Characterisation of Cyanobacterial Strains Originating from the Baltic Sea with Emphasis on *Nodularia* and its Toxin, Nodularin

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Cover figure: *Nodularia* and *Aphanizomenon* cyanobacteria.
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List of original articles

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The author's contribution in articles

I. Jaana Lehtimäki wrote the paper, did the laboratory work, and analysed the data.

II. Jaana Lehtimäki wrote the paper. She analysed growth (three different growth parameters) and toxin concentrations from cells and growth media. Jaana Lehtimäki performed statistical analyses of the data with Pia Moisander, who counted bacterial numbers and heterocyst frequencies, measured bacterial production and filament length, and detected nitrogen fixation.

III. Jaana Lehtimäki cultivated *Nodularia* and isolated nodularin from it using HPLC.

IV. Jaana Lehtimäki wrote the paper and carried out all of the phenotypic tests and most of the genotypic experiments including RFLP of 16S rRNA gene, ribotyping, and genomic fingerprinting with REP- and ERIC-sequences. Genomic fingerprinting with STRR sequence was done by Leo Rouhiainen, and the 16S rRNA genes were sequenced by Päivi Sundman. Jaana Lehtimäki analysed the ribotyping, and REP- and ERIC-PCR data while the 16S rRNA RFLP, and 16S rRNA sequence data were analysed by Christina Lyra.

Dictionary containing abbreviations

Adda	3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid.
Angiosperms	Flowering plants in which ovules are enclosed by an ovary.
Arg, R	Arginine.
Asp, D	Aspartic acid (aspartate).
Benthic	Aquatic organisms living on or in the bottom of a water body.
Bryophytes	A group of nonvascular plants comprising the mosses, hornworts, and liverworts.
Calyculin A	An octamethyl polyhydroxylated C28 fatty acid toxin isolated from marine sponges.
Cantharidin	A bicyclic terpenoid toxin isolated from beetles.
Cys, C	Cysteine.
Cytotoxin	A substance having a toxic effect on cells.
DGGE	Denaturing gradient gel electrophoresis.
ERIC -PCR	Enterobacterial repetitive intergenic consensus sequences -PCR.
Euplankton	Aquatic organisms living in open waters.
Glu, G	Glutamic acid (glutamate).
Gymnosperms	Four phyla of seed plants in which the ovules are carried naked on the cone scales.
Hepatotoxin	A substance having an adverse effect on the liver. Cyanobacterial hepatotoxins consist of cyclic peptides, microcystins and nodularins, and an alkaloid, cylindrospermopsin.
HIP1	Highly iterated palindrome.
K_s	The half saturation constant for phosphorus uptake. The level of phosphorus which produces half of maximum growth.
LD_{50}	The lethal dose 50 test. In this acute toxicity test, a substance is administrated to a group of organisms at increasing doses in order to determine the dose that kills 50**%** of the test organisms.
MeAsp	D-*erythro*-β-methylaspartic acid.
Metaphyton	Photosynthetic organisms found aggregated in the littoral zone. Synonym to the term tychoplankton used earlier.
Microcystin-LA, -LR, -YA, -YR, and - RR	Hepatotoxic heptapeptides, which have the general structure: cyclo-(D-Ala-L-***X***-D-MeAsp-L-***Y***-Adda-D-Glu-Mdha). ***X*** = leucine (L), tyrosine (Y), arginine (R). ***Y*** = arginine (R), alanine (A).
MLD_{100}	The minimum lethal dose 100 test. The test determines the minimum dose that kills 100% of the test organisms.
MU	Mouse unit. The estimated dose that kills a mouse.
Neurotoxin	A substance having a toxic effect on the nervous system. Known cyanobacterial neurotoxins consist of anatoxin-a, homoanatoxin-a, anatoxin-a(*S*), and paralytic shellfish poisons.
Nodularin	Nodularin is an hepatotoxic pentapeptide, which is produced by *Nodularia* cyanobacteria.
Periphyton	Aquatic microscopic organisms attached to objects projecting above the bottom sediment.
Phytoplankton	Microscopic aquatic organisms that float freely or have weak swimming abilities.
Protist	A single- or few-celled protoctist, which is any organisms classified into the kingdom Protoctista.
Pteridophytes	The ferns and so-called fern allies.
rbcLX	An intergenic spacer, and the gene *rbcX* and the 3´end of the *rbcL*.
REP-PCR	Repetitive extragenic palindromic –PCR
RFLP	Restriction fragment length polymorphism.
Tautomycin	A polyketide toxin produced by a soil bacterium, *Streptomyces spiroverticullatus*.

Abstract

In the Baltic Sea, *Nodularia* has been confirmed as the only toxin-producing cyanobacterium so far, but the role of environmental factors on nodularin production is not known yet. Hepatotoxic *Nodularia* blooms have been observed in almost all parts of the Baltic Sea. In these blooms, the concentration of nodularin often rises high enough to cause a health risk for animals through adverse effects on the liver. In addition to *Nodularia*, the blooms are dominated also by *Aphanizomenon*, which is not known to be toxic. These two genera are capable of forming blooms in the nitrogen-depleted water mass of the Baltic Sea in late summer due to their ability to fix nitrogen. However, they differ from each other in physiology. For example, *Nodularia* is absent from surface waters during most of the year, while *Aphanizomenon* is found all year round.

In order to understand toxin production in *Nodularia* under different environmental conditions, we studied the effect of several growth factors on intracellular and extracellular concentrations of nodularin of two batch-cultures of *Nodularia* using high performance liquid chromatography (I, II). The non-toxic *Nodularia* strain was cultured under the same growth conditions as the toxic strains in order to reveal physiological differences between toxic and non-toxic strains (I). The growth and nitrogen fixation rates of *Nodularia* and *Aphanizomenon* under different growth conditions were studied using batch-culture experiments in order to obtain information on the co-dominance of these two cyanobacterial genera in late summer blooms (II). The three-dimensional structure of nodularin in water was determined by nuclear magnetic resonance spectroscopy and molecular dynamics simulations in order to understand the inhibition of protein phosphatases by nodularin, the mechanism underlying its hepatotoxicity (III). The taxonomy of *Nodularia* from different geographical origins and with different toxin production abilities was studied using several molecular methods based on the 16S rRNA gene and whole genome (IV). In addition, the morphology of strains was examined using light microscopy (IV).

Nodularin concentrations under different growth conditions were studied using non-axenic (I) and axenic (II) *Nodularia* strains. Toxin concentrations in cells and in growth media were generally highest under conditions that promoted growth. Intracellular nodularin concentrations of the axenic *Nodularia* strain studied increased with increases in temperature, phosphate concentration, and irradiance (II). They decreased at low and high salinities and high inorganic nitrogen concentrations. The associated bacteria of non-axenic cultures had no effect on nodularin concentration. According to our studies, growth at different temperature, light, salinity, and phosphorus conditions as well as growth stage may have an effect on the release of nodularin from cells into the growth medium (I, II). When comparing the growth responses of the toxic strains and the non-toxic strain it was shown that the non-toxic strain grew poorer than the toxic ones under all conditions except at the lowest temperature and phosphate concentration tested (I).

Nitrogen fixation of *Aphanizomenon* and *Nodularia* was often, but not always, highest under conditions which promoted the growth and lowest in cultures with poor growth. Differences in growth and nitrogen fixation rates of *Nodularia* and *Aphanizomenon* were observed (II). *Aphanizomenon* preferred lower irradiances (test range 2-155 µmol $m^{-2}s^{-1}$), temperatures (7-28ºC), and salinities (0-30‰) than *Nodularia*. The different responses *of Nodularia* and *Aphanizomenon* may explain the different vertical, horizontal and temporal distribution of the two genera in the Baltic Sea. The preference of *Aphanizomenon* for low light and that of *Nodularia* for high light mirrored their vertical distribution patterns in the field; *Aphanizomenon* is more homogeneously distributed in the water column than *Nodularia*, which usually forms scum on the water surface. The ability of *Aphanizomenon* to grow at low temperatures shown in this study may explain why it is abundant in the water mass during most of the year. *Nodularia* showed a capacity to tolerate much higher temperatures than it experiences in its natural environment. The growth and nitrogen fixation rates of *Nodularia* were highest in the same salinity range (5 to 20‰) in which the genus forms mass occurrences in the Baltic Sea and other brackish waters. The incapability of *Aphanizomenon* to tolerate salinities higher than 10‰ suggests that salinity is an important factor restricting the distribution of this genus. The different salinity optima of the two genera is also seen in their different horizontal distribution patterns in the Baltic Sea: with increasing salinity from freshwater in the north to

approximately 15‰ salinity in the southern Baltic Proper, the abundance of *Aphanizomenon* decreases while the abundance of *Nodularia* increases. High phosphorus and low nitrogen concentrations have been linked to mass occurrences of *Aphanizomenon* and *Nodularia* in the Baltic Sea. Similarly, in these laboratory studies, high phosphorus and low nitrogen concentrations increased the growth of *Nodularia* and *Aphanizomenon*. Furthermore, the growth was increased with the presence of accompanying bacteria.

The solution conformation of nodularin was remarkably similar to the three-dimensional structure of microcystin-LR, which implies that nodularin inhibits protein phosphatases in the same way as microcystin-LR (III). Both toxins had a saddle-shaped backbone conformation, but microcystin-LR was more buckled than nodularin. In particular, the backbone fold in the conserved region of MeAsp-Arg-Adda-Glu was almost identical between nodularin and microcystin-LR. The molecular dynamics simulations, nevertheless, reveal a certain degree of sway for the *trans* peptide bonds. The proximal part of the Adda's side-chain was also very similar. The remote parts of Adda and Arg were not structurally defined and they were also mobile in both peptides.

No groups of *Nodularia* strains could be recognised on the basis of cell size whereas toxin production separated the strains into two groups. In this study, nodularin production of *Nodularia* strains was consistent with the genotypic analysis. Therefore, this character may be useful when identifying *Nodularia* strains. The toxic *Nodularia* strains were separated from non-toxic strains by RFLP of the 16S rRNA gene, 16S rRNA gene sequencing, REP- and ERIC-PCR, and ribotyping (IV). All strains were closely related despite their different abilities to produce toxin or geographical origins. The profiles of REP- and ERIC-sequences indicated high genetic homogeneity among toxic *Nodularia* strains from the Baltic Sea. These strains were found to be different from the toxic *Nodularia* strains from Australia and France by 16S rRNA-based methods and by REP- and ERIC-PCR. Our results indicated that two closely related *Nodularia* genotypes are found in the Baltic Sea. One genotype consists of only non-toxic strains. 16S rRNA gene sequencing showed that these strains were identical to the proposed type strain of *Nodularia spumigena* PCC 73104/1, which is not a typical *N. spumigena* strain according to morphological taxonomy. All the genetic markers separate the proposed type strain, and other non-toxic strains, from toxic strains. The toxic Baltic Sea strains form another genotype, which most closely fits the descriptions of *Nodularia baltica* and *N. spumigena* whereas the morphological characters of non-toxic *Nodularia* strains fit most closely to the description of *Nodularia sphaerocarpa*.

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1. Introduction

1.1. Cyanobacteria

Cyanobacterial evolution begun at least 2000 million years ago according to paleontological data (Hofmann, 1976 and Knoll & Golubic, 1991: cited by Golubic *et al*., 1995). Cyanobacteria are a morphologically diverse group of oxygenic photosynthetic prokaryotes (Rippka *et al*., 1979), which are phylogenetically closely related to each other and to chloroplasts (Giovannoni *et al*., 1988). Chloroplasts have originated from cyanobacteria by one or more endosymbiotic events between non-photosynthetic eukaryotic organisms and cyanobacteria (Douglas, 1994).

Cyanobacteria and oxygenic Eukaryotes, algae and plants, have generated oxygen in the earth's atmosphere. Atmospheric oxygen concentrations were high enough to promote the selection of heterocystous cyanobacteria approximately 2100 million years ago based on chemical analysis of ancient weathering profiles (Holland & Beukes, 1990: cited by Golubic *et al*., 1995). The molecular phylogenetic study of Giovannoni *et al.* (1988) has shown that the last diverging cyanobacterial groups were the heterocystous groups in which the nitrogen (N) fixing enzyme, nitrogenase, was protected from oxygen in heterocysts.

1.1.1. Geographic and ecological distribution of cyanobacteria

Due to their early evolutionary history, cyanobacteria occur abundantly in a wide range of habitats (Schopf, 1994). Cyanobacteria are known as freshwater (Hoffmann, 1996) and marine (Hoffmann, 1994) organisms, and in addition, especially the filamentous forms, also occupy a variety of terrestrial habitats (Hoffmann, 1989). Cyanobacteria form symbiotic associations with a wide range of organisms: algae, fungi, pteridophytes, gymnosperms, angiosperms, some animals, bacteria, and non-photosynthetic protists. In these associations, the cyanobacterial symbionts (cyanobionts) are usually heterocystous and filamentous forms. *Nostoc* is commonly found, but also *Anabaena*, *Calothrix*, *Fischerella*, and *Scytonema* spp. have been observed in symbiotic associations (Rowell & Kerby, 1991).

The filamentous heterocystous *Nodularia*, the main object of this thesis, occurs in saline and brackish waters and in soil all over the world (Table 1). *Aphanizomenon*, which was also studied in this thesis, lives in both fresh (Sivonen *et al*., 1990; Paerl, 1996) and brackish waters (Hällfors, 1979; Edler *et al*., 1984).

Table 1. Geographical and ecological distribution of the cyanobacterium *Nodularia*

Location	**Description of the source** (identification of species)	**Reference**
<u>Saline aquatic environments</u>		
Africa:		
- Banagher Pan, South-Africa	Saline pond (*N. spumigena* var. *vacuolaria*)	Hutchinson *et al.*, 1932
- South Africa, Namibia, Botswana, Zimbabwe, and Mozambique	Saline lakes (*N. spumigena*)	Seaman *et al.*, 1991
Antarctica:		
- South Shetland Islands	Unknown source (*N. harveyana*)	Komárek *et al.*, 1999
- Wilkes	Saline lakes and ponds (*Nodularia* sp.)	Wright & Burton, 1981
Australia:		
- Lake Alexandrina, and Lake Albert, South Australia	Saline lakes (*N. spumigena*)	Francis 1878; Baker & Humpage, 1994
- Lake Ellesmere, New Zealand	Saline lake (*N. spumigena*)	Carmichael *et al.*, 1988
- Lake Corangamite and Lake Coraculac, Victoria	Saline lakes (*N. spumigena*)	Hammer, 1981
- Orielton Lagoon, Tasmania	Coastal embayment (*N. spumigena*)	Jones *et al.*, 1994b
- Peel-Harvey Estuary, Western Australia	Estuarine (*Nodularia* sp.)	Lukatelich & McComb, 1986
Central America:		
- Alchichica, Puebla State, Mexico	Volcanic saline lake (*N.* cf. *spumigena*)	Tavera & Komárek, 1996
North America:		
- Walker Lake, Nevada, USA	Terminal saline lake (*N. spumigena*)	Cooper & Koch, 1984
- Pyramid Lake, Nevada, USA	Terminal desert lake (*N. spumigena*)	Galat *et al.*, 1981
- British Columbia and Saskatchewan, Canada	Saline lakes and ponds (*N. spumigena*)	Hammer *et al.*, 1983
- Basin, Redberry, Deadmoose, Whiteshore, and Manito lakes, Saskatchewan, Canada	Saline lakes (*N. spumigena*, *N. harveyana*)	Nordin & Stein, 1980
- Big Quill Lake, Saskatchewan, Canada	Saline lake (*N. spumigena*)	Nordin & Stein, 1980
- Riefel Wildlife Refuge, British Columbia, Canada	Coastal water (*N. harveyana*)	Nordin & Stein, 1980
- Sande Fuca and Devis Lake, Washington, USA	Coastal waters (*N. spumigena*, *N. harveyana*)	Nordin & Stein, 1980
- Great Salt Lake, Utah, USA	Terminal desert lake (*N. spumigena*)	Stephens, 1990
South America:		
- Castillos lagoon, Uruguay	Coastal water (*N. baltica-spumigena* type)	del Carmen Pérez *et al.*, 1999
Central and Southwest Asia:		
- Balkash Lake and Lake Aral, Kazakhstan/Uzbekistan	Saline tectonic lakes (*N. spumigena*)	Ergashev, 1979
- the Sea of Azov, Ukraina	Brackish water (*N. spumigena* f. *typica*)	Zenkevitch, 1963
- the Caspian Sea	Brackish water (*N. spumigena*, *N. harveyana*)	Zenkevitch, 1963
Europe:		
- Bafra Balik Gölleri (Balik Gölü, Uzun Göl), Turkey	Unknown source (*N. spumigena*, *N. harveyana*)	Gönülol & Comak, 1992
- the Baltic Sea, Europe	Brackish water (*N. spumigena*, *N. harveyana*)	Hällfors, 1979; Edler *et al.*, 1984
- Banter See, Wilhelmshaven, Germany	Brackish lake (*N. spumigena*)	Nehring, 1993
- The Gulf of St. Eufemia, the Mediterranean Sea, Italy	Brackish water (*N. harveyana*)	Pushparaj *et al.*, 1994
- Kattegat, The North Sea	Marine water (*N. spumigena*)	Smayda, 1998
- Barrow Ski Club Lake, England	Brackish lake (*N. spumigena*)	Twist & Codd, 1997

Table 1, continued

Other locations		
- Osoyoos, British Columbia, Canada	Alkaline soil near saline ponds (*N. spumigena*)	Camm & Stein, 1974; Nordin & Stein, 1980; www.pasteur.fr
- Lucknow, India	Alkaline soil (*N. harveyana*)	Dixit *et al.*, 1985
- Debari, India	Alkaline soil (*N. spumigena*)	Gopal *et al.*, 1975
- Svätý Jur, Slovakia	Littoral of brook (*N. sphaerocarpa*)	Hindàk, 1999
- the Gulf of Bothnia, the Baltic Sea	Ice (*N. spumigena*)	Laamanen, 1996
- Lindåspollene, Norway	Littoral zone of fjord (*Nodularia* sp.)	Lännergren, 1980
- Simpon's Gap, Australia	Water hole, soil (*N. spumigena*)	Nordin & Stein, 1980
- São Paulo, Brazil	Littoral zone of river (*N. willei*)	Sant´Anna, 1991
- Guadarrama mountain and Coto de Doñana, Spain	Freshwater brook and lagoon (*Nodularia* sp.)	Sanz-Alférez & del Campo, 1994
- Allahabad, India	Soil near lake (*N. harveyana*)	Tiwari & Pandey, 1976
- Buerto Princesa, Palawan, Philippines	On intertidal rocks (*N. harveyana)*	Umezaki, 1995
- Gibraltar Point, Lincolnshire, UK	Salt marsh (*N. harveyana*)	Warr *et al.*, 1984
- Dax, France	Thermal spring (*N. sphaerocarpa*/*harveyana*)	www.pasteur.fr (strain PCC7804)

1.1.2. Cyanobacterial blooms in the Baltic Sea

In the Baltic Sea, late-summer blooms of nitrogen fixing filamentous cyanobacteria are an annual phenomenon (Kononen, 1992). The geographical extent and the magnitude of these population explosions of cyanobacteria has been suspected to increase (Kahru *et al.*, 1994) due to the increased nutrient concentrations (Wulff *et al.*, 1990) and changed nutrient ratios in the Baltic Sea (Smayda, 1990).

In most open sea areas of the Baltic Sea, the blooms in July-August are dominated by *Nodularia* and *Aphanizomenon*. In addition, *Anabaena* is present in the blooms (Kononen & Niemi, 1984; Sivonen *et al.*, 1989a,b; Kononen *et al.*, 1993b). Several other cyanobacterial genera, e.g. *Aphanothece*, *Chroococcus*, *Snowella, Merismopedia*, *Microcystis*, *Lyngbya*, and *Planktothrix* have been recorded especially from coastal areas of the Baltic Sea (Hällfors, 1979; Edler *et al.*, 1984). In addition, several studies (Jochem, 1988; Kuosa, 1991; Heinänen *et al.*, 1995) have described the importance of cyanobacterial picoplankton, identified as *Synechococcus*, in the phytoplankton community of the Baltic Sea.

In the Baltic Sea, *Nodularia* has been confirmed as the only toxin-producing cyanobacterial genus so far (Sivonen *et al.* 1989a,b; Kononen *et al.*, 1993b), and animal poisonings have been associated with blooms of this genus (Nehring, 1993).

