Dynamics of freshwater cyanobacteria and
their bioactive oligopeptides

- role of abiotic environmental factors and population structure

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a naturalist's life would be a happy one if he had only to observe and never to write"

(Charles Darwin)

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ABSTRACT

Cyanobacteria produce bioactive compounds of diverse chemical structure, and an important group among these are the non-ribosomal cyanobacterial oligopeptides. The majority of studies on oligopeptides have focused on the class of microcystins, which pose a risk to human health. Little is known about the environmental control of production of oligopeptides other than microcystins, whereas this study considered the abundance of the five most important classes of oligopeptides produced by the same cyanobacterial population; aeruginosins, anabaenopeptins, cyanopeptolins, microcystins and microginins.

The main objective of the present study was to improve the understanding of how toxin-producing cyanobacteria and their main classes of bioactive oligopeptides vary by depth and through the season, and to evaluate the role of abiotic environmental factors and population structure. During a field study in the period 2001-2004, the population of *Planktothrix* sp. in the mesotrophic Lake Steinsfjorden, South-eastern Norway was used as a model to investigate how abiotic environmental factors affect the abundance and occurrence of oligopeptides. The *Planktothrix* sp. population biovolume varied from a minimum of 0.6 cm^3 m^{-2} in August 2002 up to a maximum of 73 cm^3 m^{-2} in November 2003, and the net specific growth rate was estimated to a maximum of 0.07 d^{-1}. The maximum specific growth rate obtained in culture experiments with *Planktothrix* sp. was 0.75 day^{-1}, thus giving clear indications of growth limitations for the population in Lake Steinsfjorden. The metalimnetic population of *Planktothrix* sp. in Lake Steinsfjorden were assumed not to be limited by nutrients; however a lowering of nutrient supplies over time could in addition to the suboptimal light and temperature conditions prevent bloom formation. A total of 46 clonal *Planktothrix* cultures isolated from Lake Steinsfjorden in the period 1965 to 2004 were analysed for their production of oligopeptides, and 33 different oligopeptides were found. Based on the composition of oligopeptides, four different chemotypes where identified. Changes in the relative abundance of chemotypes occurred almost constantly and could not be explained with fluctuations in light, temperature or concentration of macronutrients. The analysis of field samples revealed considerable variations in the abundance of oligopeptides per biomass of *Planktothrix* sp. By applying multivariate regression models, called generalized additive models (GAMs), the effect of temperature, irradiance, macronutrients, depth, and date on this variance could be evaluated. Taken together these factors explained between 50 and 94 % of the oligopeptide variance observed and as factors were ranked according to their contribution to this variance, date was clearly the most important one,

explained by the waxing and waning of the four chemotypes of *Planktothrix* sp. found in Lake Steinsfjorden. There was an overall trend of increase in oligopeptide abundances per *Planktothrix* sp. biomass by depth. Surprisingly, both in field and culture studies of *Planktothrix* sp. the growth resources temperature, irradiance and macronutrients seemed to have only a minor effect on the variance in oligopeptide concentration per biomass. Two strains of *Planktothrix* sp. isolated from Lake Steinsfjorden, was grown in batch cultures with nitrogen limitation and with a gradual light limitation, to examine whether the most abundant oligopeptides were affected in a similar manner by abiotic environmental factors. The production of all the oligopeptides studied was persistent throughout the experiments and significant changes were within a factor of 5, despite that the cells were experiencing large differences in light and nitrogen availability. The various classes of oligopeptides appeared to be influenced by abiotic environmental factors in a similar manner, suggesting that oligopeptides may have a similar function.

LIST OF PAPERS

This thesis is based on the following papers, which will be referred to in the text by their roman numerals (**I-VI**):

Paper I:

Halstvedt, C.B., Rohrlack, T., Andersen, T., Skulberg, O. and Edvardsen, B. (2007) Seasonal dynamics and depth distribution of *Planktothrix* spp. in Lake Steinsfjorden (Norway) related to environmental factors. *J. Plankton Res.*, **29**, 471-482.

Paper II:

Halstvedt, C.B., Rohrlack, T., Ptacnik, R. and Edvardsen, B. (2008) On the effect of abiotic environmental factors on production of bioactive oligopeptides in field populations of *Planktothrix* spp. (Cyanobacteria). *J. Plankton Res.,* **30**, 607-617.

Paper III:

Halstvedt, C.B., Rohrlack, T., Utkilen, H.C. and Edvardsen, B. Effect of abiotic environmental factors on production of all major bioactive oligopeptide classes in *Planktothrix* spp. (Cyanobacteria). (Manuscript in preparation).

Paper IV:

Rohrlack, T., Edvardsen, B., Skulberg, R., Halstvedt, C.B., Utkilen, H.C., Ptacnik, R. and Skulberg, O.M. (2008) Oligopeptide chemotypes of the toxic freshwater cyanobacterium *Planktothrix* can form subpopulations with dissimilar ecological traits. *Limnol. Oceanogr.,* **53 (4)**, 1279-1293.

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INTRODUCTION

Freshwater cyanobacterial blooms have become a worldwide phenomenon, and are associated with a number of problems, especially when occurring in waters used as drinking water resources. Cyanobacteria are known to produce toxic substances that have adverse effects on aquatic animals, terrestrial animals and even humans (Chorus and Bartram, 1999). The adverse effects of cyanobacterial toxins were first reported as cattle stock deaths at Lake Alexandrina, South Australia in 1878 (Francis, 1878). Since then, cyanobacterial poisonings have been reported around the world, involving several types of animals. Human illnesses associated with exposure to cyanobacterial toxins have been reported from Australia, Europe, Asia, North America and South America (Codd, 1995; Carmichael, 1997; Chorus and Bartram, 1999). One of the most serious human poisonings caused by cyanobacterial toxins was the death from liver failure of more than 50 patients receiving contaminated dialysis water in Caruaru, Brazil in 1996 (Jochimsen *et al.*, 1998). *Microcystis, Anabaena* and *Planktothrix* are among the most common toxin-producing cyanobacterial genera in the Northern Hemisphere (Sivonen and Jones, 1999), and in a survey of German fresh water bodies blooms dominated by *Planktothrix* had the highest toxin content (Fastner *et al.*, 1999). Most studies regarding the environmental control of *Planktothrix* populations have been done in central-Europe. However, differences in climatic conditions raise the question of whether the growth and distribution of such populations in Norwegian lakes are regulated in the same manner (Fig. 1).

Fig. 1 Cyanobacterial bloom in Lake Årungen, Norway, 2007 (Photo: Sigrid Haande, NIVA)

Cyanobacteria

Cyanobacteria (or blue-green algae) have been present on earth since at least 3500 million years ago and are known to inhabit a number of habitats including terrestrial, marine and freshwater biotopes and even some of the most extreme environments on earth (deserts, hot springs, salt marches, ice, snow). They can be distinguished from all other eubacteria by their ability to carry out oxygenic photosynthesis similar to that of eukaryotic photoautotrophs, because they possess the photosynthetic pigment chlorophyll *a* with both photosystem I (PSI) and photosystem II (PSII). Cyanobacteria are thought to have been the first producers of oxygen on earth and the ancestors of today's eukaryotic phytoplankton, macro algae and terrestrial plants. The light harvesting complexes (LHC) that capture the incident photosynthetic active radiation (PAR) are quite different in eukaryotic photoautotrophs and cyanobacteria. In eukaryotic photoautotrophs, LHC are integral to the thylakoid membrane and comprise accessory chlorophylls (*b* or *c*), whereas the major LHC in cyanobacteria is the phycobilisome (PBS), a hemispherical structure attached to the periphery of the thylakoid membranes. The PBS of cyanobacteria contain accessory pigments of the type phycobiliproteins, including allophycocyanin and phycocyanin, possessed by all cyanobacteria and responsible for the typical blue-green colour, and in some species phycoerythrin, which colours the cells red. These pigments allow cyanobacteria to use light of a much wider range of wavelengths than eukaryotic photoautotrophs (Graham and Wilcox, 2000; Oliver and Ganf, 2000). In general, growth rates of cyanobacteria are low compared to other phytoplankton algae (Reynolds, 1984; Oliver and Ganf, 2000).
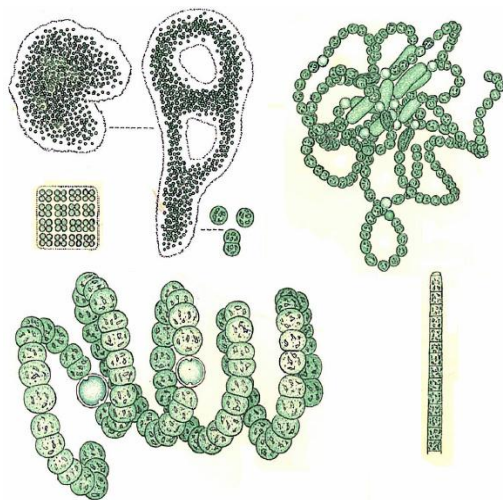
The morphology of cyano-bacteria is diverse, and species are sometimes difficult to identify because of a high phenotypic plasticity (Fig. 2). They may be unicellular or may have cells arranged in colonies. Some cyanobacteria form filaments with none, false or true branches (Graham and Wilcox, 2000).

