktonic and benthic *Anabaena* strains from the Gulf of Finland and their identification

In this study, a total of 49 planktonic *Anabaena* strains were isolated from the Gulf of Finland in 2004 (**Table 3**). Furthermore, we included in our phylogenetic analyses six previously isolated planktonic *Anabaena* strains (BIR2, BIR3, BIR4, BIR5, 315, and 318) and 15 benthic *Anabaena* strains (**Table 3**). These strains were isolated from the Gulf of Finland between 1999 and 2003 (**Table 3**, **Fig. 7**). Planktonic strains were characterized at the genus level by microscopy according the criteria of Tikkanen (1986), and identification of benthic strains was based on the criteria of Castenholz (2001). The compilation of photomicrographs in **Fig. 9** illustrates morphological diversity in these planktonic and benthic *Anabaena* strains. Subsequently, their 16S rRNA genes were sequenced for BLAST comparisons (www.ncbi.nlm.nih.gov/BLAST). Strains were not purified to axenic at the time of further analyses.

The isolation of Baltic Sea *Anabaena* cultures made it possible to systematically study microcystin production and inactivation of microcystin synthetase genes in *Anabaena* strains, as well as to construct a multi-gene phylogeny of these organisms. This information would have been impossible to obtain using only culture-independent molecular markers *in situ*. Only a few planktonic *Anabaena* strains have been previously isolated from the Baltic Sea. To the best of my knowledge, only the following isolated *Anabaena* strains exist: one strain, *Anabaena* KAC16, in the Kalmar Algae Collection; strains *Anabaena* ast8, am14-2, and am14-4, studied in Janson and Granéli (2002); strains *Anabaena* BIR2, BIR3, BIR4, BIR5, 301, 315 and 318 from Kaarina Sivonen's laboratory, originally isolated from the Gulf of Finland. Although it would be possible to gain insights using only a few strains, collective analyses of these Baltic Sea strains have not been performed. Thus, previous knowledge of Baltic Sea *Anabaena* populations based on the existing strains was comprised of fragmentary information.

Another approach to collecting data and circumventing possible limitations imposed by strain isolation would have been picking single filaments of *Anabaena.* This method was applied to population genetic studies of Baltic Sea *Nodularia* (Hayes & Barker, 1997; Barker *et al*., 1999; Laamanen *et al*., 2001) and *Aphanizomenon* (Barker *et al*., 2000b; Laamanen *et al*., 2002). Single filament or colony picking is feasible for *Nodularia* and *Aphanizomenon* because of their strong filament or filament bundle structures (see **Fig. 3**). Interestingly, Hayes and Barker (1997) found higher PC-IGS sequence diversity in *Nodularia* cultures than by culture-independent single filament picking method, demonstrating that cultures do not necessarily provide a narrow view of genetic diversity. However, the brittleness of *Anabaena* filaments hindered the use of this approach. There is also value in isolation of cultures, because molecular data obtained by culture-independent methods requires known organisms as anchors in subsequent data interpretation (Cohan, 2001; Giovannoni and Stingl, 2007; Garcia-Pichel, 2008). Furthermore, a single filament does not offer enough material for extensive toxin analyses.

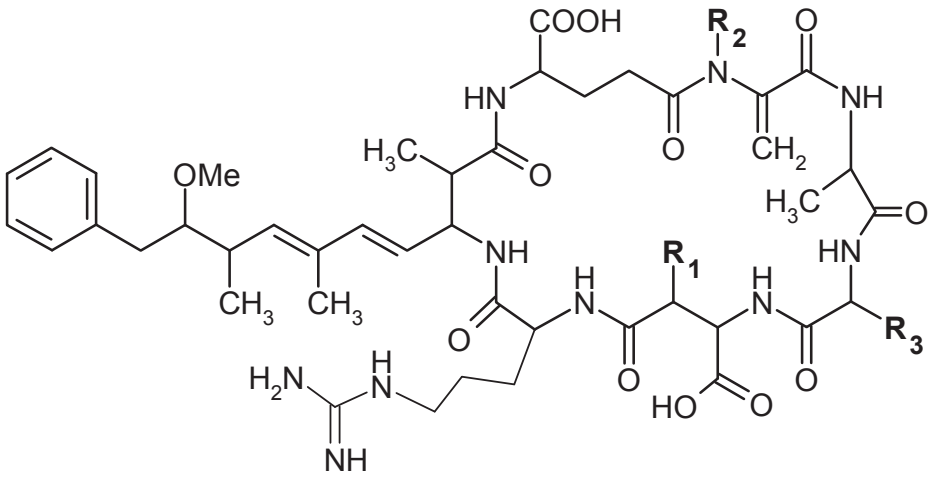
**Fig. 9.** Photomicrographs of *Anabaena* strains showing some of their morphological diversity. A) BIR2; B) BIR4; C) BIR5; D) BIR49; E) BIR52; F) BIR361; G) BIR162; H) BIR361; I) BECID22; J) BECID32. Some morphological features were measured (data not shown) for strain identification as shown in pictures F, G, and H.


A
B
C
D
E
F
G
H
I
J
20 µm
10 µm

### 4.2 Microcystin production of planktonic *Anabaena* strains

We isolated 49 *Anabaena* strains from open-sea areas of the Gulf of Finland, and five strains proved to produce microcystins in LC-MS screening of isolated cultures (**I**). The mean toxin content for these strains was 2.1 μg $mg^{-1}$ dw (**I**). Furthermore, we analyzed microcystins from two previously isolated *Anabaena* strains, 315 and 318 (Vaitomaa *et al*., 2003; Herfindal *et al*., 2005). These seven *Anabaena* strains produced microcystin MCYST-LR as well as [D-$Asp^3$]MCYST-LR, [D-$Asp^3$]MCYST-HtyR, MCYST-HtyR, [D-$Asp^3$, $Dha^7$]MCYST-HtyR and [$Dha^7$]MCYST-HtyR as their main variants **(Fig. 10)**. A number of other microcystin variants were also identified based on the principal characteristics of the microcystins (e.g. $MH^+$ values, intensities of ions *m/z* 599 and 375 or 361), but the amounts of these variants produced were too low for subsequent assignment of their structures.

According to the literature, all six identified dominant microcystin variants are toxic. $LD_{50}$ toxicity values for MCYST-LR is 50 μg $kg^{-1}$, [D-$Asp^3$]MCYST-LR and [D-$Asp^3$]MCYST-HtyR 160-300 μg $kg^{-1}$, and MCYST-HtyR 80-100 μg $kg^{-1}$ (Sivonen and Jones, 1999). Variants [D-$Asp^3$, $Dha^7$]MCYST-HtyR and [$Dha^7$]MCYST-HtyR have also been shown to be toxic in non-quantitative mouse bioassays (Sivonen and Jones, 1999). The presence of microcystins in the Gulf of Finland was verified by analysing filtered water samples by LC-MS (**I**, **III**). The seawater samples taken in years 2003 and 2004 contained variants MCYST-HtyR and MCYST-LR (**I**, **III**) together with nodularin (**III**).