1.1.3. Toxicosis and exposure routes of cyanobacterial hepatotoxins

Hepatotoxins have most frequently been associated with incidents of animal poisonings. The first scientific report on a cyanobacterial poisoning is that by Francis (1878). The author reported that stock deaths in Australia had occurred as a result of drinking from a lake infested by a

bloom of *Nodularia spumigena*. Since the finding of Francis, there have been numerous reports of animal poisonings that have been associated with *Nodularia* blooms (Table 2) and with other cyanobacterial genera (Sivonen, 1990a; Ressom *et al.*, 1994; Kuiper-Goodman *et al.*, 1999). There have also been several reports of human illness - even deaths - attributed to toxic cyanobacteria (Ressom *et al.*, 1994; Hunter, 1998; Codd *et al.*, 1999b; Kuiper-Goodman *et al.*, 1999). The poisoning episodes and monitoring surveys of toxic blooms (Sivonen, 1998; Codd *et al.*, 1999b; Sivonen & Jones, 1999) have revealed that cyanobacterial toxins are common and pose serious health hazards for animals and human beings throughout the world.

Cyanobacterial peptide toxins are linked to incidences of different human illnesses, including skin and eye irritation, allergy-like symptoms, gastro-enteritis, and hepatoenteritis caused by acute exposure to toxins (Carmichael & Falconer, 1993; Ressom *et al.*, 1994; Codd *et al.*, 1999b; Kuiper-Goodman *et al.*, 1999). Chronic exposure to microcystins through drinking water may increase incidences of human liver cancer (Codd, 1998; Codd *et al.,* 1999b; Kuiper-Goodman *et al.*, 1999). A severe hepatoenteritis of the population in Palm Island, Queensland, Australia was also associated with drinking water contaminated with *Cylindrospermopsis raciborskii* (Byth, 1980; Hawkins *et al.*, 1985). In Brazil, a major human poisoning episode was attributed to water used for dialysis containing microcystins in 1996 (Jochimsen *et al.*, 1998; Pouria *et al.*, 1998). This poisoning caused the death of at least 55 people due to severe liver damage.

These episodes showed that hemodialysis water is an important exposure route for cyanobacterial toxins together with the consumption of drinking water. Furthermore, the recreational use of waters has caused illness in individuals who have been in skin contact with blooms (Carmichael & Falconer, 1993; Hunter, 1998; Codd, 1998; Codd *et al.*, 1999b). For example, in Australia, people have had skin, eye and respiratory symptoms after contact with a bloom of *Nodularia* (Soong *et al.*, 1992). Consumption of contaminated cyanobacterial cell dietary supplements, plant products, shellfish and fish may lead to minor exposure to cyanobacterial toxins (Codd *et al.*, 1999b). For example, cyanobacterial hepatotoxins have been found from salad lettuce (Codd *et al.*, 1999a) and from mussels (Chen *et al.*, 1993; Falconer *et al.*, 1992). In addition, showering and bathing are possible routes of exposure (Codd, 1998; Codd *et al.*, 1999b).

Table 2. Toxic *Nodularia* blooms observed in monitoring surveys and due to poisoning episodes

The descriptions of clinical, pathological, and histological findings due to cyanobacterial hepatotoxins are described in section 1.3.2. *Intoxication by *Nodularia* was suspected, but no toxicity tests or pathological surveys were made.

Location and date	Affected animal (findings of hepatoxicity) and/or bioassays (animal, administration, findings)	*Nodularia* and/or nodularin (detection method)	Reference
Australia:			
- Lake Alexandrina, 1878	Sheep (pathological findings)	*Nodularia* in water	Francis, 1878
- Broomehill dam, 1974	Sheep (pathological findings), bioassays (sheep and pigs, oral, clinical and histological findings)	*Nodularia* in water	Main *et al.*, 1977
- Lake Alexandrina and Lake Albert, 1990-93	Hepatotoxicity of most *Nodularia*-dominated samples (mouse, i.p., MLD_{100})	Nodularin in several bloom samples (HPLC)	Baker & Humbage, 1994
- Orielton Lagoon, Tasmania, 1992-93		5-3500 µg nodularin g^{-1} dw (HPLC)	Jones *et al.*, 1994b; Blackburn & Jones, 1995
- Lake Alexandrina and Lake Albert, 1994-95		0.02-1.7 µg nodularin l^{-1} (HPLC)	Heresztyn & Nicholson, 1997
Europe/ the Baltic Sea:			
- Kleiner Jasmunder Bodden, Germany, 1963	Ducks (pathological findings)	*Nodularia* in water	Kalbe & Thieß, 1964
- Gotland, Simrishamn, Karlshamn, and Ystad, Sweden, 1982	Dogs (clinical and pathological findings), bioassay (mouse, i.p., > 40 MU g^{-1} dw^{-1})	*Nodularia* in water	Lind *et al.*, 1983; Lundberg *et al.*, 1983; Edler *et al.*, 1985
- Strelasund, Germany, 1983	Cattle (clinical and pathological findings)	*Nodularia* in water	von Gußmann *et al.*, 1985
- Porvoo, Finland, 1984	Dogs (pathological findings)	*Nodularia* in water	Persson *et al.*, 1984
- Saarenmaa, Estonia, 1984	Bull calves*	*Nodularia* in water	Olli, 1996
- Arkona Sea, 1986	Bioassay (mouse, i.p., LD_{50} 95-455 mg kg^{-1})	Nodularin detected in all hepatotoxic *Nodularia* bloom samples (HPLC)	Sivonen *et al.*, 1989a
- Arkona Basin, Baltic Proper, and Gulf of Finland, 1985-87	Bioassay (mouse, i.p., MLD_{100} 125-2500 mg kg^{-1} in most of the samples)	0.1-2.4 mg nodularin g^{-1} dry $weight^{-1}$ (dw^{-1}) (HPLC)	Sivonen *et al.*, 1989b
- Gulf of Finland, and Gulf of Bothnia, 1990-91	Bioassay (mouse, i.p., MLD_{100}), samples collected from the Bothnian Sea were hepatotoxic	0.3-18.1 mg g^{-1} nodularin dw^{-1} of hepatotoxic blooms samples and 0.01-4.9 mg nodularin l^{-1} in all water samples (HPLC)	Kononen *et al.*, 1993b
Europe/ the North Sea:			
- Århusbugten and Kaløvigområdet, Danemark, 1975	Dogs (clinical and pathological findings)	*Nodularia* in water	Lindstrøm, 1976
- Banter See, Wihelmshaven, Germany, 1990	Dogs (clinical and pathological findings)	*Nodularia* in water	Nehring, 1993
Africa:			
- Malmesbury, South Africa, 1993-94	Calves (clinical findings), bioassay (mouse, i.p.), cows (pathological findings), and sheep (pathological findings, and blood chemistry)	*Nodularia spumigena* in water	van Halderen *et al.*, 1995
- Lake Zeekoeivlei, Cape Town, South Africa, 1994	Dog (clinical and histological findings), bioassay (mouse, i.p., hepatotoxicity)	*Nodularia spumigena* accounted for about 95% of the bloom containing 3.5 µg nodularin mg^{-1} (HPLC)	Harding *et al.*, 1995

1.1.4. Ecological effects of cyanobacterial toxins

The ecological role of cyanobacterial toxins is still not understood despite the numerous studies on physiological and ecological bases for toxin production. Toxin production is most likely related to the physical, chemical, and biotic environment, in which cyanobacteria compete with other organisms to maximise their growth and reproduction (Ressom *et al.*, 1994; Paerl & Millie, 1996). For example, cyanobacteria are known to have toxic effects on phytoplankton and aquatic macrophytes (reviewed by Ressom *et al.*, 1994) and *Nodularia* blooms have been suspected to reduce fish abundance (Potter *et al.*, 1983; Lenanton *et al.*, 1985). In addition, field observations have revealed that zooplankton (Watanabe *et al.*, 1992; Kotak *et al.*, 1996), macroinvertebrates (Zurawell *et al.*, 1999), and mussels (Lindholm *et al.*, 1989; Falconer *et al.*, 1992; Chen *et al.*, 1993) accumulate microcystins and nodularins. These observations indicated that cyanobacterial toxins can have effects on higher trophic levels, thus they may be transfered in aquatic food chains.

Toxin production can also be a defence mechanism against grazers (see Ressom *et al.*, 1994; Hanazato, 1996). Toxic *Microcystis* and *Nodularia* (Reinikainen *et al.*, 1994; Hietala *et al.*, 1995; Walls *et al.*, 1997; Koski *et al.*, 1999) and toxins produced by these cyanobacteria (DeMott *et al.*, 1991) have been demonstrated to have harmful effects on zooplankton grazers in the laboratory. In field studies of the Baltic Sea, cyanobacterial blooms were not consumed by copepods and exposure of copepods to a bloom depressed their egg production (Sellner *et al.*, 1994, 1996). In some of these studies, also non-toxic cyanobacteria had negative effects on zooplankton, thus, there might be other factors than toxicity which affect food quality. For example, cell size and morphology affect ingestibility and assimilability of food (Koski, 1999).

Cyanobacteria are known to produce a wide range of compounds with dissimilar bioactivities (see section 1.3). For example, in *Microcystis*, different compounds have been suggested to be toxic to *Daphnia* and inhibit its filtering (Jungmann *et al.*, 1991). Recently, two variants of *Microcystis* PCC7806 strains, a wild-type strain and a mutant not able to produce toxin, were used to study the role of microcystins in the defence of *Microcystis* strains against *Daphnia galeata* (Rohrlack *et al.*, 1999). The wild-type strain was toxic to *D. galeata*, whereas the mutant was not lethal. Yet, both types of PCC7806 were able to reduce the *Daphnia* ingestion rate. The study gave strong evidence that microcystins were responsible for the poisoning of *Daphnia*, but they were not responsible for inhibition of the ingestion process.

1.2. Factors leading to cyanobacterial success

Cyanobacterial blooms have been thought to be a consequence of increased nutrient concentrations of fresh and marine waters where they occur seasonally during warm water temperatures. Although nutrients and temperature are regarded as the most important factors for the success of cyanobacteria (Paerl, 1988, 1996) several different factors have been presented to explain cyanobacterial success in aquatic environments (Hyenstrand *et al.*, 1998).

According to the review of Hyenstrand (1998), cyanobacteria are favoured by a low ratio of total N to total phosphorus (P) and by a high water temperature. Non-nitrogen fixing cyanobacteria require ammonium (NH_4^+) whereas a scarcity of N benefits the development of population of nitrogen fixing cyanobacteria, which differ from non-nitrogen fixing cyanobacteria for high requirements of trace elements (e.g. iron). Furthermore, cyanobacteria gain competitive advantage by storing P and by regulating their buoyancy. With the help of these properties, they can survive during periods of P-deficiency and regulate their vertical position in the water column. Moreover, cyanobacteria can replace eukaryotic phytoplankton in high pH or low CO_2 situations, and under low light conditions. Cyanobacteria can avoid grazing e.g. by producing toxins.

1.2.1. Factors favouring bloom formation and growth of Nodularia and Aphanizomenon

In Australia and the Baltic Sea, low N and high P concentrations have been related to *Nodularia* blooms (Table 3). In the Baltic Sea, a low N:P-ratio has also been observed to favour the dominance of *Aphanizomenon*. In addition, P and in some cases N stimulated the growth of heterocystous cyanobacteria in most of the enrichment experiments *in situ* (Table 3).

Blooms of *Nodularia* are common in the N-deficient parts of the Baltic Sea, and they only occur exceptionally in the Kattegat which is connected to the Baltic Proper (the Baltic Sea). The absence of *Nodularia* in Kattegat, which has equally low N:P-ratios, is probably related to its high salinity (Granéli *et al.*, 1990). High salinities have been observed to depress the growth of *Nodularia* in Peel-Harvey Estuary, Orielton Lagoon and Great Salt Lake (Table 3). In addition, laboratory studies with cultures have shown that salinities higher than 30 ‰ repressed the growth (Horstmann, 1975; Nordin & Stein, 1980; Warr *et al.*, 1984; Blackburn *et al.*, 1996) and promoted the sporulation of *Nodularia* (Nordin & Stein, 1980; Jones *et al.*, 1994b).

In addition to the chemical controlling factors, cyanobacterial blooms are also controlled by physical factors such as temperature, light, and water stability (Paerl, 1996; Hyenstrand, 1998). High water temperatures have been reported to support *Nodularia* blooms in saline and in

brackish waters (Table 3). Cold weather depresses initiation of cyanobacterial blooms, and cloudy and windy weather prevents the cyanobacteria from accumulating at the water surface (Table 3). The ecology and the physiology of heterocystous cyanobacterial blooms in the Baltic Sea have thoroughly been reviewed by Kononen (1992), Kononen and Leppänen (1997), and Sellner (1997).

In addition to enrichment tests done with natural communities *in situ* and in the laboratory (Table 3), unialgal cultures have been used to study the effects of several factors on cyanobacterial growth in the laboratory. The study of Nordin and Stein (1980) demonstrated that maximum growth of *Nodularia* strains occurred at salinities of 5-10‰ (test range 1-60‰), temperatures of 25-30°C (test range 15-35°C), pHs of 10.0-10.5 (test range 6.0-11.0), and light intensities of 6000 lx (test range 240-6000 lx). No preference was shown for dominant anions (Na^+, Mg_2^+) or cations (Cl^-, CO_3^{2-}, SO_4^{2-}). All strains grew well with various levels of nitrate (0.25-1.0 g NO_3^--N l^{-1}) but poor growth resulted when NH_4^+ or urea was the N source (Nordin & Stein, 1980). Later, Huber (1986b) found no stimulation of growth of *Nodularia* by NH_4^+ or NO_3^- at concentrations from 0 to 20 mg N l^{-1}. Horstman's study (1975) showed that *Nodularia* strains grew best at salinities of 5-15‰ (test range 0-30‰). Warr *et al.* (1984) studied the effect of different salinities and N sources on the growth of *Nodularia harveyana*. They found that growth was highest at 0-35‰ (test range 0-70‰). Moreover, the growth on NH_4^+ (20 mg N l^{-1}) was higher than on NO_3^- (20 mg N l^{-1}) and on gaseous N. The study of Blackburn *et al.* (1996) showed that growth of *Nodularia* strains at 0 and 35‰ was lower than at 12 and 24‰. According to Melin and Lindahl (1973), the growth of the Baltic Sea *Aphanizomenon* culture was stimulated by the addition of PO_4^{3-}, chelated trace elements or a combination of these. The highest biomass accumulations of *N. spumigena* and *Aphanizomenon* sp. occurred at the highest studied light intensity (test range 1-100 µmol m^{-2} s^{-1}) (Holswilder, 1999). For both genera, the growth at the two lowest irradiances (1 and 6 µmol m^{-2} s^{-1}) differed significantly from the two highest irradiances (60 and 100 µmol m^{-2} s^{-1}). Furthermore, the biomass of *N. spumigena* cultures grown in moderately P-limited conditions (N:P = 32:1, molar ratio) was significantly higher than those of extremely P-limited (N:P = 64:1) and non-P-limited (N:P = 16:1) (Holswilder, 1999).

Gopal *et al.* (1975) found that an alkaline pH (test range 4-9) was most suitable for growth and that nutrients stimulated growth of *N. spumigena* strain. Growth increased when phosphate (PO_4^{3-}) and NO_3^- were added. This soil strain was found to tolerate high concentrations of copper (1.5 µg l^{-1}) and zinc (2.0 µg l^{-1}). Dixit *et al.* (1985) studied the growth of *Nodularia* with different N sources. Ammonium chloride (0.5 mM) was highly toxic to this soil strain, while sodium nitrate (2 mM) and nitrite (2 mM) supported

optimal growth. Furthermore, his studies indicated maximum growth at 20-25°C, pH 7.5-9.0 and light intensity 1000-5000 lx. Camm and Stein (1974) reported that urea was toxic at high concentrations of 8.8 mM and 4.4 mM to soil strains of *N. spumigena* whereas concentrations lower than those inhibited growth on N-free medium and on NO_3^- -medium.

1.2.2. Factors controlling nitrogen fixation in Nodularia and *Aphanizomenon*

Many cyanobacteria have been experimentally shown to perform nitrogen fixation under anaerobic conditions, but fewer are able to grow at the expense of atmospheric N_2 under aerobic conditions (see Flores & Herrero, 1994). Certain cyanobacteria, which are able to perform aerobic nitrogen fixation, have developed heterocysts as a protective structure for nitrogen fixation for the oxygen-sensitive enzyme, nitrogenase. Heterocysts are present within order *Nostocales* in three families: *Nostocaceae*, *Scytonemataceae*, and *Rivulariaceae* (Castenholz, 1989b).

The progress of cyanobacterial nitrogen fixation research until 1988 has been reviewed by Gallon and Chaplin (1988). Most estimates of nitrogen fixation are derived using the acetylene reduction method. This method is based on the property of nitrogenase to reduce compounds with a triplet bond such as dinitrogen (N_2) and acetylene. The end product of the acetylene reduction, ethylene, can be measured using gas chromatography. The acetylene reduction method has been widely adopted as a method for quantifying nitrogen fixation, since it was first introduced by Stewart and co-authors (Stewart *et al.*, 1967). Most of the estimates of nitrogen fixation in field populations in the Baltic Sea are based on this method (see references in Table 3), but the ^{15}N method (Moisander *et al.*, 1996) and laser photoacoustic detection of ethylene (Zuckermann *et al.*, 1997) have been also used. The nitrogen fixation system in cyanobacteria has been intensively studied by molecular techniques. These techniques have made it possible to study the environmental regulation of cyanobacterial nitrogen fixation at a genetic level (see Flores & Herrero, 1994).

Nitrogen fixing cyanobacteria have a selective advantage in N-limited environments such as the open Baltic Sea. In the Baltic Sea, cyanobacterial nitrogen fixation has been estimated to be about 9% of the total N input (Larsson *et al.*, 1985). The estimated N inputs through nitrogen fixation vary in different areas of the Baltic Sea (Larsson *et al.*, 1985; Howart *et al.*, 1988a; Sellner, 1997). Estimations for nitrogen fixation rates and the importance for lakes, estuaries, and oceans have been reviewed by Howarth *et al.* (1988a). Nitrogen fixation rates are controlled by many chemical and physical factors (Bothe, 1982; Howarth *et al.*, 1988b). Light, temperature, nutrients and salinity are known to control nitrogen fixation in *Nodularia* and *Aphanizomenon* blooms (Table 3).

Table 3. Factors promoting and inhibiting growth and nitrogen fixation of the planktonic cyanobacteria *Nodularia* and *Aphanizomenon* in brackish and saline waters according to field observations and experimental studies with natural phytoplankton communities

Abbreviations: N, nitrogen; NO_3^-, nitrate; DIN, dissolved inorganic nitrogen; P, phosphorus; PO_4^{3-}, phosphate; SRP, soluble reactive phosphorus.