Fig. 2 Drawings by G. Nygaard showing different morphologies of cyanobacteria.

Some cyanobacteria are able to produce specialized cells to survive unfavourable growth conditions: heterocysts, which contain nitrogenase, an enzyme capable of fixing atmospheric nitrogen (N_2), and akinetes, that function as resting cells (Graham and Wilcox, 2000). The cytoplasm may contain structures for storage of certain resources, including glycogen granules (carbohydrates), lipid globules, cyanophycin granules (nitrogen), and polyphosphate bodies (phosphate) (Whitton and Potts, 2000). Cyanobacteria are the only phytoplankton group that may produce gas vesicles. They are gas-filled, hollow cylinders capped at either end by a cone and surrounded by a proteinacous wall that allows for a free passage of gases, but not water. Gas vesicles are not randomly dispersed throughout the cytoplasm, but are packed into gas vacuoles to occupy minimal space and provide maximum buoyancy (Walsby, 1994). The average cell density is reduced below that of the suspending water, providing up drift. This, combined with a variable amount of polysaccharides as ballast enables a cyanobacterium to regulate its buoyancy and to maintain a vertical position in thermally stratified waters (Reynolds, 1984, 1987).

Cyanobacterial bioactive oligopeptides and chemotypes

Cyanobacteria produce bioactive compounds of diverse chemical structure, and an important group among these are the non-ribosomal cyanobacterial oligopeptides, hereafter called oligopeptides. They are produced by large multi-enzyme complexes according to the thio-template mechanism (Welker and von Döhren, 2006). Over 600 structural variants have been described and most can be assigned to seven classes, the aeruginosins, microginins, anabaenopeptins, cyanopeptolins, microcystins, microviridins and cyclamides. Most of the oligopeptide classes have a cyclic structure, while some are linear (Table 1, Fig. 3), The majority of studies on oligopeptides have focused on the class of microcystins, first isolated from the wide-spread species *Microcystis aeruginosa* (Carmichael, 1997; Vezie *et al.*, 1998; Sivonen and Jones, 1999). The structure of microcystins is cyclic with a typical ADDA-group, involved in the bioactive mechanism of the oligopeptide (Fig. 3). The most widely studied microcystin is microcystin-LR, and the World Health Organization (WHO) has established a guideline value for it (Chorus and Bartram, 1999).

Table 1 Classes of cyanobacterial oligopeptides with structure and bioactivity (Welker and von Döhren, 2006).

Oligopeptide	Structure	Main bioactivity
Aeruginosins	Linear	Protease inhibitors
Anabaenopeptins	Cyclic	Protein phosphatase inhibitors, carboxypeptidase inhibitors
Cyanopeptolins	Cyclic	Protease inhibitors
Cyclamides	Multicyclic	
Microcystins	Cyclic with ADDA-	Protein phosphatase inhibitors
Microginins	Linear	Protease inhibitors, ACE-inhibitors
Microviridins	Cyclic	Protease inhibitors, tyrosinase inhibitors

The biosynthesis of non-ribosomal peptides requires a considerable amount of energy and nutrient resources (Welker and von Döhren, 2006). Once produced, oligopeptides stay largely inside the cyanobacterial cells and are only released in significant amount after cell death (Berg *et al.*, 1987). Isolated cyanobacterial strains held in culture over time continue to produce the same set of oligopeptides (Welker and von Döhren, 2006), and on the basis of this oligopeptide composition or pattern, so-called chemotypes can be defined (Fastner *et al.*, 2001; Welker *et al.*, 2004a,b). A cyanobacterial population may consist of several co-existing chemotypes (Vezie *et al.*, 1998; Kurmayer *et al.*, 2004).

Bioactivity and health risks posed by oligopeptides

The mechanism of most bioactive oligopeptides is inhibition of vital eukaryotic enzymes (Table 1, Honkanen *et al.*, 1994; Namikoshi and Rinehart, 1996; Ishida *et al.*, 1999). A number of compounds in cyanobacteria are inhibitors of proteases, e.g. cyanopeptolins are effective trypsin and chymotrypsin inhibitors and are responsible for the low digestibility of *Microcystis* by crustacean grazers (Namikoshi and Rinehart, 1996). Protease inhibition has been reported for aeruginosins and microviridins (Shin *et al.*, 1996; Shin *et al.*, 1997; Ishida *et al.*, 1999). Oscillapeptin J isolated from *Planktothrix rubescens* from Lake Zürich was shown to be a highly potent crustacean grazer toxin (Blom *et al.*, 2003). Rohrlack and co-workers (2004) showed that inhibition of the digestive enzyme trypsin by cyanobacterial metabolites can result in the death of *Daphnia*, and also inhibition of moulting of *Daphnia* and thus might reduce the grazing pressure of a population efficiently without directly killing the grazers.

Some oligopeptides, mainly the microcystins, pose a risk to human health and have therefore attracted attention over decades (Chorus and Bartram, 1999). In mammals, microcystin cause inhibition of protein phosphatases 1 and 2a of hepatocytes, leading to liver damage (Honkanen *et al.*, 1994; Dawson, 1998). Other cyanobacterial peptides have

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been reported to inhibit protein phosphatases too, but respective IC50-values were at least an order of magnitude higher compared to those of microcystins (Welker and von Döhren, 2006).

Aeruginosin

Microginin

Anabaenopeptin

Cyanopeptolin

Microcystin
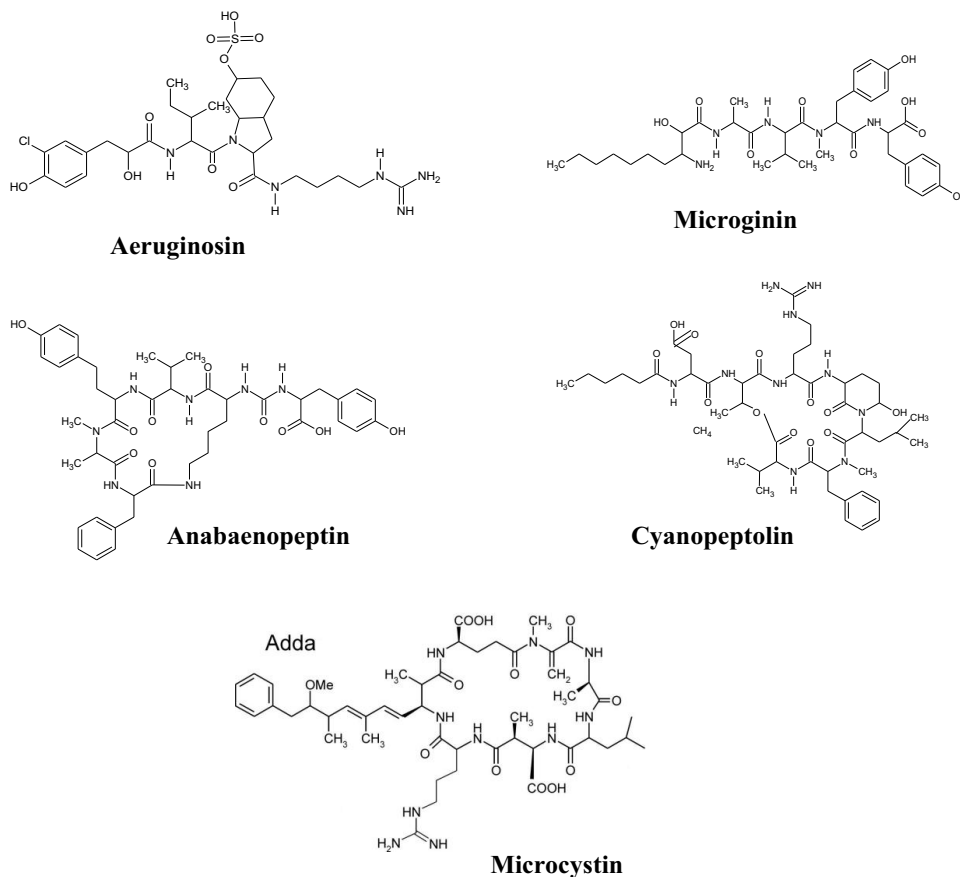
Fig. 3 General structure of the five oligopeptide classes studied in Lake Steinsfjorden (from Welker and von Döhren, 2006).