[D-$Asp^3$]MCYST-HtyR, $MH^+$ 1045: $R_1$=H; $R_2$=$CH_3$; $R_3$=$(CH_2)_2$ArOH **(A)**

MCYST-HtyR, $MH^+$ 1059: $R_1$=$CH_3$; $R_2$=$CH_3$; $R_3$=$(CH_2)_2$ArOH **(B)**

[D-$Asp^3$]MCYST-LR, $MH^+$ 981: $R_1$=H; $R_2$=$CH_3$; $R_3$=$CH_2CH(CH_2)_2$ **(C)**

MCYST-LR, $MH^+$ 995: $R_1$=$CH_3$; $R_2$=$CH_3$; $R_3$=$CH_2CH(CH_2)_2$ **(D)**

[D-$Asp^3$, $Dha^7$]MCYST-HtyR, $MH^+$ 1031: $R_1$=H; $R_2$=H; $R_3$=$(CH_2)_2$ArOH **(E)**

[$Dha^7$]MCYST-HtyR, $MH^+$ 1045: $R_1$=$CH_3$; $R_2$=H; $R_3$=$(CH_2)_2$ArOH **(F)**

**Fig. 10.** The main microcystin variants produced (A-F) by the seven studied *Anabaena* strains from the Gulf of Finland.

We were able to unambiguously prove for the first time that *Anabaena* in the Gulf of Finland is able to produce hepatotoxic microcystins. Previously, microcystins have been detected in coastal waters of the southern Baltic proper (Mazur and Pliński, 2003; Luckas *et al.*, 2005). Blooms containing MCYST-LR were detected at the entrance of the Gulf of Finland in 2003 (Karlsson *et al.*, 2005). At that time, *Cyanodictyon imperfectum, Anabaena* spp. (including among others *A. flos-aquae*, *A. inaequalis*, *A. lemmermannii*), and *Aphanizomenon flos-aquae* dominated the blooms, whereas the proportion of *Nodularia spumigena* was minor (Karlsson *et al.*, 2005). However, in all of these cases the microcystin-producer remained unidentified. Although it has been speculated that Baltic Sea *Anabaena* spp. may produce toxins, direct evidence to relate *Anabaena* to microcystin-production has not been presented before this study.

All seven hepatotoxic Baltic Sea *Anabaena* strains produced a number of microcystin variants (**I**). There are several cases of freshwater *Anabaena* blooms and isolated *Anabaena* strains from the northern hemisphere that showed production of a range of microcystins (Sivonen and Jones, 1999), usually with three to seven dominant microcystin variants (Harada *et al.*, 1991; Namikoshi *et al.*, 1992; Sivonen *et al.*, 1992). Genetic variation in *mcy* genes has been related to the variability of microcystins produced by *Anabaena* (Fewer *et al*., 2008), *Microcystis* (Mikalsen *et al.*, 2003), and *Planktothrix* strains (Kurmayer *et al.*, 2005). Growth conditions have been linked to the production of different microcystin variants in *Anabaena*: Temperature affected the production of MCYST-LR and MCYST-RR in a hepatotoxic *Anabaena* strain (Rapala *et al.*, 1997; Rapala and Sivonen, 1998), whereas light regulated the production of MCYST-LR (Rapala and Sivonen, 1998). Similarly, photon irradiance affected the production of microcystin variants in a *Planktothrix agardhii* strain, since MCYST-RR was replaced by the more toxic variant MCYST-LR when photon irradiance increased (Tonk *et al.*, 2005).

### 4.3 The use of DGGE as a method to detect hepatotoxin producers

In this study, we designed a culture-independent DGGE method to identify different putative microcystin and nodularin producers in bloom samples from the Gulf of Finland (**III**). We designed primers to specifically detect *Anabaena mcyE* genes and *Nodularia ndaF* genes. Primer design and gel running conditions were optimized to obtain the most distinct banding pattern possible (that is, each band should be well-separated along the DGGE gel; see Fig. 2 in paper **III**). We were able to detect 12 different *mcyE* and 3 different *ndaF* gene sequences (Table 3 in paper **III**). The presence of microcystin-producing *Anabaena* populations was associated with the presence of microcystins in water samples screened by LC-MS (Table 1 in paper **III**). For one station, the *mcyE* gene was identified, while microcystins were not detected in LC-MS. This may be explained by the presence of inactivated *mcy* gene clusters (see section 4.4; **IV**), which may even be quite common in the Gulf of Finland (**IV**). Interestingly, *mcyE* genes were detected in *Anabaena* populations in both years, 2003 and 2004. This excluded the possibility that the presence of microcystin-producing *Anabaena*, detected by isolating strains in 2004 (**I**), was a single phenomenon in the Gulf of Finland. Microcystin-producing *Anabaena* strains in paper **I** were also isolated only from the eastern part of the Gulf of Finland, but based on DGGE analysis (**III**) *Anabaena* populations containing *mcyE* genes were present widely in the Gulf of Finland (**Fig. 8**).

Seven microcystin-producing Baltic Sea *Anabaena* strains carried four different 16S rRNA (**Fig. 12**), *rbcL*, and *rpoC1* gene sequences (**I**, **II**). In DGGE, 12 different *Anabaena mcyE* gene sequences were obtained (**Fig. 13**). Thus, the genetic diversity detected within

the microcystin-producing *Anabaena* populations in the Gulf of Finland was much higher based on DGGE than expected based on the isolates. It is known that the use of cultures can yield a limited view of bacterial diversity in the environment (Giovannoni and Rappé, 2000; Garcia-Pichel, 2008), because culture conditions may be selective (Castenholz, 1992; Ernst *et al*., 2005). Thus, culture-independent molecular genetic markers have been used for *in situ* detection, fingerprinting, and functional studies of bacterial populations (Rappé and Giovannoni, 2003). DGGE circumvents the disadvantages of strain isolation, and in this case, it revealed a high genetic diversity for the microcystin-producing *Anabaena* populations in the Gulf of Finland.

DGGE also has shortcomings, which have been reviewed e.g. by Muyzer and Smalla (1998). According to the theory, each unique sequence should migrate to its specific location in the DGGE gel (Muyzer *et al.*, 1993; Muyzer, 1999). However, in our study, bands having identical sequences showed fragmented migration in DGGE gels; e.g. bands EB20 and EB22 shared the haplotype F although they migrated differently in DGGE gel (Fig. 2 in paper **III**). On the other hand, bands EB1 and EB4 in Fig. 2 in paper **III** migrated to the same position although their sequences were not identical (band EB1 belonged to haplotype group A, whereas band EB4 belonged to haplotype group N). These described cases may lead to an under- or overestimation of the genetic diversity of a community. Tuomainen *et al*. (2006) also noticed that dissimilar DNA sequences may co-migrate in the gel. Furthermore, the presence of artefactual bands may hamper the interpretation of DGGE analysis (Janse *et al*., 2004a) and may lead to an overestimation of the true diversity of a microbial community.

DGGE is a commonly-used fingerprinting method for detecting e.g. shifts in the biodiversity of cyanobacterial communities along environmental gradients, their seasonal variation, or to study the cyanobacterial community structure in previously unexplored environments (West and Scanlan, 1999; Zeidner and Béjà, 2004; Hongmei *et al*., 2005; Janse *et al*., 2005; Zwart *et al*., 2005; Roeselers *et al*., 2007a and 2007b). Our use of DGGE for the separation of hepatotoxin-producers from each other and from non-producers was justified, since cyanobacterial blooms are usually formed by several genera in the Baltic Sea (e.g. Kanoshina *et al*., 2003; Stal *et al*., 2003; Seppälä *et al*., 2007) and blooms in general may contain a number of toxin-producing genera (Rantala *et al*., 2006). Furthermore, microcystin producers are impossible to identify by microscopy. In our work, identification of microcystin/nodularin producers was based on the toxin synthetase genes, *mcyE* and *ndaF*, which are more consistent markers for toxin production than e.g. house-keeping genes (see section 1.6.5 for an explanation).