Type of study Location and date	Results	Reference
Field observations		
Australia:		
- Lake Alexandrina, 1975-78	High turbulence and turbidity ended blooms of nitrogen fixing cyanobacteria.	Geddes, 1984
- Peel-Harvey Estuary, 1977-83	Summer *Nodularia* blooms were related to high P concentration and fresh water loading from river flow during the previous winter. At the time of P loading, *Nodularia* growth was limited by temperature. In the years with no *Nodularia* blooms, the salinity exceeded 15‰ by the time water temperatures reached 18ºC.	Hillman *et al.*, 1990
- Peel-Harvey Estuary, 1977-83	Nitrogen fixation of *N. spumigena* blooms decreased with depth in response to light, and with increasing bloom age. Furthermore, high N concentrations, salinites, and water temperatures decreased nitrogen fixation. A positive correlation between nitrogen fixation and biomass was found.	Huber, 1986a
- Orielton Lagoon, Tasmania, 1992-93	*Nodularia* blooms were favoured by low salinity (< 24 g kg^{-1}) and high water temperature (~ 18-24ºC).	Jones *et al.*, 1994b; Blackburn & Jones, 1995
- Peel-Harvey Estuary, 1977-83	*Nodularia* blooms were related to high P concentration (100-200 µg PO_4^{3-} l^{-1}), low N:P-ratio (< 20:1), and low salinity (< 30‰).	Lukatelich & McComb, 1986
Canada:		
- 41 saline lakes, Saskatchewan, 1959-82	Growth of *Nodularia* was observed in ten lakes ranging in salinity from 10-20 to 204 g l^{-1}.	Hammer *et al.*, 983
USA:		
- Waler Lake, Nevada, 1975-77	*Nodularia spumigena* blooms were associated with low levels of inorganic N. The maximum numbers of *Nodularia* occurred at depth of one meter.	Cooper & Koch, 1984
- Pyramid Lake, Nevada, 1972-86	Low N:P-ratios, as well as climatic forcing of fluvial discharge and lake mixing patterns were important for seasonal succession of *N. spumigena.*	Galat & Verdin, 1988
- Pyramid Lake, Nevada, 1986	*Nodularia* blooms were initiated by high SRP and low DIN concentrations and dispersed by strong winds.	Galat & Verdin, 1989
- Pyramid Lake, Nevada, 1972-86	Adequate P, but limited N supplies triggered *Nodularia* blooms, which were restricted to summer and autumn when the lake was thermally stratified.	Galat *et al.*, 1990
- Pyramid Lake, Nevada, 1979	Nitrogen fixation depended on calm periods when *Nodularia* floated to the surface. Near-surface dependence of *Nodularia* distinguished it from most nitrogen fixing genera which were damaged in high light conditions. Also, low N concentration and high P concentration were controlling factors for nitrogen fixation.	Horne & Galat, 1985
- Pyramid Lake, Nevada, 1989-92	High water temperature and column stability favoured *Nodularia* blooms.	Lebo *et al.*, 1994
- Great Salt Lake, Utah, 1847-1987	The proportion of *N. spumigena* increased with the decrease in salinity, which has decreased from ~28 to 16% in the north part and from ~21 to 6% in the south part.	Stephens, 1990

Table 3, continued

Europe/ the Baltic Sea:		
- Gulf of Bothnia, 1991	*Nodulalaria spumigena* and *Aphanizomenon* sp. did not occur in the Bothnian Bay, where phosphorus limited the growth of the phytoplankton.	Andersson *et al.*, 1996
- Gulf of Finland, 1990	A bloom of *A. flos-aquae* was promoted by a nutrient pulse with a DIN:DIP-ratio of 15.	Grönlund *et al.*, 1996
- Gulf of Riga and Gulf of Finland, 1994	There was a clear difference in biomass and sinking loss of *Aphanizomenon* between the study areas. In the Gulf of Riga, the deep water rich in N invoked biomass increase and promoted the buoyancy control of *Aphanizomenon*. Also, light controlled the vertical distribution and sinking loss rates of *Aphanizomenon*.	Heiskanen & Olli, 1996
- Arkona Sea, 1974	The highest nitrogenase activity per heterocyst as well as the highest biomass and the number of heterocysts were observed from the surface water layers with the highest *Nodularia* bloom densities. Blooms were initiated through P enrichment of warm, stratified surface waters low in N.	Hübel & Hübel, 1980
- Baltic Proper, 1982	Phytoplankton growth was increased in fronts resulting from nutrient enrichment by upwelling.	Kahru *et al.*, 1984
- Gulf of Finland, 1972-85	Cyanobacteria were associated with high water temperatures.	Kononen, 1988
- Baltic Sea, 1979-88	The monitoring data showed a rather similar distribution of *A. flos-aquae* and *N. spumigena* in relation to salinity whereas they have clearly different temperature optima.	Kononen, 1992
- Gulf of Finland, 1968-81	High P concentrations and low N:P-ratios favoured heterocystous cyanobacterial species.	Kononen & Niemi, 1984
- Gulf of Finland, 1990	*A. flos-aquae* bloom formation was associated with frontal upwelling bringing up deep waters with a low N:P-ratio.	Kononen & Nõmmann, 1992
- Gulf of Finland, 1993	Cyanobacterial growth in the frontal zone was stimulated by PO_4^{3-} -replenishment zones (*A. flos-aquae*) and warming and shallowing of the surface layer (*N. spumigena*) under calm weather conditions.	Kononen & Leppänen, 1997
- Baltic Sea	*Nodularia spumigena* and *A. flos-aquae* were dissimilar regarding their temperature, salinity, and light optima, as well as in the affinity for PO_4^{3-}. The initiation of *Nodularia* blooms was not restricted to the frontal zone, but took place on a basin-wide scale, as a result of warming and shallowing of the surface layers under calm weather conditions. *A. flos-aquae* benefited from P pulses.	Kononen *et al.*, 1996
- Gulf of Finland, 1994	*Nodularia* was concentrated in the near-surface layer, while the maximum abundance of *Aphanizomenon* was observed in the top 7- to 10-m layer throughout the day.	Kononen *et al.*, 1998
- Baltic Sea	The upwelling water with a low N:P-ratio and a warm stable water column were the most important variables controlling blooms of heterocystous cyanobacteria.	Leppänen *et al.*, 1988
- Bothnian Sea, 1977-78	*Aphanizomenon flos-aquae* occurred from mid-June to the end of December. In the Bothnian Bay, the mean values of heterocyst activity (1332-1880 nmol C_2H_4 x 10^{-7} het^{-1} h^{-1}) and heterocyst frequency (0.5-2.3 mm^{-1}) were low compared to values in the other parts of the Baltic Sea due to low P concentrations. The heterocyst activity and frequency increased with increasing concentration of P.	Lindahl & Wallström, 1985
- Baltic Proper, 1976	The heterocyst frequency of *A. flos-aquae* decreased during the study, probably due to high N concentrations in water. Upwelling of bottom water caused the disappearance of cyanobacteria and reduced the number of heterocysts.	Lindahl *et al.*, 1978
- Baltic Proper, 1975	At the beginning of August, *Nodularia* declined and *Aphanizomenon* became the dominant nitrogen fixing genera. While *Aphanizomenon* was distributed within the whole euphotic layer, the *Nodularia* population was mainly floating on the surface. The nitrogen fixation showed principally the same vertical variation as the number of heterocysts, and was to a minor extent determined by the heterocyst activity.	Lindahl *et al.*, 1980

Table 3, continued

- Gulf of Finland, 1994	The nitrogen fixation activity decreased during the study with the heterocyst frequency of *Nodularia* and *Aphanizomenon.* The highest nitrogen fixation rates were found in the samples grown at the highest irradiance, and nitrogen fixation took place also in the dark. Under the highest irradiance, nitrogen fixation activity correlated positively with heterocyst frequency of *Nodularia.*	Moisander *et al.*, 1996
- Gulf of Finland, 1992	In the early phase of the bloom, a salinity front had a marked effect on the spatial distribution of *Nodularia* and *Aphanizomenon.* Their biomass decreased steeply at the low salinity side of the front. During later bloom stages, spatial separations of *Nodularia* and *Aphanizomenon* disappeared.	Moisander *et al.*, 1997
- Baltic Sea	A low N:P-ratio promoted cyanobacterial blooms, especially in areas characterized by upwelling of P-rich bottom water. In the Bothnian Bay, where P rather than N limits phytoplankton production, no blooms exist.	Niemi, 1979
- Baltic Proper and Gulf of Finland 1980, -82, and –84	*Nodularia* was found in the uppermost five meters, while *Aphanizomenon* was distributed throughout the euphotic water layer. The dominance of *Nodularia* increased whereas the proportion of *Aphanizomenon* decreased towards the southern Baltic Sea. *Nodularia* occurs generally in the open sea area, while *Aphanizomenon* occurs in high abundance also in coastal areas. Cyanobacterial biomass and nitrogen fixation fluctuated strongly in space and time owing to patchiness and movement of the water masses. No clear connection was found between bloom development and nutrient concentrations.	Niemistö *et al.*, 1989
- Gulf of Gdansk, 1992-94	A decrease of the N:P-ratio was favourable for nitrogen fixing cyanobacteria. *N. spumigena* preferred higher temperatures for growth than *A. flos-aquae.*	Pliński & Józwiak, 1996
- Gulf of Finland, 1993	*Nodularia spumigena* was a common species in all the areas of the Gulf of Finland except the Neva Estuary where the oversupply of N promotes the growth of non-heterocystous species such as *Planktothrix agardhii.*	Rantajärvi *et al.*, 1998
- Gulf of Finland, 1978	Total P and total N correlated positively with *Nodularia* biomass in eutrophic waters, while positive correlation between total P and *Nodularia* biomass was found in oligotrophic waters.	Rinne & Tarkiainen, 1978
- Gulf of Bothnia, 1978-79	The nitrogen fixation, the number of heterocysts, and the biomass of *A. flos-aquae* and *N. spumigena* showed the same horizontal distribution. The level of nitrogen fixation was negligible in the Bothnian Bay. In the central and southern Bothnian Sea they were markedly lower (1/10) than in the northern Baltic Proper and the Gulf of Finland. The absence of blooms of heterocystous species and nitrogen fixation from the north of the Gulf of Finland is apparently connected with the high inorganic N:P-ratio in that area. Also, low temperature may prevent development of blooms. The vertical abundance of heterocystous genera differed: the vertical maximum of *Aphanizomenon* occurred at 10-20 m, while the highest abundance of *Nodularia* was near the surface.	Rinne *et al.*, 1981
- Southern Baltic Proper, 1993	In surface waters, the increased irradiance experienced by *A. flos-aquae* supported increased rate of nitrogen fixation.	Stal *et al.*, 1998
- Southern Baltic Sea, 1993, -95	Nitrogenase activity was saturated at about 600 µmol photons m^{-2} s^{-1}, but considerable activity was found also in the dark.	Stal *et al.*, 1999
- Baltic Sea, 1992-94	*Nodularia spumigena* and *A. flos-aguae* were mostly restricted to the water above the thermocline, and in calm conditions their concentration increased towards the top of the water column. The highest concentrations were within the top 10 m of the water column.	Walsby *et al.*, 1995
- Baltic Proper, 1983-85	The biomass of *A. flos-aquae* varied between the years mainly due to variation in water temperature. Also, the biomass and the number of heterocysts increased with increasing distance from the sewage treatment plant due to a low N:P-ratio. The biomass of *Aphanizomenon* was highest in 1984 when the P loading from the plant was highest.	Wallström, 1988

Table 3, continued

Experiments performed *in situ* and in the laboratory		
- Gulf of Bothnia, Baltic Sea, 1991	Phytoplankton was P limited in the Bothnian Bay and the coastal area of the Bothnian Sea, and N limited in the offshore Bothnian Sea.	Andersson *et al.*, 1996
- Arkona Sea, Baltic Sea, 1978-79	Addition of N or N and P increased the biomass and changed the composition of phytoplankton. *N. spumigena* was one of the phytoplankton species which reacted most strongly to the enrichment.	Granéli, 1981
- Harvey Estuary, 1981-84 and Peel Inlet 1982-84, Australia	The *Nodularia* biomass was higher in the P treatment than in the iron and N treatments.	Hamel & Huber, 1985
- Baltic Proper, Baltic Sea, 1972	Cyanobacterial growth was stimulated by addition of PO_4^{3-}, and PO_4^{3-} and NO_3^-. When N became deficient, an increase in the frequency of heterocysts was observed, which resulted in higher nitrogen fixing ability.	Horstmann, 1975
- Peel-Harvey Estuary, Australia	Light controlled nitrogen fixation in *Nodularia*. Nitrogen fixation was higher in the light than in the dark, where it was related to the length of prior exposure to light.	Huber, 1986b
- Peel-Harvey Estuary, Australia, 1981	When P was added in the laboratory to natural *Nodularia* populations, there was a distinct inverse relationship between phosphatase activity and P taken up. The addition of P had little effect on biomass.	Huber & Hamel, 1985
- Gulf of Finland, Baltic Sea, 1990, -92	The nutrient additions benefited the growth of smaller phytoplankton species over the filamentous heterocystous cyanobacteria.	Kononen *et al.*, 1993a
- Pyramid Lake, Nevada, USA, 1989-92	Only time when N addition did not stimulate phytoplankton growth was during periods of winter mixing and *N. spumigena* bloom.	Lebo *et al.,* 1994
- Baltic Proper, Baltic Sea, 1975	Phosphate alone and in combination with chelated metals increased the heterocyst frequency of *A. flos-aquae*. Combined additions of NO_3^- and PO_4^{3-} together stimulated the ^{14}C-uptake.	Lindahl *et al.*, 1980
- Pyramid Lake, Nevada, USA, 1989-90	Phosphate enrichment, when added singly or in combination with DIN, had no effect on chlorophyll-*a* production. Nitrogen enrichment had a positive response at all times of the year except during a bloom of *N. spumigena* and after complete lake mixing.	Reuter *et al.*, 1993
- Gulf of Finland, Baltic Sea, 1974-75	Phosphorus was more important for the growth of *Nodularia* than N. In the very eutrophic areas, also N had a growth-increasing effect.	Rinne & Tarkiainen, 1978
- Arkona Basin, Baltic Sea, 1981	Temperature appeared to be the main factor influencing growth of *Nodularia*, which was also stimulated by N deficiency.	Schiewer *et al.*, 1986
- Southern Baltic Sea, 1993, -95	*Nodularia* was limited by iron, which stimulated both growth and nitrogenase activity**.** Phosphate stimulated nitrogenase activity to the same extent as iron but did not stimulate growth. Nitrogenous compounds and molybdate did not affect nitrogenase activity whereas elimination of sulfate stimulated it strongly.	Stal *et al.*, 1999
- Gulf of Finland, Baltic Sea, 1982	Ammonium was the main limiting nutrient for the phytoplankton community as a whole, but the nitrogen fixing cyanobacteria were P-limited.	Tamminen *et al.*, 1985
- Baltic Proper, Baltic Sea, 1988	Phosphorus had no effect on the biomasses of cyanobacterial species. During a mesocosm experiment, *A. flos-aquae* almost disappeared when N was present and the population of *N. spumigena* increased slowly probably due to the low water temperatures. On the basis of growth rates of *N. spumigena* at different nutrient conditions, *N. spumigena* may be superior to *A. flos-aquae* in competition for P.	Wallström *et al.*, 1992

In laboratory experiments done with *Nodularia* cultures, nitrogen fixation was highest at salinities of 0- 35‰ and salinities higher than 35‰ decreased the nitrogen fixation (Warr *et al*., 1984). In another study, highest nitrogen fixation was detected at salinity of 5-10‰ and lower salinities than 5‰ decreased the nitrogen fixation (Huber, 1986b). Phosphorus addition stimulated nitrogen fixation in P-starved *Nodularia* cultures (Huber, 1986b), while NH_4^+ addition inhibited nitrogen fixation (Sanz-Alférez & del Campo, 1994). A very high NH_4^+ concentration (42 mg l^{-1}) inhibited nitrogen fixation by both *Aphanizomenon* and *Nodularia* (Moisander *et al*., 1996). According to Huber (1986b), NH_4^+ completely inhibited nitrogenase activity whereas NO_3^- did not. Light has also been demonstrated to control nitrogen fixation of *Nodularia* cultures (Huber, 1986b; Sanz-Alférez & del Campo, 1994; Zuckermann *et al*., 1997). In outdoor cultures of *N. harveyana* nitrogenase activity was influenced by the growth rate and not by high light intensity (Pushparaj *et al*., 1994).

1.2.3. Factors influencing cyanobacterial hepatotoxin production

There is no information available about environmental conditions that influence hepatotoxin production in *Nodularia* blooms in the field whereas factors affecting toxin concentrations in *Microcystis*-blooms in eutrophic lakes have been examined, and the results are contradictionary. In the Hartbeespoort Dam (South Africa), microcystin-YR, -LR, -YA and -LA concentrations correlated positively with solar radiation and water temperature, and microcystin-YR, and –YA correlated negatively with PO_4^{3-} concentration (Wicks & Thiel, 1990). In the Coal, Driedmeat, and Little Beaver lakes (Canada), no correlation between microcystin-LR and water temperature was discovered, whereas the toxin concentration correlated positively with total and dissolved P and negatively with NO_3^- (Kotak *et al*., 1995). Earlier, in Coal Lake microcystin-LR was positively correlated with water temperature and P concentration (Kotak *et al*., 1993). In Babtiste, Narrow, Skeleton, Steele, the Coal, Little Beaver and Driedmeat lakes (Canada), microcystin-LR correlated positively with total P and total N (Zurawell *et al*., 1999). In Lake Grand-Lieu (France), microcystin-LR and one of microcystin–RR variants correlated negatively with solar radiation, and both microcystin-RR variants correlated positively with dissolved P and NO_3^- (Vezie *et al*., 1998). In Lake Tuusulanjärvi (Finland), microcystin-LR correlated positively with total N and total P and negatively with dissolved NO_3^- (Lahti *et al*., 1997). A corresponding analysis of field data on environmental factors during 78 water blooms from 72 Finnish lakes revealed that hepatotoxic *Microcystis* blooms correlated with high PO_4^{3-} concentrations (Rapala & Sivonen, 1998).

Growth and nodularin levels in response to salinity changes have been studied in laboratory experiments (Blackburn *et al.*, 1996). Nodularin concentrations on both gravimetric and cellular basis decreased at the highest studied salinity (35 ‰). In addition, nodularin production has been demonstrated to be controlled by light and by P (Holswilder, 1999). Nodularin production was reduced under light limitation and increased under P limitation. In addition, N addition was shown to promote growth and to increase the nodularin content of a *Nodularia*-culture, which was grown on N-containing medium (Carmichael *et al.*, 1988).

Variation of cellular toxin levels under different growth conditions have been studied in the laboratory mainly with batch-cultures of hepatotoxic *Microcystis*, *Oscillatoria*, *Anabaena*, and neurotoxic *Anabaena*, *Aphanizomenon*, and *Planktothrix* (reviewed by Rapala, 1998 and by Sivonen & Jones, 1999). In these laboratory experiments, temperature, light, and nutrients were most frequently examined. In addition, the effects of pH, carbon dioxide, salinity, and micronutrients were examined. The majority of studies indicated that cellular toxin levels were highest under conditions most favourable for growth. Differences in responses of hepatotoxic and neurotoxic strains have been observed, e.g. P had a pronounced effect on hepatotoxin levels, but not on neurotoxin levels. Nitrogen had no effect on toxin production by nitrogen fixing species, while non-heterocystous species, such as *Microcystis* and *Oscillatoria*, produced more toxins under N-rich media. All these laboratory studies have provided evidence of environmental regulation of gravimetric toxin concentration. Orr and Jones (1998) suggested that toxin concentration in *Microcystis* is controlled by the effects of environmental factors on the rate of cell division, not through any direct effect on the metabolic pathways on toxin production. This suggestion was based on laboratory experiments with *Microcystis aeruginosa* cultures and on re-evaluation of data made on microcystin-producing *Anabaena* and *Oscillatoria* cultures presented by others (Sivonen, 1990b; Rapala *et al.*, 1997).

Cyanobacterial hepatotoxins are so-called intracellular toxins, and they are released from cells when they are damaged or lysing (see Sivonen & Jones, 1999). Chemical, physical, and biological degradation of cyanobacterial toxins have been demonstrated to occur (Hrudey *et al.*, 1999; Sivonen & Jones, 1999). Twist and Codd (1997) studied degradation of nodularin under light and dark conditions and found photochemical breakdown of nodularin. In addition, nodularin was degraded when components of *Nodularia* cells were present. These components most likely contained nodularin degrading and metabolising bacteria. In water of Lake Alexandrina, where *N. spumigena* blooms have occurred for months, nodularin was degraded by bacterial communities within 48-50 h (half-life 24 h) (Heresztyn & Nicholson, 1997).

Furthermore, three bacterial strains, which have been isolated from sediments of the Lake Tuusulanjärvi (Finland), were able to degrade nodularin (Lahti *et al.*, 1998). On the contrary, the bacterial strain isolated from water sample treated with *Microcystis aeruginosa* extract, was not able to degrade nodularin, although it has degradative activity against microcystins (Jones *et al.*, 1994a).

1.3. Bioactive peptides from cyanobacteria

Cyanobacteria contain a large number of secondary metabolites, compounds not essential for growth and reproduction. These include peptides, macrolides, and glycosides (Patterson *et al.*, 1994; Namikoshi & Rinehart, 1996). These compounds have been reported to possess a number of bioactivities: antiviral (Pattersson *et al.*, 1993, 1994), antifungal (Patterson *et al.*, 1994), cytotoxic (Patterson *et al.,* 1991), protein phosphatase inhibitory (Honkanen *et al.*, 1995) and antineoplastic activities (Moore *et al.*, 1996). For example, *Nodularia harveyana* has antifungal, antibacterial, and alleopathic activities (Pushparajat *et al.*, 1999). Many of these secondary metabolites are toxic to animals and humans (Carmichael & Falconer, 1993; Falconer, 1996; Hunter, 1995, 1998; Codd *et al.*, 1999b).