The terms 'toxin content' and 'toxicity' are often used synonymous. However, 'toxin concentration' or 'toxin content' can refer either to the amount of toxin per litre of water or the amount of toxin per biomass of cyanobacteria. The measurement units in which toxin content are reported are not always carefully considered, as several units are used including per cyanobacterial biomass, per protein weight, per dry weight and per bloom material biomass. The term 'toxicity' on the other hand, refers to animal testing data and is expressed as the amount of cyanobacteria lethal to an animal (sometimes normalized per kilogram of body weight) (Chorus and Bartram, 1999). In earlier studies (pre-1990), the toxicity of

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bloom samples was determined by mouse bioassays, but this method is unsuitable for measuring the low concentrations of cyanobacterial oligopeptides that usually prevail in cyanobacterial populations. The development of better analytical methods, in the first instance HPLC and ELISA, but also LC-MS has made the quantification of total and individual toxins possible (Chorus and Bartram, 1999).

Freshwater cyanobacterial blooms

Because cyanobacteria can accumulate at a certain depth due to their buoyancy regulation, the populations are often observed as blooms in a stratified water column (Reynolds, 1987; Reynolds *et al.*, 1987; Walsby, 1994). In such a context, one (or a few) species dominate(s) the phytoplankton community and its biomass increases significantly over a relatively short period of time (within a few days to one or two weeks). Blooms of cyanobacteria usually occur in eutrophic environments, but stability of the water column is in most cases another pre-requisite for bloom development. Hence, most blooms occur in summer after a long period of sunshine, with calm and relatively warm conditions.

According to their behaviour in the water column, Mur and co-workers (1993) divided the planktonic cyanobacteria into four ecotypes;

1. Species able to fix atmospheric nitrogen (e.g. *Aphanizomenon flos-aqua, Cylindrospermopsis raciborskii)*

2. Stratifying species often accumulating at a depth with a combination of favourable light and nutrient conditions (e.g. *Planktothrix rubescens).*

3. Turbulent species, which are usually well-mixed in the epilimnion (e.g. *Planktothrix agardhii*).

4. Colony or aggregate forming species (e.g. *Microcystis*).

Microcystis, Anabaena and *Planktothrix* are among the most common toxin-producing genera in the Northern Hemisphere (Sivonen and Jones, 1999), and in a survey of German fresh water bodies blooms dominated by *Planktothrix* had the highest microcystin content (Fastner *et al.*, 1999). The filamentous species of the genus *Planktothrix,* adapted to low light conditions, and especially the red variants containing the pigment phycoerythrin often dominate blooms in the metalimnion, the boundary layer formed by the thermocline. The accumulation in the metalimnion during summer is attributable to the presence of very strong gas vesicles which lighten the cells while polysaccharide accumulation acts as ballast (Utkilen *et al.*, 1985; Walsby, 1994). They also compete well in late autumn and winter when low insolation and deep circulation combine to decrease available light, thereby

maintaining a population all year round in contrast to phytoplankton species that sink to the bottom in the autumn (Micheletti *et al.*, 1998; Davis *et al.*, 2003). When the epilimnion no longer is supporting a dense phytoplankton population, its transparency increases and the metalimnion border receives more irradiance (Reynolds, 1984; Buergi and Stadelmann, 2000; Jacquet *et al.*, 2005), hence metalimnetic blooms are sometimes first reported as a response to water quality restoration (Feuillade and Druart, 1994; Buergi and Stadelmann, 2000; Ernst *et al.*, 2001; Jacquet *et al.*, 2005).

      Planktothrix forming metalimnetic populations is typically encountered in sub-alpine lakes in Europe, exemplified by Lake Zürich (Thomas and Märki, 1949) and Lake Lucern (Zimmermann, 1969) in Switzerland, Lake Mondsee (Dokulil and Jagsch, 1992) in Austria, Lac du Bourget (Jacquet *et al.*, 2005) and Lake Nantua in France (Feuillade, 1994), but also in several lakes in Germany (Wiedner *et al.*, 2001; Padisák *et al.*, 2003) and Scandinavia (Berg *et al.*, 1986; Skulberg *et al.*, 1994). This phenomenon is also known in some American lakes (Edmondson, 1970; Klemer, 1976; Konopka, 1982; Konopka *et al.*, 1993).

Lake Steinsfjorden and *Planktothrix* sp.

Cyanobacteria occur in almost all Norwegian lakes, but the amount and species composition varies. About 20 species of toxin-producing cyanobacteria have been recorded and *Anabaena* is the most common toxin producing genus found in Norwegian lakes (Skulberg, 1980; Berg *et al.*, 1986; Skulberg *et al.*, 1994).

      Lake Steinsfjorden is situated in the South-eastern part of Norway (60°05´N, 63 m altitude). It is a dimictic, mesotrophic and elongated lake (7.9 km long and 2.6 km wide) with an area of 13.9 km^2, a total volume of 142×10^6 m^3, maximum and average depths of 24 and 10.2 m, respectively, and with a water retention time of 4.6 years (Strøm, 1932; Skogheim and Rognerud, 1978). During the period of thermal stratification, the lake is divided into three layers: epilimnion (0-7 m), metalimnion (8-14 m) and hypolimnion (15-20 m) which hold, in average, 60, 29 and 11 %, respectively, of the total lake volume. The catchment area is 63.7 km^2. Lake Steinsfjorden is connected to the larger and deeper Lake Tyrifjorden (121 km^2, maximum depth of 295 m) through a narrow and shallow passage with a low rate of water exchange (typically 1 m^3 s^{-1}). Lake Steinsfjorden has been the object of extensive limnological investigations (Strøm, 1932; Skogheim and Rognerud, 1978; Skulberg, 1978). The lake is no longer used as drinking water supply, but agricultural irrigation, fishing and several recreational purposes are important. Lake Steinsfjorden has a natural population of *Planktothrix* sp. which as a rule forms blooms in the metalimnion (8-

14 m) annually. The first mass occurrence was observed in association with the ice cover during the winter of 1961 (Skulberg, 1964). In 1997 blooms were also encountered in the metalimnic layer (Skulberg, 1998) and cyanobacterial oligopeptides of the type microcystins were detected (Hormazabal *et al.*, 2000). A monitoring programme was initiated by the Norwegian Institute for Water Research (NIVA) in 1997 to observe the occurrence of cyanobacteria and cyanobacterial oligopeptides, and by two occasions (June 2000 and July 2002) the population of *Planktothrix* sp. has caused restricted use of the lake because of too high microcystin concentrations (Edvardsen, 2002; **I**). In Lake Steinsfjorden *Planktothrix* sp. dominated the phytoplankton community during the period 2002-2004, allowing the present study to disregard other potential producers of bioactive oligopeptides (Fig. 4).
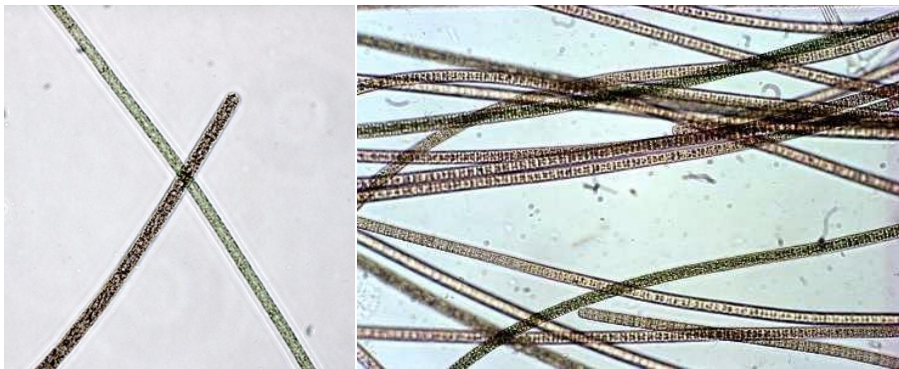

Fig. 4 Light micrograph of *Planktothrix* sp. from Lake Steinsfjorden (Photo: NIVA).