### 4.4 Inactivation of *mcy* genes in *Anabaena* populations in the Gulf of Finland

We found two non-microcystin-producing *Anabaena* strains, BIR256 and BIR259 (**Table 3**), which were shown to have a full complement of *mcy* genes in their *mcy* gene clusters (**IV)**. Consequently the inability of these strains to produce microcystins could not be explained by the absence of any of the *mcy* genes. The inability to produce microcystins was verified with LC-MS analyses (**I**). We postulated that the inability to produce microcystins was linked to the presence of insertions or deletions in essential *mcy* genes. In order to identify such insertions or deletions, we screened the entire *mcy* gene clusters of strains BIR256 and BIR259 and compared the sizes of their PCR products to the PCR products of microcystin-producing *Anabaena* sp. strain 90 [the accession number for its *mcy* gene cluster is AJ536156; Rouhiainen *et al*. (2004)]. Differences in the sizes of the

PCR products in comparison with those from the reference, *Anabaena* sp. strain 90, were presumed to indicate potential insertions or deletions in strains BIR256 and BIR259, and these PCR products were subsequently sequenced. Altogether, we detected two insertions and one deletion in strain BIR259 and one insertion in strain BIR256 (**Fig. 11**). In addition to the insertions described we cannot rule out the possible presence of point mutations or short insertions or deletions in *Anabaena* strains BIR256 and BIR259. Short insertions or deletions within the *mcy* genes could cause frame-shift errors, and point mutations may create stop codons within genes or such substitution of one base pair for another may result in nonfunctional protein.

In *Anabaena* strain BIR259, we found an insertion of 207 bp (ImcyD, **Fig. 11**) in the gene *mcyD* (**IV**). ImcyD was located between nucleotides 25801 and 25802, according to the reference sequence of *Anabaena* sp. strain 90 (AJ536156). ImcyD was flanked by 16-bp inverted repeats, and five stop codons in locations 853, 856, 857, 863 and 902 of the encoded amino acid sequence of the *mcyD* gene. A second insertion in strain BIR259 was found in the *mcyE* gene (**Fig. 11**). It was located between nucleotides 9968 and 9969, according to the sequence of *Anabaena* sp. strain 90, and its length was 126 bp. It contained three stop codons in positions 1907, 1912, and 1921 of the encoded amino acid sequence of the *mcyE*. Furthermore, in strain BIR259, an in-frame deletion of 1236 bp (DEmcyA, **Fig. 11**) was detected in *mcyA*. Such a deletion has been previously characterized in *Anabaena* strains by Fewer *et al*. (2008). The deletion removes almost the entire *N*-methyltransferase (NMT) domain (Fewer *et al*., 2008; **IV**) and thus does not explain the inactivation of the *mcy* genes.

Strain BIR256 contained a 392 bp insertion (ImcyGA, **Fig. 11**) between the *mcyA* and *mcyG* genes (**IV**). Insertion ImcyGA was also detected in strain BIR257 (Fig. 2 in paper **IV**), which is a microcystin-producing *Anabaena* strain isolated from the Gulf of Finland (**I**). Thus, ImcyGA cannot be related to the loss of microcystin production. Furthermore, ImcyGA is located in an intergenic region, which is unlikely to contribute to gene expression. The ImcyGA insertion sequence was identical in both *Anabaena* strains BIR256 and BIR257, and was located between nucleotides 36976 and 36977, according to the sequence of *Anabaena* sp. strain 90 (AJ536156).

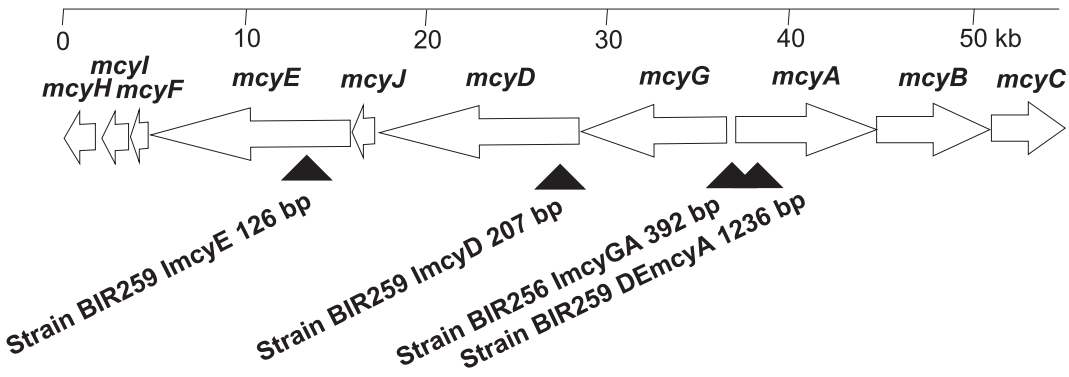



**Fig. 11.** Diagram depicting the mcy gene cluster (according to *Anabaena* strain 90; Rouhiainen *et al*., 2004) and locations of the insertions and deletions in *Anabaena* strains BIR256 and BIR259.

In our study, two out of 43 non-microcystin-producing *Anabaena* strains proved to carry *mcyE* genes (Table 1 in Paper **IV**). Although this study was the first documentation of *mcy* gene inactivation in *Anabaena*, there are several cases of such inactivation reported in strains of other cyanobacterial genera. *Microcystis* strain N-C 143 contained *mcyA* and *mcyB* genes detected by specific PCR and it also synthesized the corresponding mRNA, confirming the transcriptional functionality of the *mcyABC* gene cluster (Mikalsen *et al*., 2003). However, this strain did not produce detectable levels of microcystins (Mikalsen *et al.*, 2003). *Microcystis* strain MRC was toxic at the time of sampling, but later proved to be non-toxic (Kaebernick *et al*., 2001). It still contained all of the *mcy* genes (*mcyA-J*), and there were no mutations in promoter sites (Kaebernick *et al.*, 2001). Furthermore, 2.5 % of *Microcystis* colonies isolated from 13 freshwater bodies of water from nine European countries contained *mcy* genes but did not produce detectable levels of microcystins (Via-Ordorika *et al.*, 2004). Similarly, the use of *mcyB* and *mcyA* sequences did not allow the discrimination of microcystin-producing and non-producing *Planktothrix* strains (Kurmayer and Gumpenberger, 2006). Christiansen *et al.* (2006) found three insertions ranging from 1429 bp to 1433 bp in three different *mcy* gene locations (*mcyA*, *mcyD*, and the intergenic region of *mcyE* and *mcyG*), and two deletions of lengths 400 bp (in *mcyB*) and 1869 bp (in *mcyHA*) in *Planktothrix* strains. The insertions were found to have a conserved domain assigned to transposable elements (Christiansen *et al*., 2006). By designing mutation-specific PCR primers, Christiansen *et al*. (2006) also found insertions and one type of deletion in *Planktothrix* populations in lake samples. So far, natural insertions have been documented only in *Anabaena* and *Planktothrix* strains.

