Toxic and Bioactive Peptides in Cyanobacteria

PEPCY

Final Report

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1 Introduction

Cyanobacteria produce toxic compounds which have caused fatalities for wild animals, livestock and even humans. A large group among the diverse cyanobacterial substances are the oligopeptides (termed “cyanopeptides” in the following). The strongly hepatotoxic and probably tumour-promoting microcystins and nodularins were the first oligopeptides to be intensively studied. These are very potent toxins for eukaryotes by inhibition of the serine/threonine proteinphosphatase group (Runnegar et al. 1993). While most of the effects caused by cyanobacteria could be attributed to microcystins, nodularins, or to the alkaloid cyanotoxins, cyanobacterial extracts show effects which are more pronounced than can be explained by the sum of the known cyanotoxins contained in the respective extract (Keil et al. 2002; Mankiewicz et al. 2002; Teneva et al. 2003; Herfindal et al. 2005; Surakka et al. 2005). Therefore other substances produced by cyanobacterial species must cause this additional toxicity. One of the compounds assumed to responsible for the stronger toxicity of cyanobacterial extracts is LPS (Best et al. 2002; Best et al. 2003), however subsequent analyses were not sufficiently convincing to substantiate this assumption.

In consequence of the development of analytical methods for chemicals, particularly MALDI-TOF-MS (Erhard et al. 1997) as sensitive and efficient tool to screen cyanobacteria for their content of nonribosomal peptides, it became increasingly evident that cyanobacteria contain a wide array of peptides in addition to microcystins and nodularins. These are synthesized by non-ribosomal peptide synthesis, similar to the microcystins. While there is some debate about categorizing these cyanopeptides as primary or secondary metabolites (see also section 1.3 below), an argument for classifying them as secondary metabolites is that genotypes including mutants of a species differing significantly in the absence/presence of a specific peptide class do not show a readily apparent selective advantage/disadvantage to the producing organism (Kurmayer et al. 2004; Welker & von Döhren 2006).

Cyanopeptides are classified into distinct peptide classes, such as the anabaenopeptins, aeruginosins, cyanopeptolins, microginins, characterized by a conserved backbone of the molecule, while a number of amino acid positions are variable and cause the occurrence of numerous isoforms in a specific peptide class (see Welker & von Döhren 2006 for a comprehensive overview developed within PEPCY). Many cyanopeptides have been found to show bioactivity, such as inhibition of specific enzymes in vitro. For the microcystins, toxicity to vertrebrates and crustacea is well studied, and it remains doubtful that microcystin production evolved due to a competitive advantage of toxicity to animals. Although a number of hypotheses have been brought forward particularly for microcystins, the biological function of cyanopeptide production for the cells themselves remains puzzling.

Assays with non-microcystin cyanopeptides from laboratory strains demonstrated that they vary in biological activity (Czarnecki et al. 2006). An overview of the state of knowledge in Annex 1) shows that they limit a range of enzymes, e.g.trypsinase, thrombin, plasmin, chymotrypsin, elastase, papain, angiotensin converting enzyme, leucine aminopeptidase, carboxypeptidase, and some are cytotoxic to leukemia cells. Biological activities identified include those with potential pharmaceutical application e.g. in the treatment of high blood pressure by vasoactive substances (e.g. microginins) or conditions such as asthma and viral infection by serine protease inhibitors (e.g. cyanopeptolins) and those with toxic properties e.g. protein phosphatase inhibition (e.g. anabaenopeptins, and lethal inhibition of molting in *Daphnia* (e.g. microviridin J; Rohrlack et al. 2004). While none of these cyanopeptides has been proposed to cause overt acute or chronic toxicity, data are generally missing.

The potential for such a large range of bioactive substances to be produced in a cyanobacterial bloom demonstrates the need for knowledge on the contribution of cyanobacterial peptides to the overall biological impact of a bloom, both on other aquatic organisms and on vertebrates – including humans – that are exposed to the water, particularly through oral ingestion. The research results on potential pharmaceutical applications of cyanopeptides highlight the likelihood of environmental or health effects on aquatic biota and humans. The concentrations at which they may be encountered in aquatic environments to date had only been determined for microcystins, but not yet for the other peptide groups. For an assessment of their potential impact on human health and on aquatic biota, information is lacking regarding both their toxicological potential and the frequency and concentrations with which they occur.