1.3.1. Structure of nodularin

Cyanobacteria produce two main types of toxins: neurototoxins and hepatotoxins (Carmichael, 1992, 1994; Codd, 1998; Sivonen, 1998). Hepatotoxins are produced by several genera: *Anabaena*, *Aphanizomenon*, *Cylindrospermopsis*, *Microcystis, Nodularia*, *Nostoc*, and *Oscillatoria* (Carmichael, 1994; Sivonen, 1998; Sivonen & Jones, 1999). Hepatotoxins are divided into three groups: alkaloids (cylindrospermopsin), heptapeptides (microcystins) and pentapeptides (nodularins). Cylindrospermopsin is produced by *Cylindrospermopsis raciborskii* (Hawkins *et al*., 1985, 1997), *Aphanizomenon ovalisporun* (Banker *et al*., 1997), *and* by *Umezakia natans* (Harada *et al*., 1994). Structurally related cyclic peptides, microcystins and nodularins, differ from each other in their number and type of certain amino acids (Carmichael, 1992; Sivonen, 1998). Microcystins, which are named after the genus *Microcystis* from which they were first identified, are produced by several cyanobacterial genera and consist of seven amino acids. The main differences between the different variants of microcystins are in the two variable L-amino acids, and methylation or non-methylation of certain amino acids (Sivonen & Jones, 1999). Nodularin is produced only by *Nodularia* and is composed of five amino acids (Rinehart *et al*., 1988). Three of those amino acids are the same as in microcystins: D-MeAsp[1] (D-*erythro*-β-methylaspartic acid), Adda[3], and D-Glu[4] (D-glutamic acid)(Fig. 1). In addition to these, nodularin consists of L-Arg[2] (L-arginine) and Mdhb[5] [2-(methylamino)-2-dehydrobutyric acid]. Both toxins contain a unique C_{20} amino acid, 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid, which is

abbreviated as Adda. This amino acid is essential for the hepatotoxicity (Harada *et al.*, 1990; Rinehart *et al.*, 1994). The same peptide structure, named nodularin by Rinehart *et al.* (1988), has been found in *Nodularia* blooms and strains sampled from Australia (Runnegar *et al.*, 1988), New Zealand (Carmichael *et al.*, 1988; Rinehart *et al.*, 1988) and the Baltic Sea (Eriksson *et al.*, 1988; Sivonen *et al.*, 1989a; Sandström *et al.*, 1990).

Nodularin show considerably less structural variation than microcystins (Rinehart *et al.*, 1994). To date, five different natural analogues of nodularin have been characterised (Table 4). Furthermore, the compound [L-Val^2], named motuporin, has been found from a marine sponge (de Silva *et al.*, 1992), in which the compound is probably produced by a symbiotic cyanobacterium. This analogue differs from nodularin by substitution of a valine (Val) residue for an Arg residue. In addition, two dihydronodularins have been synthesised chemically (Namikoshi *et al.*, 1993).

Most nodularins have an intraperitoneal LD_{50} of 50-150 µg kg^{-1} in mice (Table 4), which is calculated to be at least 200 times lower than the oral LD_{50} (Kiviranta *et al.*, 1990). In a similar manner as microcystins (Harada *et al.,* 1990), nodularins with an 6*Z*-Adda isomer and with modified D-Glu do not display toxicity. Furthermore, the cyclic structure is necessary for the toxicity, since linear peptides are biologically non-toxic (Choi *et al.*, 1993).

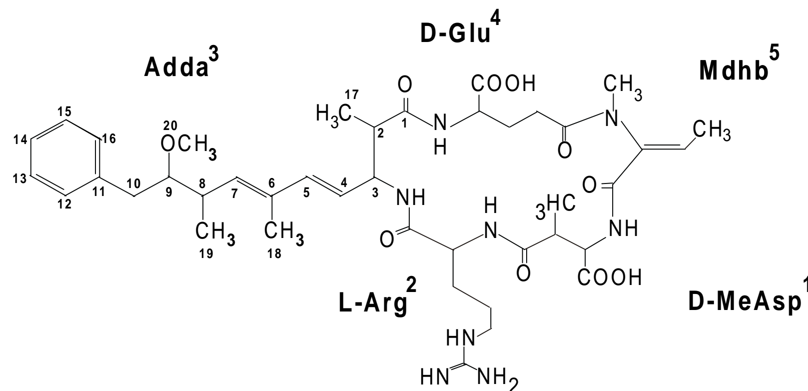

Fig. 1. Chemical structure of nodularin, a hepatotoxic pentapeptide produced by the cyanobacterium *Nodularia*

The linear peptide was first isolated when studying the biosynthesis of nodularin. This non-toxic peptide was thought to be a precursor of nodularin (Choi *et al*., 1993; Rinehart *et al*., 1994). Later, linear and cyclic peptides, spumigins and nodulapeptins, have been isolated from the toxic *Nodularia* strain AV1, but these compounds were not found from the non-toxic *Nodularia* HKVV strain (Fujii *et al*., 1997a, b). In addition, glycosidic compounds have been isolated from *Nodularia* strains (Soriente *et al*., 1992; Fujii *et al*., 1997b). The function of these compounds is not known.

The immuno-gold labelling of hepatotoxins has revealed that the toxins are primarily localised in the thylakoid area and nucleoid in *Microcystis* and *Nodularia* cells, with smaller amounts in the cell wall and sheath. In the same study, nodularin was found both in vegetative cells and heterocysts (Shi *et al*., 1995).

Table 4. Nodularin variants and their hepatotoxicity (modified from Rinehart *et al*., 1994)

ND, not determined; NK, not known

Toxin	Origin	Intraperitoneal (mouse) LD_{50} (µg kg^{-1})	Reference
nodularin	*Nodularia* strain	60	Carmichael *et al*., 1988
	Nodularia bloom	50	Eriksson *et al*., 1988
	Nodularia bloom	50	Rinehart *et al.,* 1988
	Nodularia strain	70	Runnegar *et al*., 1988
	Nodularia bloom and strain	70	Sivonen *et al*., 1989b
	Nodularia bloom	50	Sandström *et al*., 1990
[*D*-Asp^1]nodularin	*Nodularia* strain	75	Namikoshi *et al*., 1994
[$DMAdda^3$]nodularin	*Nodularia* bloom	150	Namikoshi *et al*., 1994
[(6*Z*)-$Adda^3$]nodularin	*Nodularia* bloom	> 2000	Namikoshi *et al*., 1994
[*D*-Glu-OCH_3 ^{4}]nodularin	NK	> 1200	Rinehart *et al*., 1994
[L-Val^2]nodularin (= motuporin)	*Theonella swinhoei* Gray	ND	de Silva *et al*., 1992
dihydronodularins:			
[*D*-$MeAbu^5$]nodularin	*Nodularia* strain	150	Namikoshi *et al*., 1993
[*L*-$MeAbu^5$]nodularin	*Nodularia* strain	150	Namikoshi *et al*., 1993

1.3.2. Mechanism of hepatotoxicity in mammals

After the hepatotoxins are released from the cyanobacterial cells in the digestive system and taken up in the ileum, they pass through the hepatic portal vein into the primary target organ, the liver (Carmichael & Falconer, 1993; Nishiwaki *et al.*, 1994). The liver specificity of the hepatotoxins is dependent on the active uptake of these compounds by the bile acid transporter mechanism (Carmichael, 1992; Carmichael & Falconer, 1993). In hepatocytes, they cause hyperphosphorylation of various proteins such as cytokeratin peptides 8 and 18 (Ohta *et al.*, 1992), and this leads to disturbances in the cell's cytoskeleton and in morphological changes (e.g. Eriksson *et al.*, 1990, 1992a,b; Ohta *et al.*, 1992, 1994; Toivola *et al.*, 1997). Changes in cell shape and losses of the cell's contacts with other hepatocytes and with sinusoidal capillaries lead to a lethal intrahepatic haemorrhage or hepatic insufficiency within a few hours to a few days (Carmichael, 1992, 1994; Carmichael & Falconer, 1993).

In mammals, signs of poisoning due to hepatotoxins include weakness, reluctance to move, pallor of the extremities and mucous membranes, anorexia, vomiting, cold extremities and hypovolaemic shock (Carmichael, 1992; Carmichael & Falconer, 1993). Before the animals die of intrahepatic haemorrhage and hypovolaemic shock, they frequently suffer muscle tremors and fall into a coma (Carmichael, 1992). The conclusion that death has resulted from intrahepatic haemorrhage can be confirmed by an autopsy that reveals an enlarged liver. In addition, a blood chemistry, which reveals low hepatic haemoglobin and iron concentrations, expose blood loss responsible for shock (Carmichael, 1992). Besides, necrosis and lysis of hepatocytes, accompanied by marked haemorrhages, are discovered in histological examinations (e.g. Runnegar *et al.*, 1988).

In all eukaryotic cells, intracellular signal transduction is principally linked to extracellular signals via reversible protein phosphorylation by protein kinases and phosphatases. Eukaryotic protein phosphatases (PPs) are structurally and functionally dissimilar enzymes, which belong to the PPP, PPM, and PTP gene families. PPs in the PTP family dephosphorylate phosphotyrosine whereas phosphoserine and phosphothreonine are dephosphorylated by PPMs and PPPs. The PPP family includes PP1, PP2A, and PP2B, which are the most abundant eukaryotic protein Ser/Thr phosphatases (Barford, 1996). PPs play a crucial role in a variety of cellular processes such as cell proliferation and differentiation (Shenolikar, 1994; Wera & Hemmings, 1995).

PPs have been shown to be inhibited by a variety of natural toxins. These include okadaic acid (Holmes & Boland, 1993; Honkanen *et al.*, 1994), a fatty acid polyether (Yasumoto *et al.*, 1985). This

seafood toxin, which cause diarrhoeic shellfish poisoning (DSP), is produced by marine dinoflagellates and accumulates in filter-feeding organisms (Aune & Yndestad 1993; Steidinger, 1993; Scoging 1998). Okadaic acid was the first toxin reported to inhibit PP1 and PP2A (Takai *et al.*, 1987; Bialojan & Takai, 1988; Adamson *et al.*, 1989; Haystead *et al.*, 1989, and others). Since then, microcystins and nodularin have also been found to inhibit PP1 and PP2A (MacKintosh *et. al.*, 1990; Matsushima *et al.*, 1990; Yoshizawa *et al.*, 1990; Siegl *et al.*, 1990; Honkanen *et al.*, 1990, 1991, 1994; Ohta *et al.*, 1994; Suganuma *et al.*, 1992; Runnegar *et al.*, 1993).

The toxins inhibit distinct PPs differentially. For instance, the inhibitory effects of nodularin and microcystin-LR and –LA are higher for PP2A than for PP1 (Honkanen *et al.*, 1994). The different sensitivity of PP1 and PP2A to the toxins can be due to the presence of a cysteine residue at position 269 in PP2A and a phenylalanine residue at position 276 in PP1 (Lee *et al.*, 1999)**.**

Through inhibition of PPs activities these structurally diverse toxins, which are produced by different organisms, prevent dephosphorylation of phosphoserine and phosphothreonine and therefore increase protein phosphorylation (see Quinn *et al.*, 1993). Protein phosphorylation plays a central role in many adaptive responses to environmental signals also in cyanobacteria (Tandeau de Marsac & Houmard, 1993; Mann 1994). However, cyanobacterial PPs are resistant to their own toxins, such as microcystin-LR (Shi *et al.*, 1999).

It is known that the different toxins compete for binding to PP1, since prior binding of microcystins (Matshushima *et al.*, 1990; Yoshizawa *et al.*, 1990), nodularin (Yoshizawa *et al.*, 1990), and calyculin to PP1 (Suganuma *et al.*, 1990) prevent binding of okadaic acid. Furthermore, the prior binding of okadaic acid and inhibitor-1 and inhibitor-2 prevented PP1 from interacting with microcystin (MacKintosh *et al.*, 1990).

1.3.3. Tumour promotion caused by protein phosphatase inhibitors

Masami Suganuma was the first to report that okadaic acid is a tumour promoter (Adamson *et al.*, 1989). Later, structurally various compounds including microcystins and nodularin that inhibit PP1 and PP2A were found and named as “Tumor Promoters of the Okadaic Acid Activity Class” (Fujiki & Suganuma, 1993). Further studies of these compounds revealed that inhibition of PP1 and PP2A is a general tumour promotion pathway in various organs (Fujiki & Suganuma, 1999). The next step in tumour promotion, after inhibition of PPs, is the expression of the *TNT*∝ gene and early-response genes (Sueoka *et al.*, 1997; Fujiki & Suganuma, 1999). These expressions are assumed to be associated with mRNA stabilisation.

In rat liver, repeated intraperitoneal injections of microcystin-LR and nodularin induced tumours (Nishiwaki-Matsushima

et al., 1992a; Ohta *et al.*, 1994). Skin tumour growth was promoted by oral consumption of *Microcystis* in drinking water (Falconer, 1991, 1996). Furthermore, several epidemiological studies in China have indicated that people taking their drinking water from ponds and ditches contaminated with *Microcystis* blooms have a much higher incidence of primary liver cancer than those using river or well water (Fujiki *et al.*, 1996; Hunter, 1998).

Nodularin and microcystin-LR have the same specific activity in inhibition of PP1 and PP2A (Yoshizawa *et al.*, 1990). Nodularin promotes liver tumour growth with higher activity than microcystin-LR (Ohta *et al.*, 1994; Fujiki *et al.*, 1996). In addition, nodularin is a liver carcinogen with both initiating and tumour-promoting activities, whereas microcystin-LR is a liver tumour promoter without an initiating activity (Ohta *et al.*, 1994; Fujiki *et al.*, 1996).

1.3.4. Structure-activity relationship of peptide toxins

The microcystins and nodularin inhibit PP1 and PP2A and show hepatotoxicity with similar potency. The cyclic stucture of the microcystins and nodularins share many common features. Furthermore, there are variants of microcystins and nodularin that also contain common residues in the variable regions. For instance, microcystin-LR and nodularin have Arg in common. The study of Nishiwaki-Matsushima and co-authors (1992b) with naturally occurring geometrical isomers of microcystin-LR and -LA has shown that the Arg residue did not significantly interact with the enzymes and can be substituted by other amino acids without loss of hepatotoxicity. This was further verified by the isolation of a nodularin variant, motuporin (de Silva *et al*, 1992). Motuporin is similar in structure to nodularin, but has Arg instead of Val. Despite this structural difference, both of these compounds inhibit PPs with similar potency. Furthermore, there are many naturally occurring toxic microcystins in which a number of residues replace the leucine (Leu) and/or the Arg in the peptide ring (Carmichael, 1992; Rinehart *et al.*, 1994), indicating that these residues are not critical for the inhibition of PPs. On the contrary, it has been reported that Adda, which is also common for microcystins and nodularins, is essential for the activity and that the peptides with the 6Z-Adda isomer are biologically inactive (Nishiwaki-Matsushima *et al.*, 1991; Rinehart *et al.*, 1994). Also, D-Glu has an important role in hepatotoxicity, since esterification of its free carboxyl group leads to a total loss of activity (Rinehart *et al.*, 1994). These findings suggest that only the Adda and Glu residues play important roles in the hepatotoxicity, thus they have functions in interactions with microcystins and nodularins to PP1 and PP2A.

Three-dimensional (3-D) structures of microcystins (Rudolp-Böhner *et al.*, 1994; Bagu *et al.*, 1995, 1997; Trogen *et al.*, 1996, 1998) and motuporin (Bagu *et al.*, 1995, 1997) have been determined by nuclear magnetic resonance spectroscopy (NMR). Conformational studies have

shown that microcystin-LR (Bagu *et al.*, 1995; Trogen *et al.*, 1996), microcystin-RR (Trogen *et al.*, 1998), and motuporin (Bagu *et al.*, 1995) possess a saddle-shaped peptide ring. Furthermore, the Adda side-chain in both toxins and Arg in microcystin were shown to be flexible, whereas Val in motuporin was less flexible than Arg. The conformational similarities of microcystin and nodularin imply that these peptide toxins inhibit PPs in a similar way.

The NMR results differ from the results obtained using a molecular modelling analysis of the 3-D structures of microcystin-LR and nodularin, which predicted that these toxins have planar peptide rings (Lanaras *et al.*, 1991; Taylor *et al.*, 1992). The comparison of Lanaras and co-workers was later optimised by Taylor *et al.* (1992), who used molecular modelling to calculate the energetically lowest conformations for microcystin-LR. Later, molecular modelling was expanded to include several naturally occurring toxins: cantharidin, calyculin-A, okadaic acid, tautomycin (Quinn *et al.*, 1993; Gauss *et al.*, 1997; Gupta *et al.*, 1997). The conformational studies of natural toxins have been reviewed by Quinn *et al.* (1996), who has also developed a pharmacophore model for okadaic acid, calyculin-A, and microcystin-LR (Quinn *et al.*, 1993). This model contains a conserved acidic group, two potential hydrogen-bonding sites, and a non-polar side-chain.

The 3-D structures of the inhibitors alone do not explain how PPs are inhibited, therefore, the bound conformations of inhibitors to protein phosphatases are needed. Crystal structures have been determined for rabbit PP1 complexed with microcystin-LR (Goldberg *et al.*, 1995) and for human PP1 complexed with tungsten (Egloff *et al.*, 1995). These structures clarify how PP1 dephosphorylate substrates and how it is regulated.
The study of Goldberg *et al.* (1995) showed how PP1 is inhibited by microcystin. Microcystin interacts with the PP1 in a Y-shaped groove on the surface of the enzyme at three sites: the metal-binding site, the hydrophobic groove and the edge of the C-terminal groove near the active site (Goldberg *et al.*, 1995).

Lee and co-workers (Zhang & Lee, 1997; Lee *et al.*, 1999) have mutated residues in these grooves to assess the importance of these structures in substrate recognition. Strong effects were only observed for mutation of residues involved in phosphate binding and orientation at the active site (Lee *et al.*, 1999).

In the interaction between microcystin and PP1, the Glu residue of the microcystin binds indirectly to the metals via two of the metal-liganded water molecules. The long hydrophobic Adda group is inserted into the hydrophobic groove. The Leu residue of microcystin interacts with the side-chain of tyrosine 272 (Tyr 272) within the ß12/ß13-loop of the C-terminal groove (Goldberg *et al.*, 1995). The role of the ß12/ß13-loop in toxin binding has been confirmed by studies with different mutants (Shima *et al.,* 1994; Zhang *et al.*, 1994, 1996; Lee *et al.*, 1999).

Functional differences exist between the microcystins and nodularins with respect to their interaction with PPs. These enzymes are initially bound non-covalently and inhibited by these toxins. In contrast to the microcystins (Goldberg *et al.*, 1995; MacKintosh *et al.*, 1995; Runnegar *et al.*, 1995), motuporin does not form a secondary covalent bond after inhibition of PP1 (Bagu *et al.*, 1995, 1997). The explanation why nodularins are incapable of forming a covalent linkage with PP1 is the difference in the position of N-methyldehydrobutyrine (Mdhb) residue in motuporin relative to the counterpart N-methyldehydroalanine (Mdha) in microcystin-LR (Bagu *et al.*, 1995, 1997). The different position of Mdhb at the surface of the PP1-toxin complex compared to microcystins may facilitate a chemical interaction with further macromolecules, which may explain the carcinogenic properties of nodularins (Ohta *et al.*, 1994; Bagu *et al.*, 1997). Furthermore, the higher tumour promoting activity and the carcinogenicity of nodularin are thought to be caused by smaller size of nodularin compared to microcystin. For that reason, nodularin is more easily taken into the hepatocytes than microcystin-LR (see Fujiki *et al.*, 1996).

1.3.5. Biosynthesis of peptide toxins

Microbes synthesise non-ribosomally cyclic and linear peptides by large modular multienzyme complexes, peptide synthetases. The peptide sythetases use the so-called thiotemplate mechanism for the synthesis of peptides (von Döhren *et al.*, 1997; Konz & Marahiel, 1999).

Peptide synthetase genes have been identified from several *Microcystis aeruginosa* strains (Meißner *et al.*, 1996; Dittmann *et al.*, 1996, 1997). One of these strains (PCC7806) has been transformed to non-toxic form by the mutation of a microcystin synthetase gene, which demonstrates that this gene, called *mcyB*, encodes a microcystin synthetase (Dittmann *et al.*, 1997). Recently, *mcyB* was shown to hybridise, with variable signal intensity, to DNAs from several hepatotoxic strains of *Oscillatoria*, *Microcystis*, and *Anabaena* (Neilan *et al.* 1999). On the contrary, two peptide synthetase genes encoding anabaenopeptilides (cyclic depsipeptides) in the *Anabaena* sp. strain 90 (Rouhiainen *et al.*, 2000) hybridised insignificantly to non-heterocystous microcystin-producing strains. However, this probe gave strong signals with all hepatotoxic *Anabaena* strains, and it also cross-hybridised to most of the hepatotoxic *Nostoc* and *Nodularia* strains (Neilan *et al.*, 1999). Recently, three open reading frames encoding peptide synthetases involved in microcystin synthesis were identified (Nishizawa *et al.*, 1999).