REASONS FOR AND AIMS OF THE STUDY

Most studies regarding the environmental control of *Planktothrix* populations have been done in central-Europe (see above, "Freshwater cyanobacterial blooms"). Differences in climatic conditions raised the question of whether the growth and distribution of such populations in Norwegian lakes are regulated in the same manner. Extensive knowledge exists concerning the environmental control of cyanobacterial proliferation; but mechanisms controlling the composition, abundance, distribution and seasonal dynamics of oligopeptides produced by these cyanobacteria are not clearly identified. Ever since the first reports on cyanobacterial oligopeptides, the vast variability in oligopeptide abundance among and within populations of the same cyanobacterial species has given rise to speculations (Ekman-Ekebom *et al.*, 1992; Vezie *et al.*, 1998). Reports on oligopeptide abundance in relation to cyanobacterial producer biomass are often inconsistent and/or inconclusive even regarding results from the same lake and from the same authors (de Figueiredo *et al.*, 2004; Briand *et al.*, 2005). Several attempts have been made to explain these discrepancies with a change in environmental conditions (Orr and Jones, 1998; Sivonen and Jones, 1999), however little is known about the environmental control of production of oligopeptides other than the class of microcystins.

The main objective of the present study was to improve the understanding of how toxin-producing cyanobacteria and their main classes of bioactive oligopeptides vary by depth and through the season, and to evaluate the role of abiotic environmental factors and population structure. This was accomplished by addressing the following tasks using the population of *Planktothrix* sp. in Lake Steinsfjorden, Norway, as model:

1. Describe the seasonal dynamics and depth distribution of the *Planktothrix* sp. population in Lake Steinsfjorden in relation to abiotic environmental factors (**I**).

2. Describe the variability in abundance of oligopeptides produced by *Planktothrix* sp. in Lake Steinsfjorden temporally and by depth, and assess the effect of abiotic environmental factors (**II**).

3. Reveal how the abundance of bioactive oligopeptides produced by *Planktothrix* sp. in batch cultures is affected by nitrogen and light limitation (**III**).

4. Develop a method to quantify subpopulations of *Planktothrix* sp. based on oligopeptide composition, termed chemotypes. Use this method to determine (i) their presence and variability over four decades, (ii) how they correlate to taxonomic groups and genotypes, (iii) seasonal dynamics and depth distribution in relation to abiotic environmental factors,

and (iv) whether these chemotypes interact differently with their environment and thus represent ecotypes (**IV**).

RESULTS AND DISCUSSION

The taxonomy of *Planktothrix*

The taxonomic system of Cyanophyceae (cyanobacteria) which developed during the 19th century was based almost entirely on phenotypic characters and the International Code of Botanical Nomenclature. However, the stability of these phenotypic characters and their use in taxonomy has been questioned, and since cyanobacteria are prokaryotes, bacteriological taxonomic approaches have been suggested. Here morphological, biochemical, genetic, physiological and ecological characteristics are widely used (Stanier *et al.*, 1978; Castenholz and Waterbury, 1989). Still, the taxonomy used is according to the Botanical Code (Castenholz and Waterbury, 1989). Anagnostidis and Komárek (1988) sought to revise the taxonomy of Cyanophyceae and e.g. they separated the gas-vacuolated species of the genus *Oscillatoria* into the genus *Planktothrix,* defining them as water-bloom-forming, non-heterocystous and filamentous cyanobacteria bearing many gas vacuoles. The species *Planktothrix rubescens* and *Planktothrix agardhii* were separated according to cell width and trichome colour (*P. rubescens* having phycoerythrin in addition to phycocyanin and therefore appear red, while *P. agardhii* have only phycocyanin and appear green). Suda and co-workers (2002) analysed 22 red strains and 40 green strains from algal culture collections around the world using several methods. *P. rubescens* and *P. agardhii* could not be separated on the basis of 16S rDNA sequences, fatty acid composition, or cell dimensions, but they were described as separate species based on DNA-DNA hybridization data, and pigmentation. On the basis of these findings Suda and co-workers (2002) suggested that the differentiation between these species had happened relatively recently in the evolutionary history of *Planktothrix*. Recent findings have indicated considerable overlap between the two species (Humbert and Le Berre, 2001), and that the DNA-DNA hybridization data produced by Suda and co-workers (2002) may be inconclusive (Kang *et al.*, 2007).

Planktothrix species in Lake Steinsfjorden

According to the diagnosis by Suda and co-workers (2002), two *Planktothrix* species, *Planktothrix rubescens* and *Planktothrix agardhii,* could be identified in Lake Steinsfjorden during the field program of this study (**I**). The 46 strains isolated from Lake Steinsfjorden

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from 1965 to 2004 also showed that both red and green forms of *Planktothrix* have been present in the lake for at least four decades (**IV**). However, *P. rubescens* and *P. agardhii* in Lake Steinsfjorden exhibited similar patterns of seasonal dynamics and depth distribution (**I, II**), and some of the chemotypes identified based on oligopeptide patterns fell within both of these taxa (**IV**). This in concert with the overlap in phylogenetic markers (see above) and problems with the interpretation of DNA-DNA hybridisation data used by Suda and co-workers (2002) made it necessary to reconsider the species affiliation of *Planktothrix* in Lake Steinsfjorden during the course of the present project. For this reason, *Planktothrix rubescens* and *Planktothrix agardhii* were separated in Paper **I-III**, while in Paper **IV** and this synopsis the two taxa were considered as possibly conspecific and referred to as *Planktothrix* sp.

According to the definition of ecotypes of planktonic cyanobacteria by Mur and co-workers (1993), *P. agardhii* and *P. rubescens* can be considered as different ecotypes. Further, reports of mixed populations of these species are rare, including those on *Planktothrix* in Blelham Tarn (Davis *et al.*, 2003) and Lake Garda (Salmaso, 2000). However, monitoring projects in several Norwegian lakes with *Planktothrix* (Vansjø, Lysern, Gjersjøen, Kolbotnvannet, Storvivatnet), have shown that mixed populations of red and green forms are common in this region (pers. com. Thomas Rohrlack). The green form of *Planktothrix* occurring in Norwegian lakes may be either a special ecotype of *P. agardhii,* or a green type of *P. rubescens.* A revision of the taxonomy of *Planktothrix* is thus needed.

How may abiotic environmental factors control the occurrence and abundance of oligopeptides in Lake Steinsfjorden?

Abiotic environmental factors can affect the occurrence and abundance of oligopeptides in lakes via influencing different components associated with the production of oligopeptides (Orr and Jones, 1998; Kotak *et al.*, 2000; Chorus *et al.*, 2001). Regarding the *Planktothrix* sp. population in Lake Steinsfjorden, the following components were identified:

a) The growth, seasonal dynamics and depth distribution of the *Planktothrix* sp. population (**I**),

b) the production and abundance of oligopeptides produced by *Planktothrix* sp. (**II, III**), and

c) the relative chemotype composition of the *Planktothrix* sp. population, and thereby the abundance of different oligopeptide producers (**IV**).