Interestingly, the insertions identified here, most likely leading to the loss of microcystin biosynthesis, were detected in the Baltic Sea *Anabaena* strain BIR259 and field samples, but not in freshwater *Anabaena* strains or lake water samples (**IV**). The ImcyD insertion was detected in field sample Cyano27, taken from the Gulf of Finland (**IV**), and ImcyGA was detected in two field samples, Cyano20 and Cyano27 (**IV**). This divergence between Gulf of Finland and freshwater *Anabaena* populations was examined further, and, in total, 25 microcystin-producing strains from both environments were screened for the presence of ImcyGA insertion. ImcyGA insertion was absent in all *Anabaena* strains from freshwaters, whereas some *Anabaena* strains from the Gulf of Finland contained the insertion and some did not. ImcyD, with the inverted repeats at its ends, resembles insertion sequences (Zhou *et al*., 2008).

Why do insertions exist in strains from the Gulf of Finland but probably not in strains from freshwater lakes? Insertion sequences are found in high numbers in cyanobacterial genomes, and they can move themselves within a genome or between genomes (Zhou *et al*., 2008). It is postulated that certain environments activate insertion elements (Zhou *et al*., 2008), which might explain the presence of the described insertions in *Anabaena* populations in the Gulf of Finland. The frequency of genetic rearrangement [e.g. by insertion sequences, which might induce the bacterial SOS systems (Nagy and Chandler, 2004)] is particularly high following physiological stress (Mlouka *et al*., 2004). It is possible that e.g. salinity presents such a stress factor as to activate insertion sequences in *Anabaena* communities in the Gulf of Finland.

The contribution of HGT events to the diversification of bacteria is widely acknowledged (e.g. Flores *et al*., 2008; Swingley *et al*., 2008), and it is possible that the insertions described are transferred within the *Anabaena* populations in the Gulf of Finland. Genetic exchange has been previously detected within Baltic Sea *Nodularia* populations

(Barker *et al*., 1999 and 2000a). Recently, cyanophages were isolated from the Baltic Sea, and shown to infect Baltic Sea *Nodularia* filaments (Jenkins and Hayes, 2006). Some of these cyanophages belonged to the bacteriophage family Myoviridae (Jenkins and Hayes, 2006), which are known to have a broad host range (Hess, 2008). Currently resolved Myoviridae cyanophage genomes included genes for which the closest homologs were found in cyanobacterial genomes, showing their broad spectrum of genetic transfer over the course of evolution (Hess, 2008). Cyanophages may be as abundant as $10^4$ to $10^5$ viruses per mL in seawater (Suttle and Chan, 1993; Waterbury and Valois, 1993), and some of them have broad host ranges (Waterbury and Valois, 1993; Sullivan *et al*., 2003; Chénard and Suttle, 2008; Deng and Hayes, 2008). This interchange of hosts opens up the possibility of a route for genetic exchange between strains, within species or between genera.

The *mcy* gene cluster has an ancient origin in cyanobacteria, and it is believed that *mcy* genes have been repeatedly lost in many cyanobacterial genera during evolution, explaining the sporadic distribution of this gene cluster within the clade of cyanobacteria (Rantala *et al*., 2004). Gene loss occurs where deleterious or no longer beneficial genes are lost by selection pressure due to accumulation of mutations and deletions (Lan and Reeves, 2000). This may occur e.g. in situations where a gene is not useful in a new niche (Lan and Reeves, 2000). The loss of 34kb from the *mcy* gene cluster has been described in two non-microcystin-producing *Microcystis* strains, which were originally subcultures of two microcystin-producing *Microcystis* strains (Schatz *et al*., 2005). The inactivation of the *mcy* gene cluster by insertions may act as a preliminary step to such *mcy* gene loss in *Anabaena* populations in the Gulf of Finland.

### 4.5 The genetic diversity of microcystin-producing *Anabaena* in the Gulf of Finland

Microcystin-producing *Anabaena* populations in the Gulf of Finland are genetically heterogeneous (**I**, **II**, **III**, and **IV**). In papers **I** and **II**, the seven studied microcystin-producing strains were divided into three branches in the 16S rRNA gene tree (**Fig. 12**). While *Anabaena* strains BIR250A, BIR257, BIR258, BIR260, 315 and 318 were affiliated within the previously described hepatotoxic cluster (cluster A in **Fig. 12**), strain BIR246 grouped with non-toxic freshwater *Anabaena* and *Aphanizomenon* strains (cluster D in **Fig. 12**; **I**, **II**). This genetic diversity of hepatotoxic *Anabaena* strains was confirmed by *rpoC1-rbcL* analysis (Fig. 3 in paper **II**). Further confirmation of the genetic diversity of microcystin-producing *Anabaena* populations was provided by *mcyE*-specific DGGE analysis (**III**). In DGGE analysis, 11 unique *Anabaena mcyE* haplotypes were detected in the Gulf of Finland, whilst one haplotype group included identical *mcyE* sequences from both the Gulf of Finland and freshwater lakes. The MSN analysis (**Fig. 13**) illustrates the distribution of freshwater and Baltic Sea *Anabaena mcyE* sequences. Furthermore, insertions and deletions within the *mcy* genes described in the previous chapter add to the previously described genetic diversity of *Anabaena* populations in the Gulf of Finland (**IV**).

Our results do not fully support previous studies in which all hepatotoxic *Anabaena* strain sequences from freshwater environments were confined to a single distinct group (including a few non-toxic *Anabaena* strains) when analyzed by comparison of 16S rRNA (Lyra *et al*., 2001; Gugger *et al*., 2002a; Iteman *et al*., 2002; Rajaniemi *et al*., 2005), *rpoB* (Rajaniemi *et al*., 2005), and *rbcLX* (Gugger *et al*., 2002a; Rajaniemi *et al*., 2005) gene sequences as well as the ITS region (Gugger *et al*., 2002a). In addition, the particular clustering of microcystin-producing freshwater *Anabaena* strains was also found by REP- and ERIC-PCR genomic fingerprinting of *Anabaena* strains (Lyra *et al*., 2001) as well as