The biosynthesis of nodularin has been studied by NMR analysis of nodularin obtained from experiments with several ^{13}C-labelled precursors (Choi *et al.*, 1993; Rinehart *et al.*, 1994). Carbons in Adda are derived from acetate, methionine, phenylalanine, and propionate (Moore *et al.,* 1991; Choi *et al.*, 1993; Rinehart *et al.*,

1994). Acetate and pyruvate were also incorporated into Arg and Glu. Furthermore, D-MeAsp and Mdhb were labelled with propionate and methionine, respectively. The complete synthesis of the natural nodularin variant, motuporin, has been published (Valentekovich & Schreiber, 1995; Bauer & Armstrong, 1999). In addition, synthetic analogues of nodularin have been obtained (Mehrotra & Gani, 1996). A general route for the preparation of microcystins and nodularins has been provided by Kim *et al.* (1996).

1.3.6. Analytical methods of cyanobacterial toxins

Falconer (1993) and Harada *et al.* (1999) have written reviews of biological, biochemical, and chemical methods for assays of cyanobacterial toxins. Cyanobacterial toxins in water and in cells have been detected and identified by a range of methods that differ in selectivity and sensitivity (Harada, 1996; Harada *et al.*, 1999). Early methods for the assay of cyanobacterial toxins were based on the mouse bioassay. This method was widely used for determination of outright toxicity and toxin concentration. Furthermore, a number of invertebrates have been investigated for use as bioassays for toxins (Harada *et al.*, 1999). Later, biochemical tests such as the protein phosphatase inhibition assay (An & Carmichael, 1994; Ward *et al.*, 1998; Harada *et al.*, 1999) and the enzyme-linked immuno-sorbent assay (ELISA) (Chu *et al.*, 1989, 1990; Harada, 1996; Harada *et al.*, 1999) were developed for detection of peptide hepatotoxins, microcystins and nodularins.

Due to functional groups in the molecules cyanobacterial peptide toxins have common physico-chemical properties such as molecular weight and activities. For that reason they can be analysed by the same analytical methods (Harada *et al.*, 1999). The most commonly-used analytical system for cyanobacterial peptide toxins is high performance liquid chromatography (HPLC) combined with ultraviolet (UV) detection which relies on their retention times and UV spectra for identification (Harada, 1996; Meriluoto, 1997; Harada *et al.,* 1999; Pelander, 2000). HPLC has been the most intensively used method when studying cellular toxin concentrations in cyanobacteria under different growth conditions (Sivonen & Jones, 1999).

1.4. Cyanobacterial taxonomy

Cyanobacteria were traditionally classified on the basis of their morphology. Despite the fact that their morphology is complex when compared to most other microbes, the taxonomy based on morphological characteristics does not necessarily result in a phylogenetically reliable taxonomy (Giovannoni *et al.*, 1988; Wilmotte, 1994). Nowadays, bacterial taxonomy relies on different kinds of information derived from phenotypic and genotypic data (Vandamme *et al.*, 1996).

The use of phenotypic and genotypic characteristics in cyanobacterial taxonomy was pioneered by Stanier and collaborators (e.g. Kenyon *et al.*, 1972;

Herdman *et al*., 1979a,b; Rippka *et al*., 1979). The characteristics used include morphology, pigment and fatty acid composition, photoheterotrophic growth, nitrogenase activity, DNA base composition, and genome size. The taxonomic system of Rippka *et al.* (1979), which was based on identification of 178 cyanobacterial cultures in the Pasteur Culture Collection, recognised 22 genera placed in five taxonomic sections. Section I contains unicellular cyanobacteria that reproduce either by binary fission (*Gloeobacter*, *Gloeocapsa*, *Gloeothece*, *Synechococcus*, *Synechocystis*) or by budding (*Chamaesiphon*) whereas the unicellular members in section II divide only by multiple fission, which leads to formation of motile (*Dermocarpa*) or inmotile (*Xenococcus*) baeocytes, or by both binary fission and multiple fission (*Dermocarpella*, *Myxosarcina*, *Chroococcidiopsis*, *Pleurocapsa* group). Members in sections from III to V are filamentous. The filamentous cyanobacteria in section III (*Spirulina*, *Oscillatoria*, LPP group A, *Pseudanabaena*, LPP group B) compose only of vegetative cells while the filamentous cyanobacteria of sections IV and V contain additionally heterocysts and sometimes akinetes. In section IV, filamentous cyanobacteria are distinguishable by hormogonium formation (*Nostoc*, *Scytonema*, *Calothrix*) or its absence (*Anabaena*, *Nodularia*, *Cylindrospermum*). Divisions of cells of the filamentous, heterocystous cyanobacteria belonging to sections III and IV occur in one plane whereas members in section V (*Chlorogloeopsis*, *Fischerella*) divide on more than one plane.

The bacteriological taxonomic system created for cyanobacteria by Rippka *et al*. (1979) has been modified by Castenholz (1989a, b, c), Waterbury (1989), and Waterbury and Rippka (1989) in the volume three of “Bergey’s Manual of Systematic Bacteriology”. This system also includes descriptions of cyanobacteria which are observed, but which have not been succesfully maintained in cultures. In addition, ecological features of cyanobacteria have been included. In their separate studies, Anagnostidis and Komárek (1988, 1990) and Komárek and Anagnostidis (1986, 1989) have revised the taxonomy of cyanobacteria using both bacteriological and botanical approaches. The authors made an extensive review of the literature and applied phenotypic and genotypic data concerning cyanobacterial taxonomy.

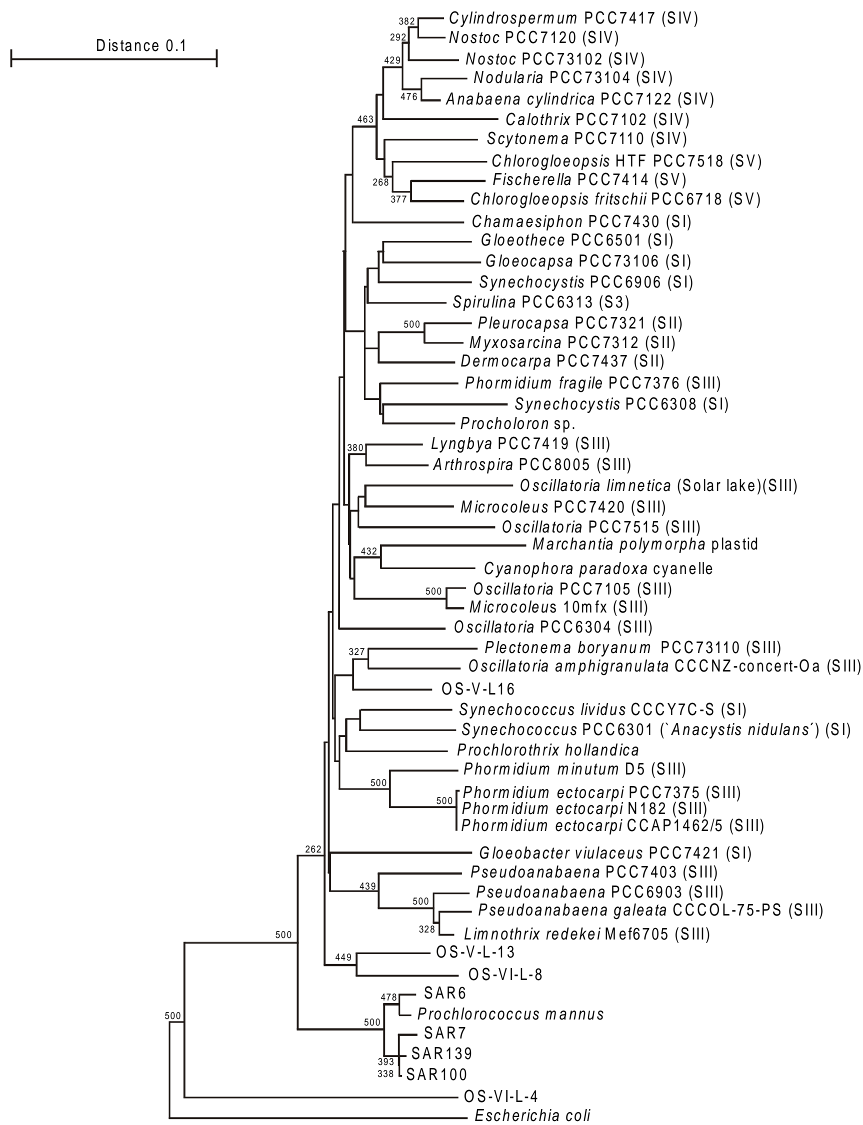

Fig. 2. Neighbor-joining tree for partial 16S rRNA gene sequences of cyanobacteria and related microorganims and organells. Bootstrap values higher than 50% of 500 resamplings are given at nodes. Scale bar expresses substitutions per nucleotide. Sections defined by Rippka *et al.* (1979) are given in parantheses alongside the strain names.

The tree was redrawn with kind permission from the copyright owner, Kluwer Academic Publishers, from Figure 3, derived from the article "Molecular Evolution and Taxonomy of Cyanobacteria" by A. Wilmotte. The article was originally published in 1994, in pp. 1-25 of *The Molecular Biology of Cyanobacteria*, edited by D. A. Bryant.

Molecular phylogenetic analysis have revealed at least ten distinct eubacterial phyla (Woese, 1987). One of these contains oxygenic photosynthetic prokaryotes, cyanobacteria and prochlorophytes, which have different pigment compositions and are genetically related on the basis of 16S rRNA sequences (Woese, 1987).

Cyanobacteria contain chlorophyll-*a* and phycobiliproteins and prochlorophytes possess chlorophyll-*a* as well as chlorophyll-*b*, but lack phycobiliproteins (Castenholz & Rippka, 1989; Lewin, 1989; Matthijs *et al*., 1994). The prochlorophytes contain three genera: *Prochloron*, *Prochlorothrix* (Lewin, 1989), and *Prochlorococcus* (Partensky *et al*., 1999). 16S rDNA sequence analysis has revealed that prochlorophytes emerged within the cyanobacteria, but on separate branches (Urbach *et al*., 1992; Wilmotte, 1994). Prochlorophytes are phylogenetically nearest to *Synechococcus* (Urbach *et al*., 1998).

The evolutionary relationships among cyanobacteria have been published by Giovannoni *et al.* (1988), Wilmotte (1994), and Turner (1997). The 16S rRNA sequence analysis (Fig. 2) were congruent to the taxonomic sections of II, III and IV defined by Rippka *et al*., (1979). On the contrary, sections I and III were scattered in different lineages and sometimes mixed. The heterocystous filamentous strains were all in the same cluster. In this cluster, the *Nodularia* strain PCC73104 grouped with the *Anabaena cylindrica* PCC7122 strain. Furthermore, it was closely related to *Nostoc* (PCC73102 and PCC7120) and to the *Cylindrospermum* (PCC7417) strains (Wilmotte, 1994).

1.4.1. Molecular methods used in cyanobacterial taxonomy

Most bacterial genomes contain genes from multiple sources, even from genetically distant ones (Doolittle, 1999). Nowadays, it is suspected that the even most trusted chronometers, rRNA genes, can be transferred (Doolittle, 1999). These genes, which are functionally constant and are composed of highly conserved as well as more variable domains, have been utilised for phylogenetic analysis in bacteria (Vandamme *et al*., 1996). Outlines of bacterial phylogenetic relationships emerged from the work of Woese (1987), who pioneered the comparison of rDNA sequences.

The first complete cyanobacterial 16S rDNA sequence was released by Tomioka and Sugiura (1983). In 1988, Giovannoni and co-authors published the first study of evolutionary relationships among cyanobacteria using partial 16S rDNA sequences (Giovannoni *et al*., 1988). The sequencing of the 16S rRNA gene have resulted in a rRNA sequence database. At the end of 1998, 171 16S rRNA genes were sequenced from cyanobacteria.

These 171 cyanobacterial rDNA sequences (81 complete sequences comprising nearly 1500 nucleotides and 90 partial sequences) serve as the backbone for modern cyanobacterial taxonomy (Wilmotte, 1994). One partial (ARB_40A3CBCC, Giovannoni *et al.*

1988) and one complete (aj22447, Hayes & Barker, 1997) 16S rDNA sequence exist from *Nodularia*.

The molecular methods for studying genotypic relationships among cyanobacteria have been reviewed by Wilmotte (1994). In the present work, only the DNA- and rRNA sequence-based studies of free-living cultured cyanobacteria have been reviewed (Table 5).

Over the recent years, techniques based on sequences of rRNA genes have become the most widely used methods for identification, classification, and phylogeny of cyanobacteria. In addition to direct comparison of the DNA sequences, other methods based on amplification of rRNA genes have been used. The amplified ribosomal genes can be digested by restriction enzymes and the resulting patterns analysed, this technique has been termed restriction fragment length polymorphism (RFLP). Before the introduction of the polymerase chain reaction (PCR) method (Lane *et al*., 1985), ribotyping based on labelled-rRNA probe have revealed the hybridised fragments generated after restriction enzyme digestion.

During the last few years, a battery of DNA-directed typing methods has been used for PCR-based genomic fingerprinting of cyanobacteria. Genomic fingerprints have been produced by PCR with random (RAPD) or by arbitrary primers (AP). In addition, fingerprints have been generated with primers corresponding to the repetitive extragenic palindromic (REP) and enterobacterial repetitive intergenic consensus (ERIC) sequences, and to the long and short tandemly repeated repetitive (LTRR and STRR) sequences. The function of repetitive and random amplified polymorphic DNA elements is not known. In addition, cyanobacterium-specific primers have been developed to identify cultured and natural samples. This method has been named “Specific PCR identification” or “Diagnostic PCR”.

DNA base composition has been determined for several cyanobacterial strains in the Pasteur Culture Collection. Generally, large differences in DNA base composition reflect the fact that strains are not closely related, whereas similar guanine and cytosine (G+C) percentages give no information about genotypic relationships, therefore the taxonomic value of this method at the species level is low (Wilmotte, 1994). The species should be delineated according to DNA-DNA hybridisation studies, which established the base composition homology between different DNA strains. For strains of the same species at least 70% hybridisation is needed (Wayne *et al*., 1987). Furthermore, protein-coding genes have been sequenced and probed. These genes can be used for identification and for classification of strains, but not for phylogeny, since they are not universally distributed and essential among bacteria (Woese, 1987), thus different molecular methods gave different taxonomic information (Vandamme *et al*., 1996).

Table 5. DNA- and rRNA sequence-based techniques applied for studying the genetic diversity of free-living cyanobacteria

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List of studied genes (their product and/or function): *apcAB,* allophycocyanin (phycobiliproteins, constituting the phycobilisome core); *cpcABC,* phycocyanin (phycobiliproteins, constituting the rod elements of phycobilisome); *cpeAB*, phycoerythrin (phycobiliproteins, constituting the rod elements of phycobilisome); *glnA,* glutamine synthetase (NH_4^+ assimilation); *gvp*A, structural gas vesicle protein (buoyancy); *nifDH,* dinitrogenase and nitrogenase reductase (nitrogenase, nitrogen fixation); *petBD,* cytochrome b_6 and subunit IV (cytochrome b_6/f complex, photosynthetic electron transport); *psbAD,* D1 and D2 proteins (photosystem II, photosynthetic electron transfer); *psbBC,* antenna polypeptides CP47 and CP43 (photosystem II, oxygen evolution); *rbcLS,* large and small subunits of RubisCo (CO_2 fixation); *rpoCD,* core enzyme and sigma factors (RNA polymerase, transcription).

Method	Reference
Sequencing:	
- 16S rRNA	Bonen & Doolittle, 1976; Tomioka & Sugiura, 1983; Giovannoni *et al.*, 1988; Ligon *et al.*, 1991; Wilmotte *et al.*, 1992, 1993, 1994; Nelissen *et al.*, 1992, 1994, 1996; Garchia-Pichel *et al.*, 1996, 1998; Palinska *et al.*, 1996; Neilan *et al.*, 1994a,b, 1997a; Ishida *et al.*, 1997; Kane *et al.*, 1997; Turner, 1997; Turner *et al.*, 1999; Otsuka *et al.*, 1998; Urbach *et al.*, 1998; Rudi *et al.*, 1997, 1998; Barker *et al.*, 1999; Honda *et al.*, 1999; Saker *et al.*, 1999; Shaw *et al.*, 1999; Lyra *et al.*, 2000
- rRNA ITS	Williamson & Doolittle, 1983; Tomioka & Sugiura, 1984; Nelissen *et al.*, 1994; Barker *et al.*, 1999; Otsuka *et al.*, 1999; Postius & Ernst, 1999
- $tRNA^{Leu}$ (UAA)	Rudi & Jakobsen, 1997, 1999
- *rpoC*	Palenik & Swift, 1996; Toledo & Palenik, 1997; Wilson *et al.*, 2000
- PC-IGS	Hayes & Barker, 1997; Barker *et al.*, 1999; Bolch *et al.*, 1999
- *psbB*, *petBD*	Urbach *et al.*, 1998
- *nifH*	Ben-Borath & Zehr, 1994; Ben-Borath *et al.*, 1993; Steppe *et al.*, 1996; Zehr *et al.*, 1997
- *rbcLX*	Rudi *et al.*, 1998
- *gvpA*-IGS	Barker *et al.*, 1999
PCR/ DGGE:	
- 16S rRNA	Garchia-Pichel *et al.*, 1996
PCR/RFLP:	
- 16S rRNA	Lyra *et al.*, 1997, 2000; Margheri *et al.*, 1999
- rRNA ITS	Neilan, 1996; Neilan *et al.*, 1997b; Lu *et al.*, 1997; West & Adams, 1997; Smith *et al.*, 1998; Scheldeman *et al.*, 1999
- PC-IGS	Neilan *et al.*, 1995; Bolch *et al.*, 1996; Neilan, 1996; Bolch *et al.*, 1999
Specific PCR identification:	
- PC-IGS	Neilan *et al.*, 1995; Hayes & Barker, 1997; Barker *et al.*, 1999
- rRNA ITS	Neilan, 1996; Neilan *et al.*, 1997b; Barker *et al.*, 1999
- *gvpA*-IGS	Barker *et al.*, 1999
- *rpoC1*	Wilson *et al.*, 2000
PCR:	
- with RAPD and AP primers	Neilan, 1995, 1996; Komárek, 1996; Nishihara *et al.*, 1997; West & Adams, 1997; Bolch *et al.*, 1999
- with repetitive DNA sequences (REP, ERIC, HIP1, STRR, LTRR)	Versalovic *et al.,* 1991; Robinson *et al.*, 1995; Rasmussen & Svenning, 1998; Smith *et al.,* 1998; Lyra *et al.*, 2000; Wilson *et al.*, 2000

Table 5, continued

Southern blot RFLP hybridization:	
Probes:	
- REPs	Asayma *et al.*, 1996
- rRNA, *cpcABC*, *psbA*, *rbcL*, *cpeAB*	Douglas & Carr, 1988
- *psbAI*	Ernst *et al.*, 1995; Postius & Ernst, 1999
- *glnA*, *rbcS*, *psbA*, *nifD* excision elements	Gebhardt & Nierzwicki-Bauer, 1991
- *psbA*, *psbCD*	Golden *et al.*, 1989
- STRR	Mazel *et al.*, 1990; Rouhiainen *et al.*, 1995
- rRNA	Golden *et al.*, 1989; Nelissen *et al.*, 1996
- 16S and 23S rRNA	Nichols *et al.*, 1982
- $tRNA^{Leu}$ (UAA)	Rudi & Jakobsen 1997, 1999
- 16S rRNA	Schönhuber *et al.*, 1999
- *psbA1*, *cpeA*, *cpeB*	Wood & Townsend, 1990
- *cpcAB*, *apcAB*, *gvpA1*, *nifH*	Zimmermann & Culley, 1991
DNA base composition:	Herdman *et al.*, 1979a; Stam, 1980; Stulp & Stam, 1984; Wilmotte & Stam, 1984
DNA-DNA hybridization:	Stam, 1980; Lachance, 1981; Stulp & Stam, 1984; Wilmotte & Stam, 1984

1.4.2. Morphological and genetic variation of Nodularia cultures and populations

Komárek and co-authors (1993) have studied the taxonomy of the genus *Nodularia*. Within this genus, two distinct groups of species, based on the ability to produce gas vesicles, were differentiated. The studies of the ultrastructure of genus *Nodularia* (Gumpert *et al.*, 1987; Šmarda *et al.*, 1988; Albertano *et al.*, 1996; Šmarda & Šmajs, 1996) have revealed large amounts of densely packed gas vesicles. Their densities in cells separated them into three distinct types (Šmarda & Šmajs, 1996). For example, the Baltic Sea *Nodularia* had three types of gas vesicles in species of *N. baltica*, *N. spumigena*, and *N. litorea* (Šmarda & Šmajs, 1996).