Improving risk management requires closing a further gap – the understanding of the driving factors that determine the occurrence of toxic cyanobacterial genotypes in natural phytoplankton populations. For microcystins, it has been well documented that producer- and non-producer genotypes co-occur within the same water-body, though their relative shares may vary between water-bodies and/or over time (e.g. Kurmayer et al. 2003). The reasons for this variability are unclear, yet cells' investment into producing substances that may constitute up to 0.5% of their mass must have some competitive advantage. In aquatic systems this advantage for producer-cyanobacteria is unlikely to be pronounced toxicity to vertebrates as shown by microcystins, and the question of the ecological benefit of peptide production is open for the other groups of cyanopeptides as well. Expanding research beyond microcystins to the broader issue of cyanopeptide occurrence is therefore promising towards elucidating the biological function that production of these substances has for the producer organisms. An understanding of this function would greatly help understand patterns of occurrence, and this in turn would provide the basis for predicting occurrence.

A major research opportunity towards this goal has arisen from the rapid development of knowledge about the genetic basis of cyanopeptide production: non-ribosomal peptide synthetase genes and polyketide synthase genes in cyanobacteria with essential function in the biosynthesis of the hepatotoxin microcystin and other peptides were discovered during the past 10 years, and this enabled the establishment of protocols to genetically manipulate cyanotoxin producing strains (Dittmann et al. 1997; Rouhiainen et al. 2000; Tillett et al. 2000). Thus, it is now possible to develop DNA-based methods for the detection of genes involved in the production of bioactive cyanopeptides. This enables the identification of cyanobacterial genotypes with the potential to synthesize toxins in environmental samples, even in single colonies and filaments directly taken from field samples. It is further the basis for developing quantitative PCR methods to investigate the share of producer- and non-producer genotypes in field populations. The manipulation of strains to knock out only the production of a specific peptide opens a wide range of opportunities for testing such mutants – e.g. to investigate the impact of peptide loss on growth rate or on their bioactivity towards other organisms. Molecular genetics is thus providing indispensable tools in cyanotoxin research.

For a comprehensive assessment of human health hazards caused by the spectrum of cyanobacterial peptides required a concerted multidisciplinary research effort using the opportunities provided by the new and emerging molecular and analytical tools.

1.1 Overarching objectives of PEPCY

The central objective of PEPCY was to improve cyanotoxin risk assessment and risk management. For this purpose, PEPCY aimed to identify the peptides occurring in cyanobacteria and to assess which ones within this wide range are important for public health because of their (i) toxicity and (ii)

frequency and concentrations of occurrence. PEPCY also aimed to improve understanding of the factors determining toxic peptide occurrence, to optimise methods for detection and analysis and to contribute to the development of appropriate risk management policies.

1.2 Understanding the occurrence of cyanopeptides and cyanopeptide producers

In face of the sparse and highly fragmentary information on cyanopeptide occurrence, a major objective of PEPCY was to provide a comprehensive understanding of the variety of cyanopeptides and the organisms that produce them. This includes knowledge of the genes encoding for the production of cyanopeptides, as this enables their identification as specific "genotypes" with molecular tools. Specific targets were to:

- identify comprehensively the potentially toxic cyanopeptides from the cyanobacterial taxa most frequently present in European waterbodies
- establish the first database on the frequencies and concentrations with which cyanopeptides occur in waterbodies, including the concurring cyanobacterial taxa and ambient environmental conditions
- using representative strains from important taxa, to compare their genetic potential to produce specific cyanopeptides with the occurrence of these peptides; i.e. to check if peptide genes are always expressed as peptide chemotypes
- detect toxin-producing cyanobacterial genotypes with new genetic tools (see section 1.6) for rapid screening.