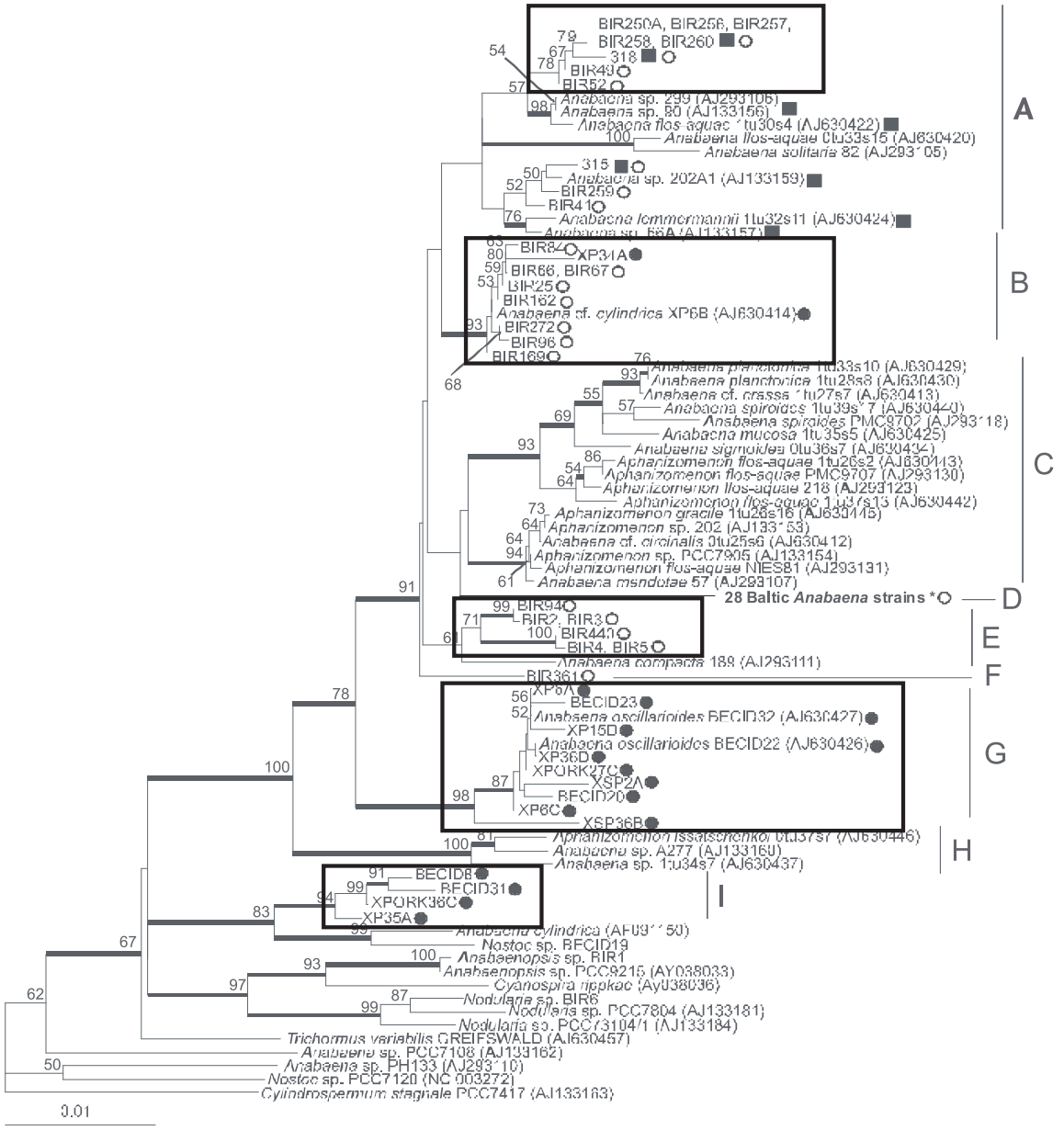



**Fig. 12.** A neighbor-joining tree based on the 16S rRNA gene sequences (1294 bp) of the *Anabaena* isolates studied (in bold). Bootstrap values over 50 % are shown at the nodes. The scale bar corresponds to a 1% difference in sequences. Putative Baltic Sea-specific *Anabaena* lineages or ecotypes in addition to branches D and F (discussed in chapter 4.8) are illustrated with boxes. ■ = microcystin-producing strain; ○= planktonic Baltic Sea *Anabaena* strain; ● = benthic Baltic Sea *Anabaena* strain. Cluster D includes strains BIR19, BIR28, BIR30, BIR31, BIR50, BIR53, BIR54, BIR56, BIR73, BIR76, BIR78, BIR130, BIR132, BIR180, BIR202, BIR208, BIR219, BIR232, BIR246■, BIR274, BIR300, BIR348, BIR358, BIR370A, BIR370B, BIR374, BIR406, BIR441.

by fatty acid analyses (Gugger *et al.*, 2002b). Such a grouping of toxic cyanobacteria has been the basis for designing diagnostic PCR to distinguish toxic from non-toxic strains (e.g. Fergusson and Saint, 2000; Laamanen *et al.*, 2001). Most of the Baltic Sea microcystin-producing *Anabaena* strain sequences grouped with this "hepatotoxic cluster" (cluster A), with only one strain, BIR246 (belonging to cluster D), falling outside of this cluster (**Fig. 12**).

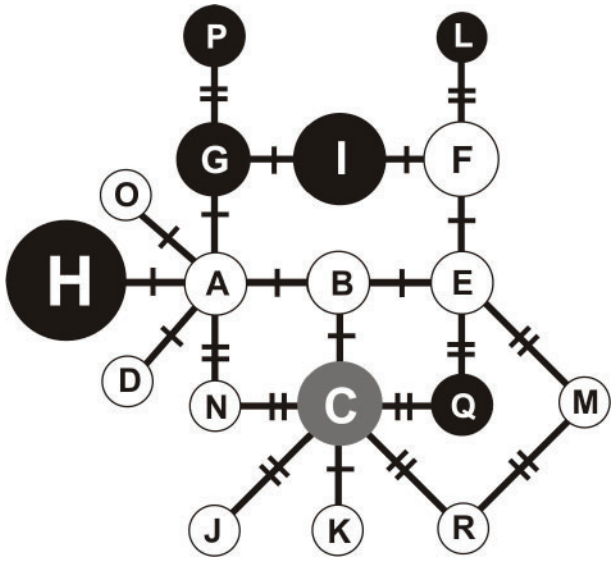



**Fig. 13.** A minimum spanning network (MSN) constructed using MINSPNET (Excoffier and Smouse, 1994) illustrating nucleotide differences between the *mcy*E haplotypes of *Anabaena*. The sizes of the circles are proportional to the number of times a haplotype was encountered. The number of substitutions separating each haplotype is illustrated with bars crossing the connecting lines. The network was constructed from 19 *mcy*E sequences originating from uncultured *Anabaena* from the Gulf of Finland (○) and 39 *mcy*E sequences from different *Anabaena* strains isolated from the Gulf of Finland and Scandinavian freshwater lakes (●). Haplotype C was found in both *Anabaena* strains and in uncultured cyanobacteria from the Gulf of Finland as detected by DGGE (●).

### 4.6 Does the salinity explain the presence of microcystin-producing *Anabaena* in the Gulf of Finland?

All microcystin-producing *Anabaena* strains were isolated either from the easternmost sampling station (station 16 in **Fig. 7**) or from coastal waters of the Gulf of Finland (**I**). Also, the *Anabaena mcyE* genes (in paper **III**) (co-occurring with microcystins) were restricted to the eastern part of the sampling area (filled triangles and squares in **Fig. 8**). This raised the question as to whether environmental factors determined the distribution of microcystin-producing *Anabaena*. The environmental physicochemical parameters considered most significant (concentrations of phosphate, total phosphorus, silicate, nitrate, nitrite, ammonium and total nitrogen as well as temperature, oxygen, and salinity) were determined for each sampling station and were used to try to identify the possible factors affecting the distribution of microcystin-producing *Anabaena* populations (**I**, **III**).

PCA analysis (Fig. 5 in paper **I**) demonstrated that microcystin-producing *Anabaena* strains were isolated from the station which had low salinity. Moreover, linear-regression analysis of DGGE data (**III**) showed that, of the environmental parameters, salinity was the best parameter to explain variation in the data (it explained 42.6 % of the variation, P = 0.0024). To investigate to what extent the variables explain the grouping and to visualize the correlation structure in multivariate data, we used canonical analysis of principle coordinates and generalised discriminant analysis (**III**). Microcystin-producing and non-microcystin-producing blooms were set as *a priori* groups (presence/absence of *mcyE*). Two first PCO axes, explaining 69.3 % and 29.9 % of the total variance, were subsequently chosen in the canonical discriminant analysis, resulting in 88 % correctly classified observations (P=0.0012). Therefore the null hypothesis, no differences between the microcystin-producing blooms (the presence of *mcy*E gene) and non-microcystin-producing blooms, was rejected.