In addition to the size and the density of gas vesicles, Komárek *et al.* (1993) have used several other markers in the intrageneric taxonomy of *Nodularia*: the size of cells of all types, the morphology of akinetes, and the ecological properties of species types. Based on these features, Komárek *et al.* (1993) suggested distinction of several types of *Nodularia* species including three benthic species (*N. harveyana*, *N. sphaerocarpa*, *N. willei*) and four planktic species (*N. baltica*, *N. litorea*, *N. spumigena*, *N. crassa*). Four of these species (Table 6: *N. harveyana*, *N. baltica*, *N. litorea*, *N. spumigena*) have been isolated from the Baltic Sea (Komárek *et al.*, 1993).

In the study of Hayes and Barker (1997), the genetic diversity of Baltic Sea *Nodularia* population seemed to be restricted to few genotypes. The diagnostic PCR study (also termed specific PCR identification) of *Nodularia* population in the Southern Baltic Sea in 1996 showed two PC-IGS (the intergenic spacer region of the phycocyanin operon) genotypes from 156 filaments. Later in 1994, the study of Barker *et al.* (1999) revealed three groups based on PC-IGS, two groups based on *gvp*A-IGS (the intergenic spacer region between two copies of *gvpA* gene), and three groups based on rDNA-ITS (the 16S-23S rRNA internal transcribed region) in thirteen clonal *Nodularia* strains. The authors could not find any correlation between the genotypic and phenotypic characters examined (trichome width, degree of coiling, and properties of gas vesicles).

The morphological and genetic variation using RFLP and DNA sequencing of the *cpcBA*-IGS region and RAPD-PCR of *Nodularia* strains from Australia and from other geographical sites have been studied (Bolch *et al*., 1996, 1999). Geographically diverse *Nodularia* strains had a near *cpcBA*-IGS (the phycocyanin intergenic spacer region) sequence identity (Bolch *et al*., 1999) suggesting that *Nodularia* is globally distributed as suggested also by Hayes and Barker (1997). However, genetically distinct geographical strains were showed by RAPD-PCR (Bolch *et al*, 1999) and by RFLP of the *cpcBA*-IGS (Bolch *et al.* 1996). Genetic groupings based on the sequence identity were supported by morphological features (size and morphology of vegetative cells, heterocysts and akinetes, and diameter and morphology of trichomes) (Bolch *et al.,* 1999).

It has been suggested that the Baltic Sea *Nodularia* populations have exchanged genetic material, since the PC-IGS, *gvpA*-IGS, and rDNA-ITS genotypic groupings of *Nodularia* strains were not congruent (Barker *et al*., 1999). The exchange of genetic material between genetically closely related cyanobacteria has been suggested also by Rudi *et al.* (1998). The authors proposed that the genetic exchange has led to the observed sequence homogeneity between these organisms and that it may also explain the similarity between the fossil and the recent species found by Schopf and others (1994). However, several studies have revealed that, for example, *Synechococcus* strains take up the DNA of any source (see Lorenz & Wackernagel, 1994) meaning that exchange may even occur between distant genera.

Table 6. Morphological and ecological properties of planktonic and benthic *Nodularia* species recorded in the Baltic Sea

Data on morphological and ecological properties were taken from Komárek *et al.* (1993) and data on gas vesicles were derived from Šmarda & Šmajs (1996). NA, Not applicable.

Characters	*N. baltica*	*N. harveyana*	*N. litorea*	*N. spumigena*
Trichome:				
Width (μm)	5-6	4-5	10-15	7-12
Cell length (μm)	2-5	2-3	2-4	2-4
Heterocysts:				
Length x width (μm)	2-5 x 5-8	3-5 x 4-6	4-7 x 10-15	4-5 x 9-14
Akinetes:				
Length x width (μm)	4-8 x 6-9	4-8 x 6-7	6-10 x 14-15	6-12 x 10-12
Observations	Sometimes solitary or in rows	In series of two to sixteen	Solitary or in short series	In series or discontinuous rows, rarely solitary or in twos
Gas vesicles:				
Present?	Yes	No	Yes	Variable
Density (μm^{-2})	140/160-180	NA	215/230-240	139/160-180
Ecology	Metaphyton or phytoplankton; marine, brackish and saline waters.	Benthic or periphyton; saline pools and lakes, thermal springs and marshes with high salinity.	Phytoplankton; marine, brackish and saline waters.	Metaphyton and euplankton; marine, brackish and saline waters.

2. Aims of the present study

Hepatotoxic *N. spumigena* blooms have been observed in all areas of the Baltic Sea except for the most northern part, the Bothnian Bay (Sivonen *et al.*, 1989a,b; Kononen *et al.*, 1993b). In these blooms, the concentration of nodularin often rises high enough to cause a health risk for animals (see Table 4). However, the role of environmental factors on nodularin production is not known yet. In order to understand toxin production in *Nodularia* under different environmental conditions, we studied the effects of several growth factors on nodularin concentrations in cells and in growth media using batch-cultures of two nodularin-producing strains (I, II). It was hoped that the study of the response of toxic *Nodularia* strains to abiotic growth factors would help understand the occurrence of hepatotoxic blooms in the Baltic Sea. For comparison, we studied the growth of the non-toxic *Nodularia* strain under the same growth conditions in order to reveal physiological differences between toxic and non-toxic strains (I).

Due to their ability to fix nitrogen, *Nodularia* and *Aphanizomenon* are capable of forming blooms in the N-depleted water mass of the Baltic Sea in late summer. However, these genera differ from each other in behavior, demonstrated by the fact that mass occurrences of *Nodularia* are absent from surface waters during most of the year, while *Aphanizomenon* is found abundantly during the whole year. Furthermore, the vertical and horizontal distribution of these two genera differ in the Baltic Sea (see Table 3). In order to obtain information on the co-dominance of these genera in late summer blooms, we used controlled laboratory experiments to study the effect of environmental factors on growth and nitrogen fixation (II). A knowledge of the individual physiological responses of these genera is needed to understand cyanobacterial bloom dynamics in the Baltic Sea.

Some protein phosphatases are inhibited by several natural toxins such as nodularins and microcystins (see section 1.3.2.). Structural variations among these toxins may explain differences in binding of these inhibitors to PPs (see section 1.3.4). In order to understand the effects of nodularin, we studied the three-dimensional structure of this molecule in water by NMR and molecular dynamics simulations (MD simulations)(III).

The genetic diversity of *Nodularia* populations in the Baltic Sea has been suggested to be low based on studies with specific PCR identification (Hayes & Barker, 1996; Barker *et al.*, 1999). Still, the phenotypic differences such as toxin production among *Nodularia* genotypes have not been examined. We examined the taxonomy of toxic and non-toxic *Nodularia* strains, originating worldwide, using morphology and different molecular methods based on the 16S rRNA gene and whole genome of *Nodularia* (IV).

3. Materials and methods

Strains. The *Nodularia* and *Aphanizomenon* strains used in this study are listed in Table 7. Most of the *Nodularia* strains were isolated from the Baltic Sea by picking filaments by microscopic examination until unialgal cultures were obtained (Sivonen *et al.*, 1989a). Both non-axenic cultures that contained bacteria and axenic cultures with no associated bacteria were used. Axenic cultures were achieved by the soft agarose-plating method (Rouhiainen *et al.*, 1995).

Table 7. *Nodularia* (Nod) and *Aphanizomenon* (Aph) strains used

Source of strains: PCC, Pasteur Culture Collection, Paris, France; NS, S. Blackburn, CSIRO, Division of Marine Research, Tasmania, Australia; the rest of the strains belong to the research group of K. Sivonen, Department of Applied Chemistry and Microbiology, University of Helsinki, Finland. Axenity: +, no cobacteria. Toxin production: -, no toxin production.

Strain	Axenity	Toxin production	Geographical origin	Date of isolation	Paper
Nod HKVV	-/+	-	Brackish water, the Baltic Sea	Not known	I, IV
Nod BY1	-/+	+	Brackish water, the Baltic Sea	13/8/86	I-IV
Nod P38 (= EIB)	-	+	Brackish water, the Baltic Sea	13/8/86	IV
Nod GDR113	-	+	Brackish water, the Baltic Sea	14/8/86	IV
Nod F81	+	+	Brackish water, the Baltic Sea	31/7/87	IV
Nod TEILI	-	+	Brackish water, the Baltic Sea	4/8/87	IV
Nod 59/22	-	+	Brackish water, the Baltic Sea	8/8/87	IV
Nod AV1	-	+	Brackish water, the Baltic Sea	8/8/87	IV
Nod AV3	+	+	Brackish water, the Baltic Sea	10/8/87	IV
Nod AV33	-	+	Brackish water, the Baltic Sea	10/8/87	IV
Nod 55/15 (= EIA)	-	+	Brackish water, the Baltic Sea	1/9/87	IV
Nod HEM	-/+	+	Brackish water, the Baltic Sea	10/9/87	I, IV
Nod SR5b	-	+	Brackish water, the Baltic Sea	14/8/91	IV
Nod GR8a	-	+	Brackish water, the Baltic Sea	3/8/92	IV
Nod GR8b	+	+	Brackish water, the Baltic Sea	4/8/92	IV
Nod TR183	-	+	Brackish water, the Baltic Sea	18/7/93	IV
Aph TR183	+	-	Brackish water, the Baltic Sea	18/7/93	II
Nod UP16a	+	-	Brackish water, the Baltic Sea	22/7/94	IV
Nod UP16f	+	-	Brackish water, the Baltic Sea	22/7/94	IV
Nod PCC73104/1	+	-	Alkaline soil, Spotted Lake, BC, Canada	1972	IV
Nod PCC7804	+	+	Thermal spring, Dax, France	1966	IV
Nod NSPI-05 (= PI9211-11)	+	+	Coastal water, Peel Inlet, Australia	11/12/92	IV
Nod NSOR-12 (= OR9301-08)	+	+	Coastal water, Orielton Lagoon, Tasmania, Australia	1/10/93	IV

Parameters studied. Toxin concentration in the cells and growth media of *Nodularia* strains BY1 (I, II) and HEM (I) under different batch-culture conditions (see Tables 1 in I and II) were measured using HPLC. Growth of toxic (BY1, HEM) and non-toxic (HKVV) *Nodularia* strains and the *Aphanizomenon* TR183 strain were investigated by measuring chlorophyll-*a*, total protein, and dry weight (I, II).

In addition, nitrogen fixation rates of *Nodularia* and *Aphanizomenon* were measured using the acetylene reduction method (II).

The three dimensional structure of nodularin in water was studied by NMR and by MD simulations (III). The toxin for these studies was isolated from *Nodularia* strain BY1 and purified by semipreparative HPLC.

Axenic *Nodularia* strains HEM, BY1, AV3, GR8b, F81, NSPI-05, NSOR-12, PCC7804, HKVV, UP16a, UP16b, and PCC73104/1 were examined using light microscopy and characterised by the 16S rRNA gene- and by total genome-based techniques (IV). In addition, their capabilities for hepatotoxin production were tested by HPLC or by ELISA. Methods used and parameters examined in this study are described in detail in the original publications and are summarised in Table 8.

Statistical analysis. Differences in growth and nodularin concentration of non-axenix *Nodularia* strains (I) were studied by multivariate analysis of variance with repeated measures (BMDP Statistical Software, Inc., version 1990), which made it possible to eliminate the time factor.

The relatedness of different parameters from the growth experiments with *Nodularia* and *Aphanizomeno*n strains (II) was tested with correlation analysis (SPSS for Windows 6.0, 1993, and Matlab for Windows 3.1, 1994). Non-parametric tests, Spearman and Kendall rank correlation coefficients, were used to evaluate the degree of correlation between chlorophyll-*a* concentrations, nitrogen fixation rates, and intracellular toxin concentrations (SPSS), because normality of data was not reached due to a large number of zero values within these parameters. In order to compare the different methods of biomass measurement, total protein, chlorophyll-*a*, and dry weight data were ln-transformed before using the parametric test (Pearson correlation coefficient). Multivariate regression analysis (Matlab) was performed for chlorophyll-*a* data for each experiment except for the experiment with accompanying bacteria. First, a transformation between original and coded variables was computed; then, interaction and quadratic terms of test variables were appended to matrices.

The GelCompar software (version 4, Applied Maths BVBA) was used for analysis patterns from RFLP of PCR-amplified 16S rRNA genes, REP-and ERIC-PCR, and ribotyping (IV). The matrix of similarities was calculated on the basis of the Dice band-matching coefficient (Dice, 1945) and the dendrogram was constructed by using the unweighted-pairs-group-method-with-averages (UPGMA) clustering algorithm (Sneath & Sokal, 1973). Phylogenetic trees based on 16S rDNA sequences were constructed by the neighbour-joining method and DNA parsimony method of Phylip (Felsenstein, 1993). In this program, bootstrap analysis was used to evaluate the tree topologies by performing 1000 resamplings.

Table 8. Parameters studied and methods used in this study

Parameters	**Methods**	**Described and used in**				**References**
Bacterial numbers	Acridine orange		II			Hobbie *et al.*, 1977
Bacterial production	Thymidine incorporation		II			Bell, 1993
Biomass	Dry weight	I	II			I
	Chlorophyll-*a*	I	II			Tandeau de Marsac & Houmard, 1988
	Total protein		II			Herbert *et al.*, 1971
Cell width	Light microcopy				IV	IV
Cell length	Light microscopy				IV	IV
Culture purity	Tryptone-glucose-yeast extract (TGY) -plates		II		IV	Atlas, 1993; IV
Culture purity	Light microscopy after Gram-staining				IV	IV
Filament length	Light microscopy after fixing with Lugol's solution		II			II
Presence of gas vesicles	Light microscopy				IV	IV
Genetic relationships	Southern blotting with STRR probe				IV	Bauer *et al.*, 1993; Rouhiainen *et al.*, 1995
	PCR-RFLP of 16S rRNA gene				IV	Weisburg *et al.*, 1991; Lyra *et al.*, 1997
	Sequencing of 16S rRNA gene				IV	Edwards *et al.*, 1989; Hultman *et al.*, 1991
	REP- and ERIC-PCR				IV	Versalovic *et al.*, 1991; IV
	Automated ribotyping				IV	Bruce, 1996; IV
Heterocyst frequency	Light microscopy after fixing with Lugol's solution		II			II
Heterocyst length	Light microscopy				IV	IV
Heterocyst width	Light microscopy				IV	IV
Nitrogen fixation rate	Acetylene reduction method		II			Moisander *et al.*, 1996
Nodularin concentration	HPLC+UV-detection	I	II		IV	Meriluoto & Eriksson, 1988; I
Nodularin concentration	ELISA				IV	IV
Nodularin purification	Semipreparative HPLC + UV-detection			III		III
Nodularin 3-D structure	NMR and molecular dynamics simulations			III		III
pH of the culture		I				I

4. Results and discussion

4.1. Phenotypic characterisation of the Baltic Sea cyanobacteria

4.1.1. Comparison of growth of toxic and non-toxic Nodularia strains

Growth of two hepatotoxic *Nodularia* strains (BY1, HEM) and one non-toxic strain (HKVV) in response to changes in temperature (test range 10-30ºC), light intensity (25-80 µmol m^{-2} s^{-1}), salinity (3-11‰), and PO_4^{3-} concentration (0.3 –1.0 mg l^{-1}) was studied in batch-cultures. All strains were isolated from the Baltic Sea (Table 7). The non-toxic strains were shown to belong to different genotype than toxic strains (see IV).

The non-toxic HKVV strain grew poorer than the toxic strains under all conditions except at the lowest temperature (I: Fig. 2a) and P concentration tested (I: Fig. 5a). Dry weights and chlorophyll-*a* contents of the non-toxic strain and toxic strains differed significantly at different temperatures ($p < 0.05$) and at P concentrations ($p < 0.02$). Similar results have been shown for neurotoxic and non-toxic strains of *Anabaena* (Rapala *et al.*, 1993).

Temperature had a statistically significant effect on dry weight and chlorophyll-*a* ($p < 0.05$). The growth of all strains was highest at 20ºC and lowest at 10ºC (I: Fig. 2a). The optimal growth temperature has been estimated to be higher than 24ºC for *Microcystis* (Gentile & Maloney, 1964; Gorham, 1964; Krüger & Eloff 1981; van der Westhuizen & Eloff, 1985; Watanabe & Oishi, 1985; Ohtake *et al.*, 1989), *Synecococcus* (Krüger & Eloff, 1981), *Oscillatoria* (Sivonen, 1990b) and *Cylindrospermopsis* (Saker *et al.*, 1999). The growth of *Anabaena* and *Aphanizomenon* was optimal at temperatures higher than 20ºC (Peary & Gorham, 1966; Rapala *et al.*, 1993, 1997; Rapala & Sivonen, 1998).

In this study, differences in light intensity had no statistically significant effect on growth or toxin concentration of *Nodularia*, probably due to the narrow test range and the effect of time. All strains grew slightly better at high light intensities than at low light intensities until day 15 (I: Fig. 3a). After that, the responses of strains to light intensities differed. The non-toxic HKVV strain grew equally well at 50 and 80 µmol $m^{-2}s^{-1}$. Growth of the toxic BY1 strain was highest at the lowest (25 µmol $m^{-2}s^{-1}$) and highest studied (80 µmol $m^{-2}s^{-1}$) light level, whereas the toxic HEM strain grew best at the highest light level. Light intensity increasing up to approximately 100 µmol $m^{-2}s^{-1}$ has been shown to increase the growth of *Anabaena* (Rapala *et al.*, 1993, 1997; Rapala & Sivonen, 1998), *Aphanizomenon* (Rapala *et al.*, 1993), *Microcystis* (Gorham, 1964; Watanabe & Oishi, 1985; van der Westhuizen & Eloff, 1985), and *Nodularia* (Holswilder, 1999). The growth of *Oscillatoria* was highest at the light intensity of 50 µmol $m^{-2}s^{-1}$ (Sivonen, 1990b).

In this study, the growth of the strain BY1 was highest at a salinity of 5‰. Differences in the growth of the toxic strain HEM and the non-toxic strain HKVV at different salinities were rather small. The lowest salinity seemed to reduce the growth of all strains (I: Fig. 3a). Similarly, Blackburn *et al.* (1996) found the lowest cell yields of *Nodularia* strains from three Australian populations at 0‰ salinity and highest at 12‰.

In this study, P had a significant effect on dry weight ($p < 0.05$). Growth was highest at the P concentration of 0.6 mg PO_4^{3-} l^{-1} (I: Fig. 5). Lower concentrations limited the growth and toxin concentration, which was normalised to biomass, of all strains, whereas higher concentrations had no additional effect. In the same way, P limitation has been demonstrated to reduce both growth and hepatotoxin concentration of *Anabaena* (Rapala *et al.*, 1997) and *Oscillatoria* (Sivonen, 1990b). No difference in neurotoxin concentration of *Anabaena* induced by P concentration was detected, although the two lowest concentrations limited growth (Rapala *et al*., 1993)

4.1.2. Physiological differences between Nodularia and Aphanizomenon

Differences in growth and nitrogen fixation rates of *Nodularia* and *Aphanizomenon* were studied in changing growth conditions. *Aphanizomenon* preferred lower irradiances (test range 2-155 µmol $m^{-2}s^{-1}$), salinities (0-30‰), and temperatures (7-28ºC) than *Nodularia* (II: Figs 2-4). The different responses *of Nodularia* and *Aphanizomenon* to different salinity, irradiance, and temperature may explain the different spatial and temporal distribution of these species in the Baltic Sea. In the Baltic Sea, the effects of temperature and light are seen in the seasonal and vertical abundance of *N. spumigena* and *Aphanizomenon* sp. The mass occurrence *of N. spumigena* occurs in the summer months (Kononen & Leppänen, 1997) when the surface water temperature reaches about 17°C (Hübel & Hübel, 1980). *Aphanizomenon* sp. is abundant in the water mass from March to September (Kononen & Leppänen, 1997), thus showing the ability to grow at low temperatures, which was also shown in this study (II: Fig. 2). In this study, *Nodularia* showed the capacity to tolerate much higher temperatures than it experiences in the Baltic Sea (Fig. 2). Field observations in the Baltic Sea have revealed that *N. spumigena* and *Aphanizomenon* sp. have different temperature optima for growth (Kononen, 1992; Kononen *et al*., 1996; Pliñski & Józwiak, 1996). The preference of *Aphanizomenon* for low light and that of *Nodularia* for high light (II: Fig. 3) mirrored their vertical distribution patterns in the field. *Aphanizomenon* is more homogeneously distributed than *Nodularia*, which usually occurs only in the upper mixed layer and forms scum on the water surface (Lindahl *et al*., 1980; Rinne *et al*., 1981; Niemistö *et al*., 1989; Kononen *et al*., 1998).