The following discussion evaluates the importance of these different ways by which abiotic environmental factors may affect the composition of oligopeptides.

Dynamics of the **Planktothrix** *sp. population related to abiotic environmental factors*

Specific physiological capabilities of *Planktothrix* enable them to compete efficiently with planktonic algae and other cyanobacteria. As other gas vacuolated planktonic cyanobacteria, *Planktothrix* can regulate their buoyancy in a stratified water column and thus position themselves in relation to nutrient and light availability. They possess accessory pigments such as phycocyanin, allophycocyanin and phycoerythrin that enable them to absorb a greater part of the visible spectra and to carry out photosynthesis under low light conditions, such as in the metalimnion during summer where at the same time nutrient concentrations are higher than in the epilimnion (Reynolds, 1984; Graham and Wilcox, 2000; Whitton and Potts, 2000).

From several years of studying populations of *Planktothrix* in lakes around the world, a good understanding has evolved on what and how environmental factors control their metalimnetic blooms. Data from several water bodies are available: Lake Zürich, Switzerland (Walsby and Schanz, 2002), Lake Pusiano, Italy (Legnani *et al.*, 2005), Lac du Bourget, France (Jacquet *et al.*, 2005), Lake Mondsee, Austria (Dokulil and Jagsch, 1992), several lakes in Germany (Wiedner *et al.*, 2001; Padisák *et al.*, 2003) and some American lakes (Klemer, 1976; Konopka, 1982; Konopka *et al.*, 1993). Metalimnetic *Planktothrix* blooms have also been observed in several Scandinavian lakes (Berg *et al.*, 1986; Skulberg *et al.*, 1994), but few studies have focused on the environmental factors associated with the occurrence of these blooms.

During the field study in Lake Steinsfjorden, the *Planktothrix* sp. population biovolume varied from a minimum of 0.6 cm^3 m^{-2} in August 2002 up to a maximum of 73 cm^3 m^{-2} in November 2003, and the net specific growth rate was estimated to a maximum of 0.07 d^{-1}. The maximum specific growth rate obtained in culture experiments with *P. rubescens* was 0.75 day^{-1} (**III**), thus giving clear indications of growth limitations for the *Planktothrix* sp. in Lake Steinsfjorden. However, cultures were running under optimal growth conditions of 20 °C and with an irradiance of about 16 μmol m^{-2} s^{-1}, conditions which are rarely experienced by the field population of *Planktothrix* sp. in Lake Steinsfjorden (**I**). Most of the population in Lake Steinsfjorden was growing at 6-20 ºC, i.e.

below optimal temperature conditions during even the warmest period in summer, which is comparable to the temperature conditions in other European lakes with *Planktothrix* (Micheletti *et al.*, 1998; Jacquet *et al.*, 2005; Legnani *et al.*, 2005). During thermal stratification when *Planktothrix* sp. had accumulated in the metalimnion (8-14 m), the light availability (about 5 µmol m^{-2} s^{-1}) was equal to or less than the irradiance needed for half of maximal growth rate, and the population experienced even lower levels of irradiances the rest of the year (**I**).

Cyanobacteria are able to survive periods with low supply of resources by using shortages of essential nutrients, probably by accumulation of reserve compounds while relevant nutrients are still available (Whitton and Potts, 2000). Cyanophycin, a non-ribosomally synthesized polypeptide of aspartate and arginine, may act as dynamic nitrogen reservoir (Mackerras *et al.*, 1990). Phycocyanin, a structural compound of light-harvesting complexes involved in transmission of light energy to photosystem II, plays an additional role as nitrogen reserve because of its proteinacous character (Carr, 1988) and polyphosphate granulae clearly serve as phosphorus storage compounds (Shively, 1988). Compared to minimum levels of nitrogen and phosphorus for maximal growth rate in culture studies (Rohrlack and Utkilen, 2007), the conditions of both the epi- and metalimnion in Lake Steinsfjorden were often below the level necessary to achieve maximal growth rate (**I**). However, these nutrient levels are required for maximal growth rate assuming optimal light and temperature conditions. As the population of *Planktothrix* sp. was experiencing the low light and temperature conditions of Lake Steinsfjorden, it was assumed not to be limited by nutrients (**I**). Reynolds and co-workers (Reynolds, 1987) suggested that the accumulation of the *Planktothrix* population in the metalimnion in summer provides a means of maintaining biomass with minimum energy expenditure through a period in which growth in the epilimnion is severely limited by nutrient deficiencies.

A 80-90 % decrease in the population of *Planktothrix* sp. in Lake Steinsfjorden was observed twice during the field study 2002-2004. In summer 2002 large amounts of *Planktothrix* sp. biomass floated to the surface and were then transported by winds to the shore, accumulating on beaches (**I**). The local authorities gave restrictions for bathing activities due to the high microcystin content. After a massive bloom of *Planktothrix* sp. during autumn 2003, large amounts of biomass were observed floating under and embedded into the ice cover in January 2004 (Fig. 5). In April 2004, after ice melting, this biomass was seen accumulating in the literal zone of Lake Steinsfjorden, probably being transported there

by currents under the ice (**I**). So why do 80-90 % of the population suddenly float to the surface? The causes of these events are still unclear, as is the case for similar events in other lakes with *Planktothrix* populations (Walsby *et al.*, 1998). A failure of buoyancy mechanism may be due to CO_2 limitation which is known to be accompanied by an increase in gas vesicles (Klemer *et al.*, 1982; Konopka, 1982a,b).

Fig. 5 Accumulations of *Planktothrix* sp. biomass during period of ice cover in Lake Steinsfjorden (Photo: NIVA).

In general, populations of *Planktohrix* and other gas vacuolated planktonic cyanobacteria are considered to take little part in loss processes. Davis and co-workers (Davis *et al.*, 2003) studied loss processes in a *Planktothrix* spp. population in Blelhamn Tarn and found that the principal losses they observed were caused by hydraulic flushing and not by sedimentation. Since *Planktothrix* is able to regulate their buoyancy, one can assume that the loss by sedimentation is small (Walsby, 1994). In general filamentous cyanobacteria are difficult to ingest for zooplankton because of the mechanical interference with the feeding apparatus (Gliwicz, 1990), and some oligopeptides produced by *Planktothrix* seems to be involved in the resistance to grazing (Kurmayer and Jüttner, 1999; Blom *et al.*, 2001). The grazing of *Planktothrix* is usually assumed to be negligible (Reynolds, 1987; Whitton and Potts, 2000), and it was not considered in this study. However, there are a few reports on heavy grazing leading to a dramatic decrease in the *Planktothrix* populations; e.g. a large population of ciliates of the genus *Nassula* were observed to contain *Planktothrix* filaments, and coincided with a 90 % decrease in the

population of *Planktothrix rubescens* in Lake Nantua, France in 1972 (Feuillade, 1994), and a similar event was observed by (Thomas, 1941) in Lake Zürich.

In summary, the growth of *Planktothrix* sp. in Lake Steinsfjorden was shown to be mainly controlled by light and temperature and to a lesser extent by macronutrients (**I**). Post and co-workers (Post *et al.*, 1985) found that temperature only influenced the growth rate under saturating light conditions, but that it was independent of temperature under low light conditions in culture experiments performed with *Planktothrix agardhii*. Hence, the *Planktothrix* sp. population in Lake Steinsfjorden was probably first of all controlled by irradiance and not by temperature when growing in the low light conditions of the metalimnion in summer, when circulating in the whole water column during autumn and spring and when growing under the ice cover in winter (**I**). Irradiance is shown to be an important factor controlling the distribution of *Planktothrix* populations in several lakes, e.g. Crooked Lake, Indiana, U.S.A. (Konopka, 1982a) and Lake Zürich, Switzerland (Walsby and Schanz, 2002). In an analysis of 55 shallow and turbid lakes with *Planktothrix,* (Scheffer *et al.*, 1997) found no correlation between the relative abundance of *Planktothrix* and nutrient concentrations or nutrient ratios, but a significant correlation with secchi depth and the degree of shading. They suggested that *Planktothrix* respond more to changes in the underwater light climate than to changes in nutrient availability.