In the literature, no data is available on the relationship between salinity and microcystin production of *Anabaena*. Salinity is known to have an impact on the bacterial community structure (Bernhard *et al*., 2005; Kaartokallio *et al*., 2005; Sahan and Muyzer, 2008), and correspondingly cyanobacteria tend to respond to salinity fluctuations as well (Nübel *et al*., 2000; Marshall *et al*., 2006; Ryan *et al*., 2008). In the future, the microcystin-producing, brackish water *Anabaena* strains isolated in this study (**I**) will provide the possibility to perform laboratory experiments to study the response to salinity of the growth and microcystin production of *Anabaena*.

As nitrogen-fixers, *Anabaena* and other heterocystous cyanobacteria have a competitive advantage in waters with a low ratio of inorganic nitrogen to phosphorus (DIN:DIP) (Vuorio *et al*., 2005). Interestingly, $PO_4$-phosphorus was not a parameter explaining the presence of microcystin-producing *Anabaena* in the Gulf of Finland (Fig. 5 in paper **I**). The culture-independent DGGE gave similar results, since linear regression analysis showed that $PO_4$ did not significantly (P = 0.1519; data not shown in paper **III**) explain the presence of microcystin-producing blooms. This is not in agreement with a study of Finnish freshwater lakes, where the presence of microcystin-producing *Anabaena* blooms was associated with low $PO_4$-phosphorus and low $NO_3$-nitrogen (Rapala and Sivonen, 1998). In paper **I**, in addition to low salinity, low silicate concentrations also corresponded to the presence of microcystin-producing *Anabaena* (Fig. 5 in paper **I**). Since sediment is a significant silicate source, Suomela *et al.* (2005) proposed that high silicate concentrations in the water column indicate upwelling. Upwelling releases nutrients such as phosphate into the water column and raises more saline deep waters. This would explain the co-occurrence of high salinity together with high silicate concentrations (and vice versa low salinity with low silicate concentrations) (Fig. 5 in paper **I**). However, cyanobacteria are not known to require silicate, so the reasons for the co-occurrence of microcystin-producing *Anabaena* and low silicate are unclear.

A number of studies have addressed the effects of chemical and physical factors on microcystin production. The factors selected include nutrients (mainly nitrogen and phosphorus) (Sivonen, 1990; Rapala *et al.*, 1997; Vécie *et al.*, 2002), temperature (Sivonen, 1990; Rapala *et al.*, 1997), and light (Sivonen, 1990; Rapala *et al.*, 1997). The general understanding is that toxin production of *Anabaena* is promoted in the conditions which are most favorable for its growth (Sivonen and Jones, 1999). Recently, after characterization of *mcy*/*nda* gene sequences, research on external regulation factors for microcystin/nodularin

production was extended to the genetic level (Kaebernick *et al*., 2000; Jonasson *et al*., 2008). Further transcriptional expression studies of *mcy*/*nda* genes may reveal more detailed information on the regulatory mechanisms of *mcy* genes in *Anabaena*.

We investigated *Anabaena* populations only in the Gulf of Finland, and thus it is not clear to what extent salinity plays a role in the occurence of microcystin-producing *Anabaena* in the Baltic Sea more generally. It is possible that ecological attributes other than salinity contribute to structuring *Anabaena* communities, and explain the presence of microcystin-producing *Anabaena* in the Gulf of Finland. Such factors may be external, such as water currents correlating with salinity (a quasi-permanent front exists at the entrance of the Gulf of Finland which separates the more saline waters of the Baltic Sea proper and the lower salinity waters of the Gulf of Finland). Influecing factors may also be internal, such as population dynamics within assemblages (e.g. effects of viral pathogens and other grazers). However, based on the results presented here, it seems likely that salinity plays a role in the distribution of microcystin-producing *Anabaena*.

### 4.7 Proposals for the origin of the microcystin-producing *Anabaena* populations in the Gulf of Finland

The introduction of microcystin-producing *Anabaena* through river discharges would explain their presence in low salinity waters (see chapter 4.6). In this case, their presence would be constrained by the increasing salinity outside the Gulf of Finland. Haplotype C in **Fig. 13** from both the Gulf of Finland and freshwater lakes shared identical *mcyE* gene sequences. This suggests that microcystin-producing *Anabaena* populations are at least partly introduced to the Gulf of Finland through freshwater inflow. It is also possible that microcystin-producing *Anabaena* populations are an indigenous part of phytoplankton assemblages in the Gulf of Finland rather than primarily being introduced only by freshwater input. This possibility is supported by the phylogenetic data, since microcystin-producing *Anabaena* strain sequences from the Gulf of Finland were not genetically identical to freshwater strain sequences (**Fig. 12**), despite being closely related. The Baltic Sea microcystin-producing *Anabaena* strain sequences were also scattered in the phylogenetic tree and were genetically more diverse than freshwater microcystin-producing *Anabaena* strain sequences (although based on only one strain, BIR246 in cluster D, being an outlier) (**Fig. 12**). Furthermore, a number of Baltic Sea and freshwater lake specific *mcyE* sequences were found in MSN analysis (**Fig. 13**), implying a genetic distinction between populations in these environments. According to empirical data, peripheral populations have less genetic variation than core populations of the same species, since population bottlenecks usually reduce genetic variation (Johannesson and André, 2006). Based on genetic diversity, Baltic Sea *Anabaena* populations cannot be considered peripheral populations of freshwater *Anabaena*. Although our results point toward the idea of microcystin-producing *Anabaena* being indigenous to the Baltic Sea, the question of its true origin remains to be studied.

### 4.8 Genetic diversity found in the Baltic Sea *Anabaena* isolates studied by 16S rRNA, *rbcL*, and *rpoC1* genes

In the context of this study, it is pertinent to mention that much of our current knowledge of *Anabaena* phylogeny is based on strains isolated from freshwater environments. Sequence analyses based on partial 16S rRNA, *rbcL*, and *rpoC1* genes revealed a high genetic diversity among the strains of Baltic Sea *Anabaena*. Planktonic and benthic Baltic Sea

*Anabaena* strain sequences could be divided into seven clusters in the 16S rRNA gene tree (**Fig. 12**), and the same grouping was also supported by the *rpoC1-rbcL* tree (Fig. 3 in paper **II**). Sequences obtained from the open-sea planktonic isolates could be divided into five clusters (A, B, D, E, and F), whereas sequences from the benthic *Anabaena* strains, which were isolated from the littoral zone of Karpilahti, Porkkala and Vuosaari (**Fig. 7**), fell into three clusters, B, G and I (**Fig. 12**). Thus, both planktonic and benthic Baltic Sea *Anabaena* strains were genetically heterogeneous.