The growth and nitrogen fixation rates of *Nodularia* strain BY1 were highest in the same salinity range, 5 to 20‰, (II: Fig. 4) in which the genus forms mass occurrences in the Baltic Sea and other brackish waters (see references in Kononen *et al*., 1996). The incapability of *Aphanizomenon* to tolerate salinities higher than 10‰ (II: Fig. 4) suggests that salinity is an important factor limiting the distribution of this genus. The different salinity optimum of the two genera is seen in their different horizontal distribution patterns in the Baltic Sea. With increasing salinity from freshwater in the north to approximately 15‰ salinity in the southern Baltic Proper, the abundance of *Aphanizomenon* sp. decreases while the abundance of *N. spumigena* increases (Niemistö *et al*., 1989). Furthermore, *Nodularia* generally occurs in the open sea area, while *Aphanizomenon* occurs abundantly also in coastal areas of the Baltic Sea (Niemistö *et al*., 1989; Tenson, 1995). In the northern part of the Gulf of Bothnia, the Bothnian Bay, where salinity approaches freshwater, *N. spumigena* is low in numbers (Andersson *et al*., 1996).

The growth of both species increased with unnaturally high PO_4^{3-} concentration (II: Fig. 5) and with accompanying bacteria (II: Fig. 1), and decreased with unnaturally high inorganic N concentrations (Fig. 3). Prior to the P experiment, the inocula were grown without P for seven days to deplete cellular P reserves. The slow growth of *Nodularia* during the P experiment (II: Fig. 5b) was probably due to the low survival of the P-starved inoculum. Phosphorus starvation did not affect the inoculum of *Aphanizomenon*, which may be a result of its higher cellular P pools or lower P demands compared to *Nodularia*. The latter is not in accordance with the estimated half saturation P uptake constants (K_s) for *Aphanizomenon* and *Nodularia*; K_s was higher for *Aphanizomenon* (Uehlinger, 1981: 1.5 µl P l^{-1}) than for *Nodularia* (Wallström *et al*., 1992: < 0.5 µl P l^{-1}). This means that higher P concentration is needed for *Aphanizomenon* than for *Nodularia* to produce half the maximum growth. The results might not be comparable, since the Ks values were estimated separately for a continuous culture of *Aphanizomenon* and for a natural population of *Nodularia*. It is possible that the latter material was not P-starved. The effective utilisation of high P concentration by *Aphanizomenon* seems to be its strategy to form blooms in frontal and upwelling regions in the Baltic Sea (Wallström, 1988; Kononen & Nõmmann, 1992; Grönlund *et al*., 1996; Kononen *et al*., 1996). The stimulation of growth of cultured strains of *Aphanizomenon* (II; Melin & Lindahl 1973) and *Nodularia* (I; II; Hamel & Huber, 1985; Huber & Hamel 1985) by high P concentration has been demonstrated. Growth was also stimulated in P enrichment studies with natural cyanobacterial populations (Horstman, 1975; Rinne & Tarkiainen, 1978; Hamel & Huber, 1985; Tamminen *et al*., 1985). High P concentration and low N:P-ratio have been linked to mass occurrences of *Aphanizomenon* and *Nodularia* in the Baltic Sea (Niemi, 1979; Hübel & Hübel, 1980; Kononen & Niemi, 1984; Leppänen

et al., 1988; Wallström, 1988; Kononen & Nômmann, 1992; Kahru *et al.*, 1994; Pliñski & Jóźwiak, 1996).

The positive impact of bacteria on the growth and nitrogen fixation rate of cyanobacteria has been reported by Love & Rawson (1986). In our study and the studies of Meffert & Overbeck (1981) and Meffert (1993), only the growth was promoted by accompanying bacteria (II: Fig. 1). In addition, non-axenic strains (see references in Paerl & Pinckney, 1996) have been noticed to be easier to maintain in cultures than axenic strains. In the presence of heterotrophic bacteria, cyanobacteria may, for example, overcome high oxygen concentrations inhibiting nitrogenase or low inorganic carbon concentrations limiting photosynthesis (Paerl, 1982; Paerl & Pinckney, 1996). Several studies have revealed that planktic *Nodularia* are frequently colonised with epiphytic bacteria (Bursa, 1968; Hoppe, 1981; Šmarda, 1985; Šmarda *et al.*, 1986; Gumpert *et al.*, 1987; Šmarda & Hübel, 1994; Albertano *et al.*, 1996). Similarly, bacterial epiphytes are associated with the Baltic Sea *Aphanizomenon* (Janson *et al.*, 1994).

The presence of NH_4^+ at concentrations of 0.5-1.0 g l^{-1} may cause poor growth (Nordin & Stein, 1980). Even low NH_4^+ concentrations (< 200 µg l^{-1}) resulted in disappearance of heterocysts (Sanz-Alférez & del Campo, 1994) and nitrogen fixation of *Nodularia* (Huber, 1986b; Sanz-Alférez & del Campo, 1994). Likewise in this study, a decrease in growth of *Nodularia*, which had been cultivated without inorganic N for years, was seen when NH_4^+ was present. The N concentration of 42 mg l^{-1} used in this experiment inhibited nitrogen fixation (Moisander *et al.*, 1996) and was detrimental to the growth of *Aphanizomenon* and *Nodularia* (Fig. 3). Similarly, addition of NO_3^- or NH_4^+ to field populations of *Nodularia* had no clear effects on nitrogenase activity or growth (Stal *et al.*, 1999) unless the added concentrations were high (< 5 mM) enough to be toxic (Stal *et al.*, 1999: unpublished data).

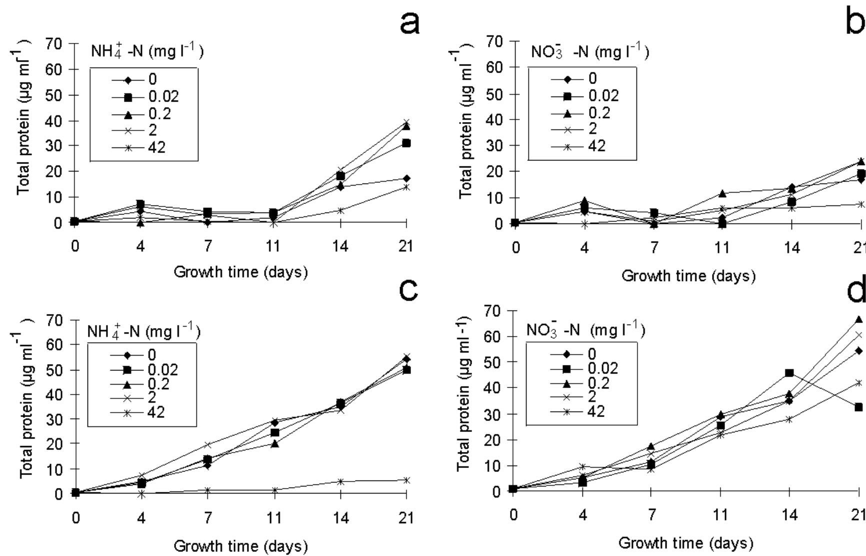

Fig. 3. Effect of ammonium and nitrate on the growth of *Nodularia* BY1 strain (a, b) and *Aphanizomenon* TR183 strain (c, d), respectively, grown under N_2 prior to the experiment.

Nitrogen fixation of *Aphanizomenon* and *Nodularia* was often, but not always, highest under conditions which promoted growth and lowest in cultures with poor growth. Growth and nitrogen fixation rates of *Aphanizomenon* were different than those of *Nodularia* in experiments comparing different temperatures (II: Fig. 2a,c), light intensities (II: Fig. 3a,c), and P concentrations (II: Fig. 5a,c). Both genera showed different responses in the experiment done with axenic and non-axenic cultures (II: Fig 1c, d). Although the heterocyst frequency of *Aphanizomenon* was lower (ca. 10 mm^{-1}) than that of *Nodularia* (30 to 40 mm^{-1}) (II: data not shown) the normalised nitrogen fixation rates [in millimoles of ethylene (gram of chlorophyll-*a*)$^{-1}$hour^{-1}] were generally higher in *Aphanizomenon* than in *Nodularia*. These observations indicate differences in nitrogen fixation potential in heterocysts of these two genera or distribution of nitrogenase in vegetative cells in *Aphanizomenon*. Janson (1995) detected low amounts of nitrogenase in the vegetative cells of *Aphanizomenon*. The higher heterocyst frequency of *Nodularia* compared to *Aphanizomenon* has been also recorded in field populations (Lindahl *et al*., 1980; Niemistö *et al*., 1989). In the present study (II), the frequency of heterocysts and normalised nitrogen fixation rate of *Aphanizomenon* were positively related to the presence of bacteria ($p < 0.05$), temperature ($p < 0.01$), P ($p < 0.05$), and NO_3^- ($p < 0.001$) treatments (not determined for light experiment). Positive correlation between the frequency of heterocysts and

normalised nitrogen fixation rate of *Nodularia* was found in salinity ($p < 0.05$)and NH_4^+ ($p < 0.01$) treatments (not determined for light and P experiments). The heterocyst frequency and the filament length of *Aphanizomenon* positively correlated with temperature ($p < 0.01$), salinity ($p < 0.01$), ammonium ($p < 0.001$), and nitrate ($p < 0.01$) experiments (II: not determined for the light experiment). A positive relationship between filament length and heterocyst frequency of *Nodularia* was found only in the salinity experiment ($p < 0.05$) (not determined for light and P experiments).

4.2. Molecular characterisation of the Baltic Sea cyanobacteria

4.2.1. Phylogenetic position of Aphanizomenon

To date, difficulties to cultivate *Aphanizomenon* strains from the Baltic Sea under laboratory conditions have limited investigations of this organism. We have succeeded in isolating one *Aphanizomenon* strain (TR183) from the Baltic Sea, which we have used in the experiment where the growth and nitrogen fixation of A*phanizomenon* and *Nodularia* in response to changes in growth conditions were investigated (II). The analysis of 16S rRNA-gene sequences has revealed that the Baltic Sea *Aphanizomenon* strain is phylogenetically highly similar to *Aphanizomenon* strains PCC7905 and 202, which have been isolated from Lake Brielse Meer (the Netherlands) and Lake Vesijärvi (Finland) respectively (Lyra *et al*., 2000). On the contrary, based on the ultrastructural study of *Aphanizomenon* sp. trichomes from the Baltic Sea, Janson *et al.* (1994) demonstrated that the Baltic Sea *Aphanizomenon* sp. differs in many respects from freshwater *Aphanizomenon flos-aquae*. In addition, Cronberg and Hajdu (1998) have stated that the Baltic Sea *Aphanizomenon* differs in many ways from freshwater *A. flos-aquae* and that it should be designated *Aphanizomenon baltica*.

4.2.2. Phylogenetic position of Nodularia

Sequence analysis of 16S rRNA gene (IV: Fig. 4) revealed that *Nodularia* is closely related to *Nostoc*, *Aphanizomenon* and *Anabaena*. This result is in line with findings based on 16S rDNA sequence analysis demonstrated that *Nodularia* is situated in the same cluster as other filamentous heterocystous species, but on a separate branch (e.g. Nelissen *et al*., 1994, 1996; Wilmotte, 1994; Wilmotte *et al*., 1993, 1994; Kane *et al*., 1997).

4.2.3. Genetic relationships between Nodularia strains

This study revealed similar genotypic diversity for *Nodularia* strains with the 16S rRNA-gene-based and fingerprinting techniques. Vinuesa *et al.* (1998) showed these techniques to be consistent when characterising *Bradyrhizobium* strains. Using these two techniques, the *Nodularia* strains were separated to two (IV: Figs 4-6) or three clusters (IV: Fig. 3). With 16S rRNA RFLP (IV: Fig. 3), toxic strains from the Baltic Sea were separated from toxic strains from different geographical origins.

All the toxic strains were separated from non-toxic strains using RFLP of the 16S rRNA gene (IV: Fig. 3), 16S rRNA gene sequencing, (IV: Fig. 4), REP- and ERIC-PCR (IV: Fig. 5), and ribotyping (IV: Fig. 6).

All *Nodularia* strains were closely related despite their different geographical origins and abilities to produce toxin. The 16S rDNA sequence difference between the toxic and non-toxic *Nodularia* clusters was low. For example, between strain PCC7804 and the non-toxic strains the difference was 1.3%. Furthermore, toxic strains from the Baltic Sea (BY1) and France (PCC7804) were 99.0% similar in 16S rRNA sequence. Similar findings have been reported for *Anabaena* (Lyra *et al.*, 2000), *Microcystis* (Neilan *et al.*, 1997a; Lyra *et al*, 2000), *Planktothrix* (Lyra *et al.*, 2000), and *Prochlorococcus* strains (Urbach *et al*, 1998). The study by Bolch *et al.* (1999) showed geographically diverse strains of *Nodularia* to be close relatives based on *cpcBA*-IGS sequences.

Whether the *Nodularia* strains in this study belong to one genospecies needs to be determined by DNA-DNA hybridisation studies since similarity of 16S rRNA sequences does not guarantee species identity (Fox *et al.*, 1992). DNA-DNA homology studies have been used to measure the degree of relatedness between organisms with high 16S rRNA sequence similarity. According to Stackebrandt & Goebel (1994), lower than 70% DNA homology is expected for species having lower than 97% sequence similarity. In this study, *Nodularia* PCC73104/1 and PCC7804 strains had 98.7% sequence similarity. According to the study of Lachance (1981), these strains had a 65% relative binding value. Therefore, these strains can be the same genospecies because the DNA relatedness of 65% is not much below the low boundary level suggested by the Ad Hoc Committee on Reconciliation of Approaches to Bacterial Systematics (Wayne *et al.*, 1987).

The profiles of REP and ERIC sequences (IV: Fig. 5), which have earlier been shown to exist in cyanobacterial genomes (Versalovic *et al.*, 1991; Rasmussen & Svenning, 1998), indicated high genetic homogeneity among toxic strains of *Nodularia* from the Baltic Sea. The toxic strains NSPI-05, NSOR-12 from Australia and PPC7804 from France were found to be different from the Baltic Sea *Nodularia* strains by 16 rRNA-based methods (IV: Figs 3,4) and by REP- and ERIC-PCR (IV: Fig. 5). Similarly, Bolch *et al.* (1996, 1999) have shown Australian strains to be different than Northern Hemisphere strains using PCR-RFLP and sequences of *cpcBA*-IGS, and PCR-RADP.

Our results indicated that two closely related *Nodularia* genotypes are found in the Baltic Sea. One genotype consists of only non-toxic strains (UP16a, UP16f, and HKVV). 16S rRNA gene sequencing (Fig. 4) showed that these strains were identical to the proposed type strain of *Nodularia spumigena* PCC73104/1 (http://www.pasteur.fr/recherche/). All genetic markers separated the proposed type strain, and other non-toxic strains,

from toxic strains. The toxic strains (HEM, BY1, AV3, GR8b, F81, NSPI-05,

NSOR-12, and PCC7804) form another genotype, which most closely fits the descriptions of *N. baltica* and *N. spumigena* (IV: Tables 2, 3). According to the nomenclature of Komárek *et al.* (1993), the proposed type strain PCC73104/1 is not typical for the strain of *N. spumigena*. The phenotypic characters of non-toxic *Nodularia* strains fit most closely to the description of *N. sphaerocarpa* (IV: Tables 2; 3) without recorded data of akinetes. When they were present in the HKVV strain, they occurred in series and were more or less spherical.

Previously, the genetic diversity of *Nodularia* populations in the Baltic Sea has been demonstrated to be low by using specific PCR identification. Two to three distinct *Nodularia* genotypes were detected based on PC-IGS sequences (Hayes & Barker, 1996; Barker *et al.*, 1999). In addition, two groups based on *gvgA*-IGS and three groups based on rDNA-ITS were assigned (Barker *et al.*, 1999).

4.2.4. Phenotypic differences between Nodularia genotypes

Nodularia strains could not be separated based on cell size whereas gas vesicle production separated the strains into two groups (IV: Table 2). All the toxic *Nodularia* strains examined produced gas vesicles, whereas the non-toxic strains were unable to produce these structures. The ability to form gas vesicles, which regulate the buoyancy of planktonic cyanobacteria, has been used to differentiate *Nodularia* species (Šmarda *et al.*, 1988; Komarek *et al.*, 1993). In the laboratory, cyanobacterial strains may lose gas vesicles as shown also in this study with strains BY1 and HEM (IV: Table 2). Therefore, this character may not be useful when identifying cultured strains. The cell size of cultured *Nodularia* strains was not reflected in the 16S rRNA gene similarity. Morphological characterisation of unicellular *Merismopedia* and *Microcystis* strains has also been unsuccessful in distinguishing genetic subclusters created by 16S rDNA sequencing (Palinska *et al.*, 1996; Otsuka *et al.*, 1998). On the contrary, the trichome morphology of *Cylindrospermopsis* strains correlated with sequences of 16S rRNA gene (Saker *et al.*, 1999) and STRR (Wilson *et al.*, 2000).

In this study, nodularin production was consistent with the genotypic analysis. With all genotypic methods used, the non-toxic *Nodularia* strains were differentiated from the toxic ones (IV: Figs 2-6). Bolch *et al.* (1999) found a genotypic distinction between most toxic and non-toxic *Nodularia* strains using RAPD-PCR and *cpcBA*-IGS sequence data. Thus, nodularin production could be used as a marker in the taxonomy of *Nodularia* as proposed by Komárek *et al.* (1993). In contrast to our study, no correlation between 16S rRNA gene evolution and *Microcystis* strain toxin production was observed (Neilan *et al.,* 1997a; Otsuka *et al.*, 1999) whereas hepatotoxic *Anabaena*

strains were phylogenetically different from neurotoxin-producing strains using 16S rDNA sequence (Lyra *et al.*, 2000). Earlier, hepatotoxic *Anabaena* strains had been distinguished from neurotoxic strains by Southern hybridisation of STRR sequences (Rouhiainen *et al.*, 1995). This method was also applied to *Nodularia* in the present study (IV: Fig. 2). Although most of the *Nodularia* strains had only a few locations with STRR sequences, this method gave the same information as the other fingerprinting methods. These STRR sequences have been detected also in the genomes of other filamentous, heterocystous genera such as *Calothrix* (Mazel *et al.*, 1990), *Cylindrospermopsis* (Wilson *et al.*, 2000), *Anabaena* and *Nostoc* (Rouhiainen *et al.*, 1995).

Since toxic *Nodularia* strains could be differentiated from non-toxic strains by using 16S rRNA gene sequencing, one could design PCR primers or probes to detect potential nodularin producers. However, the number of studied non-toxic strains in this study was low, therefore, more non-toxic strains are needed to test the validity of any difference in 16S rRNA gene sequence as a genetic marker between toxic and non-toxic *Nodularia* strains. Previously, Bolch *et al.* (1999) could not separate two non-toxic *Nodularia* strains (NSBL-03, and NSBL-05) from toxin-producing strains using *cpcBA*-IGS sequences. It is also possible that nodularin levels produced by these strains were under the detection limits. The characterisation of microcystin synthetase genes from hepatotoxic cyanobacteria will enable the design of specific PCR methods for the detection of potential hepatotoxin producers.

16S rRNA sequence analysis has shown that *Prochlorococcus* is phylogenetically related to cyanobacteria (Urbach *et al.*, 1992), and is closest to *Synechococcus* (Urbach *et al.*, 1998). The findings that pigment data for *Prochlorococcus* strains correlated with 16S rRNA sequence data (Moore *et al.*, 1988; Urbach *et al.*, 1988) and that pigment data for *Synechococcus* correlated with ITS sequence data (Postius & Ernst, 1999) showed that phylogenetically closely related strains can be physiologically distinct. *Nodularia* strains of the present study were shown to be phylogenetically highly similar but physiologically quite distinct. For example, some of the strains were able to produce nodularin whereas others were not toxic. Furthermore, their response to changes in temperature and P concentrations were different.