Although the metalimnetic population of *Planktothrix* sp. in Lake Steinsfjorden are assumed not to be limited by nutrients, a lowering of nutrient supplies over time could in addition to the suboptimal light and temperature conditions prevent bloom formation (**I**). Considering the low net growth rates of *Planktothrix* sp., it is likely that the occurrence of an additional stress factor, such as a further decrease in nutrient concentrations over time, would exceed the ability of *Planktothrix* sp. to compensate loss processes and to form metalimnetic blooms. This might be the case in Lake Steinsfjorden, as a continued monitoring program in the following years (2005-2007) of the field work of this study have reported a decline in the *Planktothrix* sp. population and the typical annual metalimnetic blooms in summer have not been observed (pers. com. Thomas Rohrlack). Successful attempts to control the populations of *Planktothrix* have been reported from several lakes around the world and often include a removal of phosphate (Dokulil and Jagsch, 1992; Buergi and Stadelmann, 2000; Jacquet *et al.*, 2005). E.g. in Lake Bled, Slovenia the population of *P. rubescens* disappeared rapidly after restoration of the lake involving a combination of artificial inflow of clean water, removal of the hypolimnetic water and the diversion of treated sewage (Vrhovsek *et al.*, 1985). In Lake Mondsee, Austria, phosphate

concentrations reached below 10 µg l^{-1} after restoration and lead to the disappearance of *P. rubescens* (Dokulil and Jagsch, 1992).

*** The cellular oligopeptide content related to abiotic environmental factors under field and culture conditions***

To examine cyanobacteria in their natural habitat, as part of populations and whole phytoplankton communities, gives the advantages of studying the organisms as they are influenced by the present environmental factors. However, in field studies it is impossible to encounter all the factors involved in the control of cyanobacterial and oligopeptide dynamics. The controlled conditions of culture experiments give the opportunity to focus on the influence of just one or a few factors at a time. To achieve a more complete picture of how abiotic environmental factors affect the composition of oligopeptides in Lake Steinsfjorden, the dynamics of oligopeptides produced by *Planktothrix* sp. were studied under both field (**II**) and culture (**III**) conditions (Fig. 6).

Fig. 6 Culture vessel with *Planktothrix* sp. biomass.

The production of oligopeptides by cyanobacteria seems to be conservative, as strains that grow under laboratory conditions for decades do not usually lose the ability to produce the oligopeptides typical for that strain (Welker and von Döhren, 2006). In general, despite marked changes in culture conditions, the production of oligopeptides per biomass is seldom reported to cease and variations in the amount of cell-bound oligopeptides per

biomass unit have not exceeded a factor of 5 (Orr and Jones, 1998; Sivonen and Jones, 1999; Rohrlack and Utkilen, 2007).

Most studies regarding oligopeptide production in cyanobacteria have been focused on toxic compounds belonging to the well-known class of microcystins. Briand and co-workers (2005) found a linear relationship between growth rate and microcystin production in both cultures of *P. rubescens* and in natural populations from Lac du Bourget. Despite the changing environmental conditions experienced by the *P. rubescens* population from April to December, there were no significant changes in the microcystin content of the cells. Under experimental conditions, attempts to demonstrate the impact on microcystin production of various growth factors have led to contradictory conclusions (Watanabe and Oishi, 1985; Sivonen, 1990; Utkilen and Gjølme, 1992; Orr and Jones, 1998; Long *et al.*, 2001; Wiedner *et al.*, 2003). Numerous reports have been published looking at individual factors such as temperature (van der Westhuizen and Eloff, 1985; Watanabe and Oishi, 1985), irradiance (Sivonen, 1990; Utkilen and Gjølme, 1992; Rapala *et al.*, 1997), major nutrients such as N and P (van der Westhuizen and Eloff, 1985; Sivonen, 1990; Rapala *et al.*, 1997), and trace elements (Lukac and Aegerter, 1993; Utkilen and Gjølme, 1995). In all these studies, which covered a range of species, the effects of environmental factors on microcystin production were highly variable. The only qualitative explanation of this variability that has been proffered is that nutrients and other growth-limiting factors indirectly influence the oligopeptide production in cyanobacteria through their effect on growth and cell division, and not through any direct effect on the oligopeptide biosynthetic or catabolic pathways (Orr and Jones, 1998; Sivonen and Jones, 1999).

This study is the first on oligopeptide production to consider the effect of environmental factors on all major oligopeptide classes produced by the same cyanobacterial strains, and it includes measurements of the classes aeruginosins and microginins, which have never been studied in culture experiments before (**III**). The analysis of field samples revealed considerable variations in the abundance of the seven oligopeptides per biomass of *Planktothrix* sp (**II**). By applying multivariate regression models, called generalized additive models (GAMs), the effect of temperature, irradiance, macronutrients, depth, and date on this variance could be evaluated (**II**). Taken together these factors explained between 50 and 94 % of the oligopeptide variance observed and as factors were ranked according to their contribution to this variance, date was clearly the most important one, explained by the waxing and waning of the four chemotypes (**II**).

Depth explained on average 20 % of the variation in oligopeptide concentration per *Planktothrix* sp. biomass in Lake Steinsfjorden (**II**). For all the seven oligopeptides studied, concentration appeared to increase linearly with increasing depth, which could not be explained by the changes in light intensity and temperature with depth (**II**). As a result of depth-dependent variations in irradiance, phytoplankton cells that move vertically, either as a result of turbulent mixing or through buoyancy regulation, will encounter changes in the underwater light climate. Not only will light intensity change quantitatively by depth, but also qualitatively by changes in the spectral distribution of photosynthetically active radiation (PAR) (Oliver and Ganf, 2000). As typical for a inland freshwater lake with low concentration of dissolved organic compounds, the red and blue light will be absorbed in the epilimnion by phytoplankton algae, leaving mostly green light to penetrate deeper. Since *Planktothrix* sp. possesses phycobiliproteins (phycocyanin, allophycocyanin and phycoerythrin) they are specialized on exploiting both low light intensities in general and green light in particular (Graham and Wilcox, 2000; Oliver and Ganf, 2000). Jüttner and Lüthi (2008) analysed the content of microcystins and cyanopeptolins in different cell constituents of the *Microcystis* strain PCC 7806 and found that oligopeptides were bound to a protein fraction primarily composed of phycobiliproteins. The observed positive correlation of oligopeptide concentrations with depth in Lake Steinsfjorden (**II**) could therefore be associated with an increase in phycobiliproteins per biomass of *Planktothrix* sp. with depth. However, whether or not *Planktothrix* sp. filaments at different depths adapt to rapidly fluctuating irradiances will depend upon the time scale for photoadaptation compared to that of turbulent mixing (Oliver and Ganf, 2000).

Surprisingly, both in field and culture studies of *Planktothrix* sp. from Lake Steinsfjorden the growth resources temperature, irradiance and macronutrients explained only a minor part of the variance in oligopeptide concentration per biomass (**II, III**). Light and macronutrients accounted for less than 10 % in the variation of cell-bound oligopeptide content per biomass in the field study (**II**), while the production of oligopeptides changed within a factor of 1-5 under culture experiments, despite both nitrogen and light conditions ranging from optimal to limiting (**III**). Böttcher and co-workers (2001) found in the study of three *Planktothrix agardhii* and two *Microcystis aeruginosa* strains, with and without microcystins, that total microcystin cell quotas are scarcely affected by photon flux densities over a wide range of irradiances down to levels almost approaching the maintenance energy requirements. Watanabe and Oishi (1985) found that decreasing the concentrations of

nitrogen and phosphorus significantly reduces the growth rate, but has comparatively little effect on toxicity.

The similarity shown for the five oligopeptide classes included in this work regarding dynamics in the field and effect of environmental factors on the cellular content (**II, III**) suggests that the production of oligopeptides in general is regulated in a similar way.