Cluster A contained Baltic Sea planktonic *Anabaena* strains that produced microcystins (BIR250A, BIR257, BIR258, BIR260, 315 and 318) as well as strains that did not (BIR41, BIR49, BIR52, BIR256 and BIR259). These strains also grouped with microcystin-producing and non-producing *Anabaena* strains from freshwater lakes. Cluster B included strains from two benthic (XP34A and XP6B) and eight planktonic (BIR25, BIR66, BIR67, BIR84, BIR96, BIR162, BIR169, BIR272) strains. This cluster contained sequences only from strains isolated from the Gulf of Finland. Clusters C and H contained sequences from *Anabaena* and *Aphanizomenon* strains that had all been previously isolated from freshwater environments. Cluster D was formed by a total of 28 identical Baltic Sea *Anabaena* strain sequences. Thus, in the Gulf of Finland, the representatives of this cluster were the most common and widely dispersed, which could merely be due to selective culturing. The sequences from six non-toxic planktonic *Anabaena* strains (BIR2, BIR3, BIR4, BIR5, BIR94 and BIR440) made up cluster E together with freshwater *Anabaena compacta* 189. *Anabaena* strain BIR361 formed branch F. Clusters G and I were formed exclusively by sequences of *Anabaena* strains isolated from different benthic habitats from the coastal Gulf of Finland.

Clusters D and I, as well as branch F, did not contain strains from freshwater environments nor previously sequenced *Anabaena* strains, and may represent novel lineages of the genus *Anabaena*. The study of Rajaniemi *et al*. (2005) demonstrated the existence of two novel Baltic Sea lineages corresponding to clusters B and G (**Fig. 12**), although only three strains were analyzed: *Anabaena* cf. *cylindrica* XP6B (cluster B in **Fig. 12**), and *Anabaena oscillarioides* BECID22, and BECID32 (cluster G in **Fig. 12**). Although the brackish water and freshwater *Anabaena* strains were closely related, they were partly distinguishable as separate clusters. By isolating new planktonic *Anabaena* strains from the Baltic Sea and analyzing their phylogeny alongside previously isolated *Anabaena* strains, we found novel *Anabaena* lineages. This highlights the importance of analysing strains from diverse environments in phylogenetic analyses

The distribution of freshwater and Baltic Sea *Anabaena* strain sequences in the phylogenetic tree (**Fig. 12**), with clusters B, D, G, and I, along with branch F, possibly being specific to brackish conditions, suggests ecotypic differentiation in *Anabaena* populations. Furthermore, in cluster A, Baltic Sea *Anabaena* strains BIR49, BIR52, BIR250A, BIR256, BIR257, BIR258, BIR260 and 318 form a "Baltic Sea specific cluster" which diverges from freshwater strain sequences, and this same phenomenon is seen in cluster E (Baltic Sea *Anabaena* strain sequences diverge from the strain *A. compacta* 189 sequence). In these cases, clusters A and E, as well as *Anabaena* strain sequences in clusters B, D, F, G and I, may be considered to be Baltic Sea *Anabaena* ecotypes (illustrated with boxes in **Fig. 12**). Cohan (2002) defines 'ecotype' as "*a set of strains using the same or similar ecological resources, such that an adaptive mutant from within the ecotype out-competes to extinction all other strains of the same ecotype; an adaptive mutant does not however,*

*drive to extinction strains from other ecotypes*". Eventually ecotype is identified as a monophyletic cluster in a sequence-based phylogeny. Cohan (2001) writes: "*an ecotype may expand its ecological diversity through accumulation of mutations and by receiving genes from other ecotypes*". Examples of ecological divergence of the Baltic Sea *Anabaena* ecotypes may be seen in clusters A, B, G, and I (**Fig. 12**). The detection of such putative ecotypic differentiation was due to the molecular methods used, since morphological differences are sometimes elusive and morphological features can change in laboratory conditions. Environmentally-driven ecotypic differentiation has been demonstrated before where genetic analysis revealed diversification of high-light adapted and low-light adapted *Prochlorococcus* marine ecotypes (Moore *et al*., 1998; Urbach *et al*., 1998; West and Scanlan, 1999; Rocap *et al*., 2002 and 2003). Sequence data from 16S rRNA genes together with physiological characters of *Synechococcus* strains isolated from Oregon hot springs showed that strains having different optima for growth temperature were grouped in different clades in phylogenetic analysis (Miller and Castenholz, 2000). To determine whether clusters B, D, F, G and I are true Baltic Sea-specific *Anabaena* lineages remains to be confirmed: laboratory experiments are needed to determine their salinity tolerance. Furthermore, culture-independent molecular studies to specifically detect the genetic structures of *Anabaena* populations both in the Baltic Sea as well as in freshwater lakes would provide additional information and reduce the extent of distortion due to limited sample size.

To exclude the influence of possible genetic exchange, we sequenced three partial genes, 16S rRNA, *rpoC1* and *rbcL*. It was important to take this into account, since the *rbcLX* gene locus has been demonstrated to be laterally transferred within *Nostoc* and *Microcystis* (Rudi *et al*., 1998). Such genetic exchange has also been documented to occur in Baltic Sea *Nodularia* populations (Barker *et al*., 1999 and 2000a). However, in this study of 68 *Anabaena* strains (**II**), the concordance of *rbcL*, *rpoC1* and 16S rRNA gene trees suggests that exchange of these genes was unlikely.

### 4.8.1 Benthic habitats maintain higher genetic diversity than planktonic habitats

Benthic *Anabaena* strain sequences were grouped into three clusters (B, G, and I) in the 16S rRNA gene tree (**Fig. 12**). In cluster B, benthic *Anabaena* strain sequences were grouped with planktonic *Anabaena* strain sequences, whereas clusters G and I were formed solely by benthic *Anabaena* strain sequences. These two clusters, G and I, were genetically diverse and distant from each other (94.9 %) and from clusters A-F (≤ 97.1 %), which contained mainly planktonic *Anabaena* strain sequences.

The intermixing of planktonic and benthic strains in cluster B (**Fig. 12**) shows that benthic and planktonic *Anabaena* strains may not always be genetically separable based on their ecological niche. Such separation according to niches appears to be the case in planktonic and benthic *Nodularia* strains (Lyra *et al*., 2005). Benthic *Anabaena* and *Nodularia* strains are morphologically separable, since gas vacuoles are absent in benthic species (Lyra *et al*., 2005; Rajaniemi *et al*., 2005). It is also possible that planktonic *Anabaena* strains in cluster B are originally from the benthos, and thus are not true planktonic strains. However, some *Anabaena* strains may live both as benthic and as planktonic forms, depending on the prevailing environmental conditions.

The similarity values and distances between benthic *Anabaena* strains were higher than between planktonic strains. This suggests that the benthic *Anabaena* populations

are genetically diverse and that benthic habitats allow for greater genetic diversity of cyanobacteria than planktonic habitats. Benthic cyanobacterial mats are known to be diverse (Hoffmann, 1999; Stal, 2000). Various cyanobacterial genera, including *Anabaen*a, *Calothrix*, *Cyanothece*, *Nodularia*, *Nostoc*, *Phormidium*, and *Rivularia*, are present in the littoral zone of the Gulf of Finland (Herfindal *et al*., 2005; Lyra *et al*., 2005; Surakka *et al*., 2005; Sihvonen *et al*., 2007). However, little is still known about their genetic diversity [excluding *Nodularia* (Lyra *et al*., 2005) as well as *Calothrix* and *Rivularia* (Sihvonen *et al*., 2007) in the Gulf of Finland]. Mat-forming cyanobacteria have been intensively studied, but they mostly originate from the littoral zone of tidal coastal regions. The fluctuations result in an extreme growth environment for mat-forming benthic cyanobacteria, with continuous changes in temperature, salinity and grazing organisms (Stal, 2000). The Baltic Sea is a non-tidal sea and thus the littoral ecology is more stable than tidal coastal regions. The genetic diversity maintained by benthic habitats may be partly explained by the presence of more nutrients, by the heterogeneity of the benthic habitats, or by fluctuating physicochemical gradients in the littoral zones (Stal, 1995). The clustering of benthic *Anabaena* strain sequences (clusters G and I in **Fig. 12**) also suggests the habitat-related distribution patterns in *Anabaena* populations.