4.3. Characterisation of nodularin

4.3.1. Nodularin concentration under different growth conditions

Nodularin concentration under different growth conditions was studied using non-axenic (I) and axenic (II) *Nodularia* strains. Nodularin concentrations in cells and in growth media were generally highest under conditions which promoted growth (I, II). In all experiments (not examined in N experiment) growth and intracellular

toxin correlated positively (II: $p < 0.005$). The association of *Nodularia* and nodularin has been demonstrated earlier by field data (Sivonen *et al*., 1989b; Jones *et al*., 1994b; Blackburn & Jones, 1995; Heresztyn & Nicholson, 1997). Furthermore, the concentrations of microcystins in phytoplankton have been positively correlated with the abundance and biomass of *Microcystis* (Kotak *et al*., 1995; Lahti *et al*., 1997; Zurawell *et al*., 1999).

Marked differences between nodularin concentrations in cells grown under different temperatures (I), salinities (II) and P concentrations (II) were observed. The differing responses of the studied strains probably resulted from different test ranges and the presence of accompanying bacteria. In the study done with non-axenic strains (I), the test ranges were perhaps too narrow to reveal differences in different treatments. However, the results revealed that toxic *Nodularia* blooms might be expected in late summer with high water temperatures in areas of the Baltic Sea with high P concentrations and moderate salinity.

Intracellular Nodularin. Intracellular nodularin concentrations in the axenic *Nodularia* strain BY1 increased with temperature (II: Fig. 2e), PO_4^{3-} concentration (II: Fig. 5e), and irradiance (II: Fig. 3e). They decreased with low and high salinities (II: Fig. 4e) and high inorganic N concentrations (Fig. 3). Associated bacteria had no effect on the nodularin concentration in *Nodularia* (II: Fig. 1e), whereas microcystin concentration in two of three axenic *Oscillatoria* strains was significantly higher than that in non-axenic clones (Sivonen, 1990b).

Effect of Temperature. In previous studies, temperature has been shown to control hepatotoxin concentrations in many cyanobacteria, such as *Anabaena* (Rapala *et al*., 1997), *Microcystis* (Gorham, 1964; Runnegar *et al*., 1983; Watanabe & Oishi, 1985; van der Westhuizen & Eloff, 1985; van der Westhuizen *et al*., 1986; Codd & Poon, 1988), and *Oscillatoria* (Sivonen, 1990b). In most of these studies, highest toxin concentrations (Sivonen, 1990b; Rapala *et al*., 1997) or toxicities (Runnegar *et al*., 1983; Watanabe & Oishi, 1985; van der Westhuizen & Eloff, 1985; van der Westhuizen *et al*., 1986; Codd & Poon, 1988; Ohtake *et al*., 1989) were detected from cyanobacterial cells grown at 18-25ºC. This was also true in this study, where the highest toxin concentrations were detected from *Nodularia* cells grown at temperatures at approximately 20ºC (I: Fig. 2b; II: Fig. 2e). A rise in the temperature increased the growth of *Nodularia* (II: positive linear regression coefficient, $p < 0.001$). During the time course, the growth of *Nodularia* decreased at low temperature and increased at high temperature (II: positive regression coefficient for the co-effect, $p < 0.001$).

Effect of Phosphorus. Low PO_4^{3-} concentrations seem to decrease the toxin concentrations in *Nodularia* (I: Fig. 5c; II: Fig. 5d). In contrast, Holswilder (1999)

reported that the highest nodularin concentrations were produced under P-limited growth conditions. The different results obtained are probably a result of different experimental conditions. In our study, *Nodularia* was grown without inorganic N, whereas in the study of Holswilder (1999) the growth media contained NO_3^- (N:P = 64:1, molar ratio) which at high concentrations has been demonstrated to decrease nodularin concentration (see below). However, our result is in line with those from batch-culture experiments with hepatotoxic *Anabaena* (Rapala *et al.*, 1997), *Microcystis* (Watanabe & Oishi, 1985) and *Oscillatoria* (Sivonen, 1990b). A continuous-culture experiment has shown that microcystins are produced in higher amounts in P-limited conditions (Oh *et al.*, 2000). Oh *et al.* (2000) have suggested two reasons for the controversial results between batch and chemostat experiments. First, in batch-cultures complete cellular P-limitation may not be induced. Second, there may be a threshold concentration for P. The latter may be true, since several studies have indicated that above a certain P-concentration there was no additional effect on toxin concentration (e.g. I; II; Watanabe & Oishi, 1985; Sivonen, 1990b; Rapala *et al.,* 1997). In contrast to the study of Oh *et al.* (2000), Utkilen & Gjølme (1995) found that P-limited condition decreased microcystin-RR content per dry weight of *Microcystis* grown in continuous culture.

Effect of Light. Low light seems to lower nodularin concentration as shown by this study and the study of Holswilder (1999). In the present study, nodularin concentration in the axenic *Nodularia* BY1 at the light intensity of 2 µmol m^{-2} s^{-1} was lower than at higher light levels (II: Fig. 3e). In the light experiment done with non-axenic *Nodularia* strains, the test range may be too narrow to show clear differences between light levels (I: Fig. 3b). Light limitation has been reported to decrease the concentration of hepatoxin in *Anabaena* (Rapala *et el.*, 1997; Rapala & Sivonen, 1998), and that of neurotoxin in *Aphanizomenon* (Rapala *et al.*, 1993). In addition, the concentration of microcystin in *Oscillatoria* (Sivonen, 1990b) decreased at high light and that of *Anabaena* at both high and low light levels (Rapala *et al.*, 1993). The results concerning toxicity of *Microcystis* in different light levels are controversial. According to the studies of Gorham (1964), van der Westhuizen & Eloff (1985), van der Westhuizen *et al.* (1986), and Codd & Poon (1988) light had no marked effect on hepatotoxicity whereas other studies noticed a considerable change in toxicity (Watanabe & Oishi, 1985) or toxin concentration (Utkilen & Gjølme, 1992) when light intensity was varied. Light controls microcystins content, but it also seems to regulate the composition of microcystin variants (e.g. Rapala *et el.*, 1997; Rapala & Sivonen, 1998). In addition, iron uptake, which has been shown to influence the toxin concentration of *Microcystis* (Lukač & Aegerter 1993; Utkilen & Gjølme, 1995;

Lyck *et al.*, 1996), is light dependent (Utkilen & Gjølme, 1995).

Effect of Salinity. The axenic *Nodularia* strain BY1 produced highest nodularin concentrations at salinities 5-20‰ (six levels tested in the range 0-30‰), and nodularin concentration was reduced at lower and higher salinities than that (II: Fig. 4e). Salinity did not have any statistically significant effect on intracellular toxin concentrations in non-axenic *Nodularia* cultures (I). High salinity (35‰) was previously found to reduce nodularin concentrations (Blackburn *et al.*, 1996) and increase the number of akinetes (Jones *et al.*, 1994b). At salinities 0 - 24 ‰, nodularin was produced in similar amounts although the lowest salinity significantly reduced growth (Blackburn *et al.*, 1996). In this study, growth and nodularin concentration were remarkably lower at salinities 0 and 30‰ than at 5-20‰.

Effect of Nitrogen. The negative effect of N on the nodularin concentration in *Nodularia* was seen only at the highest N concentration, 42 mg l^{-1} (Fig. 3). Previously, N has been shown to increase hepatotoxin concentration of non-heterocystous *Microcystis* (Codd & Poon, 1988; Utkilen & Gjølme, 1995; Watanabe & Oishi, 1985) and *Oscillatoria* (Sivonen, 1990b). Orr and Jones (1998) demonstrated that microcystin production rate by *Oscillatoria agardhii* (data originally published by Sivonen, 1990b), *Anabaena flos-aquae* (data originally published by Rapala *et al.*, 1997) and by *Microcystis aeruginosa* (Orr & Jones, 1998) was indirectly influenced by nutrients and other growth-limiting factors. These factors have an effect on growth and cell division and in that way on microcystin production rate (Orr & Jones, 1998). In *Anabaena*, addition of N (test range 0-50 mg NO_3^--N l^{-1}) changed the proportion of different microcystin variants, decreased their total intracellular concentration, and increased the concentrations of extracellular toxins in growth media (Rapala *et al.*, 1997). *Cylindrospermopsis raciborskii* produced highest concentration of cylindrospermopsin when grown with no N addition (Saker *et al.*, 1999).

Extracellular Nodularin. Nodularin concentrations in growth media increased with incubation time (I: Fig. 1c, Table 2). Even when the extracellular toxin was normalised to the biomass present, the concentration of toxin in the growth medium increased (II: Figs 1f, 2f, 3f, 4f) suggesting that the toxin was released mostly from dying cells. Similarly, microcystins have been shown to be released into the growth medium in several other studies (e.g. Berg *et al.*, 1987; Sivonen, 1990b; Kiviranta *et al.*, 1991; Watanabe *et al.*, 1992). According to our studies, growth at different temperature, light, salinity, and P conditions as well as growth stage may effect the release of nodularin. Higher toxin concentrations in growth media, which were normalised to biomass, were detected at high temperatures than at low temperatures (I: Fig. 2b; II: Fig. 2f). Salinity had a significant effect on extracellular

nodularin concentration (II: $p < 0.10$). The highest extracellular concentrations were found at salinities of 5 to 15‰ (II: Fig. 4f); these were also favourable for growth. Toxin release from non-axenic cells into the growth media was highest at the lowest PO_4^{3-} concentration (I: Fig. 5c). On the contrary, at the end of experiment, the highest concentrations released from axenic cells were found at the highest P concentrations (II: Fig. 5e) which also promoted growth. It seems that extracellular toxin concentrations are proportional to intracellular concentrations, therefore higher concentrations are found in fast-growing cultures than slow-growing cultures. Accordingly, Rapala *et al.* (1997) found that with time, high N concentration and increasing light levels significantly increased the concentrations of extracellular hepatotoxins. In this study, the concentration of nodularin (I: Fig. 1c) in cells as well as total nodularin concentration in culture (I: Table 1) increased with time. No clear differences between axenic and non-axenic cultures in intra- and extracellular toxin concentrations were observed (II: Fig. 1e,f). Furthermore, the fact that large amounts of nodularin were present in growth media of non-axenic cultures at the end of incubation (I: Fig. 1c, Table 1) implies that nodularin was not biodegraded by associated bacteria in contrast to the studies of Jones *et al.* (1994a), Twist & Codd (1997), Heresztyn & Nicholson (1997), and Lahti *et al.* (1998).

4.3.2. Three-dimensional structure of nodularin

Nodularins and microcystins, that are chemically quite similar, exihibit similar inhibitory actions against protein phosphatases. However, they show different biochemical reactions with different protein phosphatases, e.g. nodularins do not bind covalently to PP-1 like microcystins. In order to explain the biochemical differences between these two toxins, the 3-D structure of nodularin was determined by NMR and MD simulations to find similarities and differences between the chemical structures of nodularins and microcystins.

NMR-structure. In water, the peptide ring of nodularin has a saddle-shaped form with flexible Adda and Arg side-chains. This has emerged from distance geometry calculations using distance and dihedral restraints.

A detailed inspection of the first structure determinations revealed two families of solution structures for nodularin. These differed by the orientation of the Mdhb side-chain depending on the stereospecificity of the methylenes of Glu (III: data not shown). Altogether, there were initially four structural families. It was concluded that the methyls of Mdhb and MeAsp were on opposite sides with respect to the plane of the Glu-Mdhb peptide bond. Otherwise there should have been cross-peaks for the protons in the side-chain of Mdhb and $C_\gamma H_3$ of MeAsp. The stereospecificity was then deduced relying on the unequal NOES

between the CH_3- groups of Mdhb and C_γHs of Glu (III: Fig. 3). This family of conformations also had fewer restraint violations than the three other conformation sets.

A new set of 100 structures was computed in which ϕ of the Mdhb was restrained between 0 and 180º and the center of methyls of Mdhb and MeAsp were forced to be further apart than 4 Å. Out of 100 structures, 25 structures passed the acceptance test, which consisted of two criteria: at most three distance constrains below 0.3 Å, and no dihedral constraints violations above 10º. For this family, the refined distances were computed by the iterative relaxation matrix method. Based on the refined restraint set, 100 structures were computed. Forty-seven out of 100 structures were free of restraint violations. Minor violations (below 0.2 Å) were tolerated in the remote part of Adda and Arg.

Using both distance geometry and iterative relaxation matrix analysis, one family of the cyclic-saddle shaped backbone solution structure for nodularin was defined (III: Fig. 5). The long side-chains of Adda and Arg protruded from the otherwise fairly globular backbone structure.

MD-simulation-structure. The simulated nodularin conformation remained close to NMR-generated initial conformation of nodularin (III: Fig. 6). No significant violations to the experimental distance restraints were observed. The remote parts of Adda and the side-chains of Mdhb were found to be quite flexible as suggested also by NMR results. Generally, the structure of the cyclic backbone of nodularin was quite rigid, but some fluctuations were found in the peptide bonds between Mdhb-MeAsp, Arg-Adda, and Adda-Glu, which caused local flip-flop movements of the peptide bond plane (III: Fig. 7). This affected the hydrogen bonding, especially from HN of Glu. The side chain of Arg adopted multiple conformations, which did not have any effect on the cyclic backbone fold. The hydrogen-bonding pattern revealed by MD-simulation is in good agreement with the NMR data. During simulations, the rotating COO^- group of MeAsp frequently formed hydrogen bonds. In most cases, the hydrogen bonds from the COO^- group of MeAsp to the HN of Adda prevailed (80% of the time). Occasionally, the COO^- group of MeAsp was bond to the HN of Glu. The third commonly formed hydrogen bond type was detected between HN and C=O of MeAsp, which was present 50% of the simulation time. Differences in the radial distributions of the water around the backbone amides (III: Fig. 8) are in good agreement with the measured amide proton exchange rates (III: Fig. 4). The HN of MeAsp and Adda were found to be buried with a very low water density, which makes the hydrogen exchange with water slower than in the case of more solvent accessible Glu and Arg protons.

Comparison to microcystin. The conformation of nodularin was remarkably similar to the 3-D structure of microcystin-LR, which implies that nodularin will inhibit

PPs in the same way as microcystin-LR. Both toxins had a saddle-shaped backbone conformation, but microcystin-LR was more buckled than nodularin (III: Fig. 9). In particularly, the backbone fold in the conserved region of MeAsp-Arg-Adda-Glu was almost identical for nodularin and microcystin-LR. The MD-simulations, nevertheless, revealed a certain degree of sway for the *trans* peptide bonds. The proximal part of the Adda side-chain was also very similar. The remote parts of Adda and Arg were not structurally defined and they were also mobile in both peptides.

Bagu *et al.* (1995) have also compared the solution structures of microcystin-LR and a nodularin variant, motuporin. They showed that both of these peptides have saddle-shaped backbones. Furthermore, they showed that Adda was highly flexible in both peptides, whereas Val, a counterpart of Arg in nodularin, was less flexible than Arg in microcystin-LR. Also, the conformational study of Trogen *et al.* (1995) confirmed the saddle-shaped form of peptide ring in microcystin-LR. The NMR results differ from molecular dynamics calculations, which predict that these toxins have planar rings (Lanaras *et al.*, 1991; Taylor *et al.*, 1992). Here, a good agreement was found between experimental and computational data.

Although nodularins and microcystins are structurally and physiologically very similar, there is one significant difference between them in respect to their interactions with PPs. According to NMR results of Bagu *et al.* (1997) and ours (III), the side-chain of Mdhb of nodularins points to a different direction than that of Mdha of microcystin-LR. Therefore, Mdhb of bound nodularin should not reach to the Cys-272 of PP1 and consequently not form a covalent bond with the SH group. The different positions of Mdhb and Mdha in nodularins and microcystins, respectively, have no effect on toxicity, since both toxins inhibit PPs in the same way.

Bagu *et al.* (1997) have showed that the free NMR solution structures of microcystin-LR is highly similar to the bound crystal structure of microcystin-LR, indicating that this peptide will bind to PPs with only little conformational changes. Therefore, it can be assumed that the free structure of nodularin would not change dramatically upon binding.

5. General conclusions and future prospects

According to this study*:*

Variation in growth conditions influenced the growth and nodularin production of *Nodularia* strains.

- Nodularin concentrations were generally highest under conditions that promoted growth.
- Intracellular nodularin concentrations increased with increases in temperature, phosphate concentrations and irradiance. They decreased at low and high salinities and unnaturally high nitrogen concentration. Therefore, toxic *Nodularia* blooms may be expected in late summer in areas of the Baltic Sea with high phosphorus concentration and moderate salinity.

The growth rate of non-toxic *Nodularia* strain was lower than that of toxic strains under all growth conditions except the lowest temperature and phosphorus concentration tested. Therefore, high temperature and high phosphorus concentrations may favor toxic *Nodularia* blooms over non-toxic ones.

Several differences in the growth and nitrogen fixation rates of *Nodularia* and *Aphanizomenon* were observed, which may explain the different vertical, horizontal and temporal distribution of the two genera in the Baltic Sea.

- *Aphanizomenon* preferred lower irradiances, salinities, and temperatures than *Nodularia*.

The biomass of *Nodularia* and *Aphanizomenon* increased with high phosphate concentrations and with accompanying bacteria and decreased with unnaturally high nitrogen concentrations.
Nitrogen fixation was often, but not always, highest under conditions that promoted growth and lowest in cultures with poor growth.

The solution conformation of nodularin was remarkably similar to the tree-dimensional structure of microcystin-LR, which implies that nodularin inhibits protein phosphatases in the same way as microcystin-LR.

- Both toxins had a saddle-shaped bacbone conformation, but microcystin-LR was more buckled than nodularin. In particular, the backbone fold in the conserved region of MeAsp-Arg-Adda-Glu was almost identical for nodularin and microcystin-LR. The proximal part of Adda was also very similar. The remote parts of Adda and Arg were not structurally defined and they were also mobile in both peptides.

Nodularia strains could not be grouped on the basis of cell size, whereas toxin production separated the strains into two

groups. Nodularin production was consistent with the genotypic analysis.

- ❖ The toxic *Nodularia* strains were separated from non-toxic strains by RFLP of the 16S rRNA gene, 16S rRNA gene sequencing, REP- and ERIC-PCR, and ribotyping. All strains were closely related despite their different abilities to produce toxin or geographical origins.

In the Baltic Sea, two closely related *Nodularia* genotypes are found. One genotype consists of only non-toxic strains and most closely fits the morphological description of *Nodularia sphaerocarpa*. The toxic Baltic Sea strains form another genotype, which most closely fits the descriptions of *Nodularia baltica* and *Nodularia spumigena*.
The toxic *Nodularia* strains from Australia and France were found to be different from the toxin-producing strains of the Baltic Sea by all genotypic methods. The profiles of REP- and ERIC-sequences indicated high genetic homogeneity among the toxic Baltic Sea strains.

Future prospects

This study suggested that non-toxic *Nodularia* strains may need less phosphorus for growth than toxic strains. Whether this physiological difference is universal needs to be confirmed with several non-toxic and toxic strains.

Furthermore, *Nodularia* and *Aphanizomenon* genera seemed to have different capabilities to tolerate phosphorus starvation. This suggestion remains as yet unconfirmed.

During this study, attempts to isolate *Aphanizomenon* cultures from the Baltic Sea were usually unsuccessful. In the future, we must be able to grow *Aphanizomenon* in the laboratory, which is essential to study the characteristics of this cyanobacterial genus.

In this study, we have shown a clear genotypic distinction between toxic and non-toxic *Nodularia* strains. Whether this separation is valid in natural *Nodularia* populations remains to be tested. It remains also to be investigated whether non-toxic *Nodularia* strains are really benthic lacking gas vesicles, as I suggested. Therefore, the presence of gas vesicles and the natural habitat of non-toxic strains should be clarified.

Peptide synthetase genes have been genetically disrupted in order to produce non-toxic *Microcystis* mutants. These mutants are likely to reveal the role of cyanobacterial toxins to the producing organism and to the ecosystem. Furthermore, identification of peptide synthetase genes would allow the detection of potential toxin-producers in natural cyanobacterial populations. For the reasons mentioned above, peptide synthetase genes should be identified also from other toxin-producing cyanobacterial genera, such as *Nodularia*.

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