Chemotypes and oligopeptide composition

A total of 46 clonal *Planktothrix* cultures were isolated from Lake Steinsfjorden in the period 1965 to 2004. They produced 33 different oligopeptides belonging to the classes aeruginosins, microginins, microcystins, cyanopeptolins and anabaenopeptins (**IV**). Based on the composition of oligopeptides belonging to these five classes, four different chemotypes where identified and called Cht1-4. These chemotypes seem to be persistent in Lake Steinsfjorden as they were repeatedly isolated throughout several years or decades (**IV**). Several other populations of *Planktothrix* and *Microcystis* in central-Europe have been analysed regarding their chemotype composition, generally reporting a higher number of chemotypes than found in Lake Steinsfjorden (Fastner *et al.*, 2001; Welker *et al.*, 2004a,b; Saker *et al.*, 2005; Yepremian *et al.*, 2007). For example, based only on microcystin variants, 16 chemotypes were found in 41 strains isolated from a population of *Planktothrix agardhii* in Lake Viry-Châtillon, France (Yepremian *et al.*, 2007), whereas Welker and co-workers (Welker *et al.*, 2004b) found 15 chemotypes in 18 isolates, based on 24 oligopeptides in a *Planktothrix* population in Lake Maxsee near Berlin, Germany.

The quantitative chemotype composition in Lake Steinsfjorden varied greatly during the study-period 2003-2004, because of the different dynamics of the four chemotypes, and two major shifts in the relative chemotype composition were observed (**IV**). Statistical methods applied to test for the correlation between environmental factors (temperature, light and nutrient availabilities) and the dynamics of the four chemotypes found in Lake Steinsfjorden did not show any significant correlation of these factors on the relative abundance of chemotypes (**IV**).

One of the major shifts observed in chemotype composition coincided with the massive transport of *Planktothrix* sp. biomass from under the ice to the littoral zone during winter 2003/2004 (see "Dynamics of the *Planktothrix* sp. population related to abiotic environmental factors"). Isolates taken from the stranded biomass was shown to consist of 96 % Cht4, and there was a shift in dominance of Cht4 to that of Cht3 (**IV**). Most probably,

Cht4 had floated up underneath the ice, while Cht3 stayed deeper in the water column. This could be due to a difference in buoyancy regulation as found by Beard and co-workers (2000). They isolated 71 *Planktothrix* sp. strains from Nordic lakes and divided them into 12 genotypes based on gas vesicle genes, which coded for gas vesicle strength. Four strains from Lake Steinsfjorden were included, which also are among the strains analysed for bioactive oligopeptides in this work (**IV**). These strains were identified as four different gas vesicle genotypes in the work of Beard and co-workers (2000), whereas they were characterized as representatives of three of the four chemotypes in Lake Steinsfjorden (Cht1-3, **IV**).

Properties of a given population may depend on its chemotype composition, and any changes in this parameter may have an effect on whole-population characteristics. The mechanisms regulating the chemotype composition of a cyanobacterial population are poorly understood. Apparently, a high diversity of oligopeptides and chemotypes seem to be beneficial to the individual cyanobacterial cells and to the cyanobacterial population as a whole. Otherwise this effort on producing all these oligopeptides by single cells and the co-existence of several chemotypes in the same population would not be incidental.

Subpopulations

Cyanobacterial populations consisting of ecologically distinct subpopulations have recently been reported from both marine and freshwater ecosystems (Moore *et al.*, 1998; Stomp *et al.*, 2004; Oberhaus *et al.*, 2007; Stomp *et al.*, 2007)**,** challenging the traditional view in phytoplankton ecology that the species is the main ecological unit. Studies of certain cyanobacterial DNA regions often report on high sequence homogeneity, and are explained by the frequent transfer of genetic material between strains, a mechanism which is common in bacteria (Rudi *et al.*, 1998). This frequent transfer of genetic information could in theory lead to a low diversity among cyanobacteria species and strains sharing the same environment, however, competition among strains and species for the same environmental resources has led to a niche differentiation that facilitates coexistence (The paradox of the plankton, Hutchinson, 1961).

Subpopulations of cyanobacteria can be polymorphic with respect to, for example, cellular content of biologically active compounds (Fastner *et al.*, 2001; Welker *et al.*, 2004b), gas-vesicle properties (Beard *et al.*, 2000), susceptibility to grazers (Rohrlack *et al.*, 2005) and light dependent physiologies (Moore *et al.*, 1998; Stomp *et al.*, 2004; Stomp *et al.*, 2007). Stomp and co-workers (2007) sampled picocyanobacteria from 70 aquatic marine

and freshwater ecosystems. They found that red picocyanobacteria dominated in clear waters, green picocyanobacteria dominated in turbid waters and a coexistence of the two forms were found in waters of intermediate turbidity. Moore and co-workers (1998) postulated that the coexistence of *Prochlorococcus* ecotypes in marine waters were due to adaptation to different light intensities, and permits the survival of the population as a whole, covering adaptations over a broader range of environmental conditions than would be possible for a homogenous population. *Planktothrix rubescens* and *Planktothrix agardhii* isolated from two French sub-alpine lakes were shown to have different ecophysiological properties when grown in both mono and mixed cultures under different light and temperature conditions (Oberhaus *et al.*, 2007). *P. rubescens* was characterized by being well adapted to low intensities of green light, displayed strong photoinhibition under high irradiance levels and grew better at lower temperatures. *P. agardhii* appeared less specialized with regard to light quality, less sensitive to photoinhibition and grew better at high temperatures. Humbert and Le Berre (2001) tested the taxonomic validity of *P. rubescens* and *P. agardhii* by sequencing four DNA regions, including the well known conserved coding region of 16S rRNA gene and suggested that these two species probably are conspecific and may constitute two ecotypes of the same species adapted to different environmental conditions.

The red and green subpopulations of *Planktothrix* sp. in Lake Steinsfjorden were shown to follow the same depth distributions (**I**), and similar coexisting populations have also been reported from Blelham Tarn (Davis *et al.*, 2003) and Lake Garda (Salmaso, 2003), supporting the hypothesis of niche differentiation along the light spectrum promoting phytoplankton diversity (Moore *et al.*, 1998; Stomp *et al.*, 2007).

When the 33 strains isolated from Lake Steinsfjorden between 1965 and 2004 were grouped according to their oligopeptide pattern, Cht4 contained both red and green isolates (**IV**), and the chemotypes also showed differences regarding seasonal dynamics, depth distribution, and participation in loss processes. The quantitative method developed to quantify chemotype-specific biomass were shown suitable for the studies at the subpopulation level (**IV**), and demonstrated that the four chemotypes in Lake Steinsfjorden are ecological distinct subpopulations (**IV**).

In summary, our findings and those by authors who worked with other *Planktothrix* populations, *Prochlorococcus* and *Synechococcus* species suggests that populations comprising ecologically distinct subpopulations are common in cyanobacteria (Moore *et al.*, 1998; Humbert and Le Berre, 2001; Oberhaus *et al.*, 2007; Stomp *et al.*, 2007). A higher

level of understanding of cyanobacteria may thus demand studies that recognize the subpopulation level as a major basis for biological processes. A wide range of characters in cyanobacteria may be applied to study different mechanisms at the subpopulation level.

The function of cyanobacterial oligopeptides

Why are individual cyanobacterial cells producing such a high diversity of oligopeptides, and why have such a high number of coexisting chemotypes evolved in cyanobacterial populations? Although a great deal of research has been directed towards identifying toxic metabolites in relation to mammalian health, the exact physiological and ecological bases for and controls of toxin production remain unknown. Most likely, the bases for and controls of oligopeptide production is related to the immediate physical, chemical and biotic environment within which cyanobacteria exist (Carmichael, 1992; Codd, 1995; Leflaive and Ten-Hage, 2007). Cyanobacterial bioactive oligopeptides were first identified as secondary metabolites, and considered as non-essential to the cyanobacterial cells (Carmichael, 1992, 1997). Hence, the production of bioactive oligopeptides was expected to decline when resources become limiting, and maintenance metabolism or synthesis of vital compounds need to be prioritized. However, as is demonstrated repeatedly in numerous culture studies, including Paper **III**, the production of oligopeptides has hardly ever been reported to stop (Sivonen and Jones, 1999). Besides being widely produced, cyanobacterial toxins can account for a high proportion of cyanobacterial biomass, e.g. 0.1 to 0.2 µg microcystins per µg chlorophyll *a* in *Microcystis aeruginosa* (Lawton *et al.*, 1994). It is unlikely that the production of such abundant products would have been retained throughout cyanobacterial evolution unless they have an important biological function (Codd, 1995; Welker and von Döhren, 2006).