### 4.8.2 How many *Anabaena* species exist in the Gulf of Finland?

The definition of "species" in microbiology is not straightforward. However, because concepts are required to describe the populations of each organism, and e.g. to measure biodiversity, criteria are needed as working tools. According to definitions for species and genus based on 16S rRNA gene sequences, requiring similarities of ≥ 97.5 % and ≥ 95 %, respectively (Stackebrandt & Goebel, 1994; Ludwig *et al*., 1998), all planktonic Baltic Sea *Anabaena* strains would belong to the same species. However, according to the revised view of Stackebrandt and Ebers (2006) suggesting 98.7-99% 16S rRNA gene sequence similarity as the cut-off value for a preliminary species demarcation, we could specify three planktonic *Anabaena* species based on our analyses. These three *Anabaena* species would contain the following clusters in the 16S rRNA tree in **Fig. 12**: cluster A, cluster B, and clusters D-E-F.

Benthic *Anabaena* strains in clusters G and I (**Fig. 12**) would represent species separated from other planktonic Baltic Sea *Anabaena* strains, irrespective of the cut-off values (97.5 % or 98.7%) used. Clusters G and I, exclusively formed by benthic strain sequences, would represent two *Anabaena* species. The distances between cluster I and other clusters including Baltic Sea *Anabaena* strains varied from 94.6 % to 95.6 % (Table 2 in paper **II**), and thus cluster I may even represent a different genus based on a cut-off value of 95% (Ludwig *et al*., 1998). Specifying the species requires not only molecular data but also information on phenotypic features, which should be consistent within the species. In this study, we did not carry out thorough morphological and other phenotypic analyses of *Anabaena* strains, because of the methodological problems reported previously (see chapter 1.5). Supplementary analyses are required to confirm the stability of the described clustering of the Baltic Sea *Anabaena* strains. Based on molecular data of a total of 68 *Anabaena* strains (**II**), it is a valid to say that three planktonic and two benthic *Anabaena* species exists in the Gulf of Finland.

How well does the species concept defined by threshold values [e.g. similarity of 16S rRNA gene above 98.7% according to Stackebrandt and Ebers (2006)] describe the

populations? Unfortunately, while these kinds of nominal threshold values are practical tools, they do not take into account e.g. the ecology of a species (Konstantinidis and Tiedje, 2004). For example, strains in cluster A in **Fig. 12** would be considered to be one *Anabaena* species even with the threshold value of 98.7 % 16S rRNA gene similarity (Stackebrandt and Ebers, 2006), regardless of the clear ecotypic diversification within the cluster. It was demonstrated in whole-genome comparison that two *Prochorococcus* strains, which both were high-light adopted and differed only 0.8% in their 16S rRNA sequences, had 139 and 236 strain specific and ecologically useful genes (Coleman *et al*., 2006). Even 20% of the genome of one strain may be absent from the other strain of the same species (Lan and Reeves, 2000). This demonstrates that genomes may contain ecologically relevant genes (e.g. responsible for niche adaptation) of very different evolutionary history, and this information is undermined by species defined by 16S rRNA genes (Hess, 2008). Instead of analyzing the number of species, focusing on ecologically unique ecotypes would follow the true evolutionary history of each clade, which is the underlying basis of systematics (Cohan, 2002). The new species definition presented by Konstantinidis and Tiedje (2004) of average nucleotide identity (ANI) of all conserved genes would take into account the different evolutionary histories of genes as well as phenotype and the ecological potential of species. However, ANI results correlated with those from 16S rRNA gene sequence analyses (Konstantinidis and Tiedje, 2004). Thus, until new criteria for species are defined, 16S rRNA gene analysis is a serviceable method for defining cyanobacterial species.

# 5. GENERAL CONCLUSIONS AND FUTURE PROSPECTS

Is Baltic Sea microcystin-producing *Anabaena* specific to lower salinity waters? This can be the case based on finding microcystins only in the Gulf of Finland and in the coastal waters of the southern Baltic Proper. Several climate change scenarios have predicted that freshwater input to the Baltic Sea would increase due to a rise in rainfall (Myrberg *et al*., 2006). This would lead to lower salinity of the surface water in the Baltic Sea (Myrberg *et al*., 2006). If the fitness of microcystin-producing *Anabaena* is promoted in environments of lower salinity, it is possible that hepatotoxic cyanobacterial blooms will increase, especially in the coastal waters of the Baltic Sea.

Microbial ecology presents a continuum of developing understanding, and because of this, I have here and there already raised questions for future studies concerning Baltic Sea *Anabaena*. Future studies are needed to evaluate the distribution of microcystin-producing *Anabaena* populations in the Baltic Sea, and to determine the factors promoting their growth and microcystin-production. Also toxin screening and monitoring schemes should be extended to take into account the possible presence of microcystin variants produced by *Anabaena*. However, I present here conclusions drawn based on the papers brought together in this Thesis.

**1. A revision of the hepatotoxicity of cyanobacteria in the Baltic Sea is required**

The toxicity of cyanobacterial blooms in the Baltic Sea has been systematically connected to nodularin-producing *Nodularia spumigena*, which is a dominant bloom-forming cyanobacterium in the Baltic Sea (Sivonen *et al*., 1989; Laamanen *et al*., 2001). *Aphanizomenon* has been shown to be non-toxic (Sivonen *et al*., 1989; Lehtimäki *et al*., 1997; Repka *et al*., 2004). However, we were able to show that *Anabaena*, which has been considered a non-microcystin-producer in the Baltic Sea, is able to produce a range of hepatotoxic microcystins. Microcystin-producing *Anabaena* populations were found in both study years (2003 and 2004), ruling out the possibility that the presence of these microcystin-producers is a single phenomenon. These toxic *Anabaena* populations were also detected throughout the entire Gulf of Finland.

**2. Planktonic and benthic *Anabaena* populations are genetically diverse in the Baltic Sea**

This was the first study to systematically investigate genetic diversity of Baltic Sea *Anabaena*. Previous understanding of Baltic Sea *Anabaena* was poor, mainly due to lack of isolates. Based on analyses of 16S rRNA, *rpoC1*, *rbcL*, and *mcyE* gene sequences, we found *Anabaena* populations, both in the plankton as well as in the benthos, to be genetically heterogeneous. Interestingly, benthic *Anabaena* strains were genetically more diverse than strains isolated from plankton. The Baltic Sea *Anabaena* strains presented phylogenetic novelty, since we observed previously unknown clusters and branches in phylogenetic trees of the 16S rRNA gene sequences.

**3. The applicability of molecular methods to the study of toxicity of the cyanobacteria is not straightforward**

We found two non-microcystin-producing Baltic Sea *Anabaena* strains which carried the full complement of *mcy* genes. We described insertions elements in strain BIR259, which most likely caused the genetic inactivation of microcystin production. Such insertions have been previously described in *Planktothrix* strains and field samples. This study showed that genetic inactivation of *mcy* genes also occurs in *Anabaena* strains as well as in field samples from the Gulf of Finland.

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