Strains lacking the genes associated with the biosynthesis of the class of microcystins did not seem to exhibit any severe disadvantage (Hesse *et al.*, 2001; Kaebernick *et al.*, 2001). However, this apply to strains in culture where the production of just one oligopeptide are knocked-out, and where a possible benefit of that oligopeptide to the cyanobacteria in their natural environment is not verified (Repka *et al.*, 2001). Repka and co-workers (2004) emphasized that the understanding of the function of oligopeptides requires studying cyanobacterial oligopeptides as a group rather than focusing on individual compounds as has been done recently with microcystins. The similarity in regulation pattern shown for the five most important oligopeptide classes in *Planktothrix* sp. in Lake Steinsfjorden support this (**II, III**).

Putative functions of oligopeptides include an enhanced competitive ability compared to other phytoplankton algae by allelopathy (Smith and Doan, 1999; Schagerl *et al.*, 2002; Leflaive and Ten-Hage, 2007), virus defence (Rinehart *et al.*, 1981; Zainuddin *et al.*, 2002), involvement in cell metabolism (Hesse *et al.*, 2001), quorum sensing (Kehr *et al.*, 2006; Schatz *et al.*, 2007), internal storage of nitrogen (Kotak *et al.*, 2000) though most studies have focused on the possible role as chemical defence against grazing (Demott and Moxter, 1991; Kurmayer and Jüttner, 1999; Kaebernick *et al.*, 2001). The latter is in agreement with the toxicity of several oligopeptides to *Daphnia* (Jakobi *et al.*, 1996; Blom *et al.*, 2003; Rohrlack *et al.*, 2004; Rohrlack *et al.*, 2005). Microcystins are stored in high concentrations (mmolar) in cyanobacterial cells (Long *et al.*, 2001; Wiedner *et al.*, 2003). From an ecological point of view this is advantageous when they should act as defence molecules against grazers (Jüttner and Lüthi, 2008). Oscillapeptin J isolated from *Planktothrix rubescens* from Lake Zürich was shown to be a highly potent crustacean grazer toxin (Blom *et al.*, 2003). Rohrlack and co-workers (2004) showed that inhibition of the digestive enzyme trypsin by cyanobacterial metabolites can result in the death of *Daphnia*, and also inhibition of moulting of *Daphnia* and thus might reduce the grazing pressure of a population efficiently without directly killing the grazers. Later Rohrlack and co-workers (2005) demonstrated that the production of inhibitors of *Daphnia* trypsin is a common feature in the widely distributed cyanobacterial genus *Planktothrix*, of 89 strains analyzed, 70 % contained these compounds.

Consequences for risk assessment

The complex nature of cyanobacterial populations emphasizes the importance of extensive limnological surveys in order to do useful monitoring and risk assessment regarding cyanobacterial oligopeptides. As the results of this work clearly demonstrate, there is a need not only to identify the toxic cyanobacterial species present, but also the chemotype composition of the cyanobacterial population (**IV**). However, regular monitoring programs are often economically limited to a low sampling frequency with the risk of overlooking toxic bloom events. A cyanobacterial bloom is rarely evenly distributed in a lake, and taking samples from just one location and one depth, which is often the case in monitoring programs, might be insufficient. Also traditional microscopical biomass measuring methods are both time-consuming and costly and do not recognize the chemotypes present. Simply measuring the biomass of toxic cyanobacterial species present in a lake will only give a worst case estimate of the potential toxicity of a bloom (Briand *et al.*, 2008). With the

increasing number of new bioactive oligopeptides being discovered, the development of new rapid, inexpensive and sensitive enough monitoring methods to promptly screen simultaneously a great diversity of oligopeptides and also check their toxic effects are becoming necessary (de Figueiredo *et al.*, 2004).

The understanding of the adverse effects of cyanobacterial oligopeptides on other animals than humans and especially on organisms in aquatic ecosystems is fragmented (Christoffersen, 1996). Although cyanobacterial oligopeptides have adverse effect on mammals and humans, the effects on organisms in the proximate environment of toxic freshwater cyanobacteria are more likely to become encountered by these compounds (Codd, 1995; Leflaive and Ten-Hage, 2007). Aquatic organisms differ in their sensitivity to cyanobacterial oligopeptides, e.g. copepods appear more sensitive than cladoceran. As the different parts of aquatic food webs may display dissimilar reactions to cyanobacterial oligopeptides, the ecological importance of cyanobacterial oligopeptides is complex and need more attention (Christoffersen, 1996).

World Health Organization (WHO) has established a guideline value concerning the concentration of microcystin LR in drinking water (1 µg l^{-1}) and for bathing activities (10 µg l^{-1}) (Kuiper-Goodman *et al.*, 1999). However such guidelines exist to date only for microcystin LR, while the toxicology of other cyanobacterial bioactive oligopeptides are mostly unknown. This is partly due to the problematic toxicological methods necessary to establish these guideline values, especially regarding humans (Chorus and Bartram, 1999).

CONCLUSIONS AND FUTURE PERSPECTIVES

This study has shown:

- To survive in a mesotrophic lake like Lake Steinsfjorden, populations of *Planktothrix* sp. have developed adaptations to accumulate in the metalimnion during periods of thermal stratification and maintain a viable population all year round in the water column, a life strategy similar to that of other metalimnetic European populations of *Planktothrix*, despite significant differences in climatic conditions (**I**).

- Although the populations of *Planktothrix* sp. are adapted to the limiting temperature and irradiance conditions of Lake Steinsfjorden, the low net growth rates make them vulnerable to the occurrence of an additional stress factor, such as a limitation by nutrients, which would exceed the ability to compensate loss processes and to form metalimnetic blooms (**I**).

- The abundance of the most important oligopeptides produced by the population of *Planktothrix* sp., belonging to the oligopeptide classes; aeruginosins, anabaenopeptins, cyanopeptolins, microcystins and microginins, varied considerably throughout the season (**II**).

- The statistical models used to investigate the importance of temperature, irradiance, macronutrients, depth and date on the variability of oligopeptide content per *Planktothrix* sp. biomass showed that date was the most important contributor, linked to the waxing and veining of the four chemotypes found in Lake Steinsfjorden (**II**).

- The production of all the oligopeptides studied was persistent throughout the culture experiments and significant changes were within a factor of 5, despite that the cells were experiencing large differences in light and nitrogen availability. Our results suggest that the production of all major bioactive cyanobacterial oligopeptide classes in general is regulated in a similar manner (**III**).

- The population of *Planktothrix* sp. were found to be composed of four chemotypes, Cht1-4, that were distinguished on the basis of their unique cellular oligopeptide patterns, and these oligopeptide chemotypes occurred largely unaltered throughout a period of up to 33 years (**IV**).

- The four chemotypes, Cht1-4, differed with respect to seasonal dynamics, depth distribution, and participation in loss processes. The dramatic change observed in the

relative abundance of the four chemotypes could not be explained by fluctuations in irradiance, temperature or concentration of macronutrients (**IV**).

- Our findings suggest that (i) oligopeptide chemotypes can have dissimilar ecological traits and therefore interact differently with their environment, (ii) freshwater cyanobacterial populations can comprise multiple ecologically distinct sub-populations, and (iii) the relative abundance of these may vary, causing a high variability in whole-population properties (**IV**).

- Populations comprising ecologically distinct subpopulations are common in cyanobacteria (**IV**). A higher level of understanding of cyanobacteria may thus demand studies that recognize the subpopulation level as a major basis for biological processes. A wide range of characters in cyanobacteria may be applied to study different mechanisms at the subpopulation level.

- The chemotype composition of the *Planktothrix* sp. population is a key mechanism determining the oligopeptide composition in Lake Steinsfjorden (**II**, **IV**). The abiotic environmental factors light intensity, temperature and macronutrient concentrations may determine the size of a population as a whole (**I**) but not necessarily its chemotype composition (**IV**). To fully understand the dynamics of oligopeptides in a lake, the factors governing the outcome of competition between chemotypes need to be identified.

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