1.3 Understanding the regulation of cyanopeptide production

While the physiological regulation of microcystin synthesis has been studied intensively, there is no information available on the physiological regulation of other peptides. It is unknown whether their production varies with environmental conditions, or whether the production is switched on or off under certain conditions.

Specific targets in elucidating the regulation of cyanopeptide production were to:

- analyse the role of physiological regulation in the synthesis of bioactive peptides other than microcystins
- investigate whether or not genes are "switched on and off", or whether peptides are continuously found in those genotypes containing the respective peptide gene.

A prerequisite for predicting peptide production from cell numbers is that the genotype composition of a field population remains constant. However, if it changes seasonally and shifts occur between genotypes differing in peptide composition during certain periods of the year, levels of a given peptide per unit cyanobacterial biomass would also shift. Until very recently, this mechanism could not be addressed in field studies because genotypes differing in peptide production look the same and cannot be differentiated in the microscope. Thus, physiological regulation could not be distinguished from regulation through shifts in genotype composition. Targets of PEPCY work on the regulation of cyanopeptide production therefore also included:

- providing genetic methods suitable to quantify peptide genotypes and to follow their seasonal development
- first assessments of the variability of occurrence of different cyanopeptides and the producer genotypes in relation to environmental factors in the field
- first descriptions of the seasonal occurrence of peptide genotypes during the formation of algal blooms and during pre-bloom conditions.

1.4 Assessing cyanopeptide toxicity

In face of the highly fragmented evidence on toxicity of cyanopeptides (beyond the well-studied microcystins and nodularins), a major objective of PEPCY was to systematically characterise cyanopeptide toxicity, largely using sub-organismic assays. Specific targets were:

- provision of dose-response data on potentially toxic cyanopeptides
- description of modes of action and type(s) of effects
- identification of most likely exposure routes and subsequently assessment of potential human health hazards through exposure.

1.5 Testing toxicity to aquatic invertebrates with a rapid and simple bioassay

Many cyanopeptides are protease inhibitors i.e. inhibition of trypsin/chymotrypsin by aeruginosin (Kodani et al. 1998), cyanopeptolins (Jakobi et al. 1995)] or microviridins (Shin et al. 1996). Toxicity testing of crude extracts has demonstrated toxicity beyond that predicted from microcystins (Jungmann & Benndorf 1994, Keil et al. 2002). Recently, oligopeptides (oscillapeptin, microviridin J) with a toxicity to aquatic crustaceans that is comparable to that of microcystin have been reported (Agrawal et al. 2001, Blom et al. 2003, Rohrlack et al. 2003). An objective of PEPCY therefore was to test the applicability of a rapid and simple bioassay for assessing the toxicity of cyanobacterial peptides in water samples. This included elucidating the symptoms of damages characteristic for protease inhibitors by conducting physiological experiments with invertebrates (*Daphnia*).

1.6 Producing materials, developing new tools and standardising methods for cyanopeptide research

The provision of analytical standards, purified materials for toxicity testing and analytical methods were essential for the objective of improving cyanotoxin hazard characterization, risk assessment and risk management. Standard materials and methods allow both qualitative and quantitative data to be obtained on the regulation of cyanopeptide production, the toxicity of cyanopeptides and the formulation and implementation of a risk assessment and management approach.

In previous cyanobacterial toxin research, the need for standard analytical materials and methods has been demonstrated through the success of interlaboratory comparison exercises for microcystins (Fastner et al., 2002) and cylindrospermopsin (Torokne et al., 2004). During these trials the importance of optimised extraction procedures and analytical methods was demonstrated by the different toxin yields obtained from a common sample of pure toxin or dried cells. Recently, further evidence of the need for standard materials was demonstrated by the assessment of commercial

standards for microcystin-RR and the finding that only one out of three contained the correct quantity of the correct microcystin isoform (Kubwabo et al., 2004). In the field of cyanobacterial toxins, the need for reliable certified reference materials has been recognized as one of the major problems restricting the development of analytical methods (Meriluoto, 2004; Quilliam et al., 2004). Providing purified gravimentric standards for analyses and larger amounts of purified peptides for toxicity testing was therefore a major basis for work in PEPCY. This applied equally to molecular genetic tools, i.e the provision of primers and mutants as well as testing DNA extraction procedures. Specific targets in providing tools, materials and methods for cyanobacterial research were:

- peptide purification from laboratory cultures of cyanobacteria and environmental samples of cyanobacterial mass populations (blooms, scums) from European freshwater resources.
- establishment of quantitative physicochemical analytical methods for cyanobacterial peptides (MALDI-TOF MS; HPLC).
- establishment of quantitative procedures for peptide extraction from cyanobacterial cells and other matrices.
- establishment of quantitative immunoassay-based methods for cyanobacterial peptides.
- identification of genes involved in the biosynthesis of new cyanopeptides
- generating mutants lacking cyanopeptides as tools for assessment of toxicity and to elucidate their role in mass development of cyanobacteria
- development of quantitative molecular methods for assessing the genotype diversity of cyanopeptide-producing cyanobacteria in water bodies and for studying potential seasonal shifts in genotype composition
- provision of a set of standardised method guidelines, primarily for harmonisation of work in PEPCY and for dissemination beyond PEPCY.

1.7 Improving risk assessment, risk management and risk communication to professionals and to the general public

The central objective of PEPCY was to focus the scientific results gained in the project on improving cyanopeptide risk assessment and risk management. This implied using the scientific outcomes of PEPCY – i.e. an improved understanding of the extend to which cyanopeptides other than microcystins present a hazard to human health, and an assessment of the likelihood of their occurrence in different types of water bodes used by humans – as a basis for deriving potential exposure scenarios, decision trees and an Alert Levels Framework.

In parallel to PEPCY, the revision of the World Health Organisation's Guidelines for Drinking-water Quality (WHO 2004) developed a new approach that sets priorities towards optimising the protection of public health – the "Water Safety Framwork" (see section 2.7). This includes a new risk assessment and risk management concept for drinking-water, i.e. to develop a specific "Water Safety Plan" for a given drinking-water supply system. For cyanotoxins risk assessment and risk management requires particular attention to the specific conditions of a given setting, and because a Water Safety Plan is tailored to the specific setting, this approach has substantial advantages over more schematic approaches. Also, it focuses on process control rather than on end-product monitoring. This can emphase catchment management and resource protection in order to mitigate eutrophication and thus address the source of cyanobacterial proliferation. For the objective of developing an improved risk

management approach, PEPCY therefore based the guidance developed in the project on this WHO approach.

In particular, a target of PEPCY aimed to develop a tool to support practitioners in developing a risk management strategy based on the new WHO approach. This included dissemination materials for professionals, linking managagement guidance to more detailed background information on hazard characterisation and risk assessment. A further target was the provision of model materials for the general public focusing on information about the hazard and personal options for recognition and exposure avoidance.

1.8 Structural approach to the PEPCY Project

At the outset of PEPCY it was clear that a large large number of cyanopeptides occur and that *in vitro* assay results indicate a range of modes of bioactivity, suggesting toxicity. Research in PEPCY therefore needed to focus on selected representatives from each cyanopeptide class. Criteria for choosing the most relevant peptides would be (i) frequency of their occurrence in water-bodies at concentrations causing effects and (ii) their impact on human health, i.e. the criterion "relevance" depends both on occurrence and toxicity. Such information was available only for microcystins (MCYSTs). For the other cyanopeptides, quantitative screening of occurrence required producing purified gravimetric standards, and toxicity testing required large-scale peptided purification.

Optimising the choice of peptides for production and purification thus requires several iterations, as indicated in Fig. 1. As data on the environmental concentrations of cyanopeptides were completely lacking, a comprehensive representation of peptide classes was targeted for the first iteration. Close networking between partners was therefore organised in four scientific workpackages and a further workpackage addressing risk management: Fig. 1 highlights the interaction beween the 4 research-based workpackages.

WP1: New tools for characterising cyanobacterial chemotypes and genotypes

WP2: Regulation and monitoring of cyanopeptide occurrence

WP3: Quantitative analysis and purifying cyanopeptides

WP4: Toxicity and hazard characterisation

WP5: Cyanopeptide risk management.

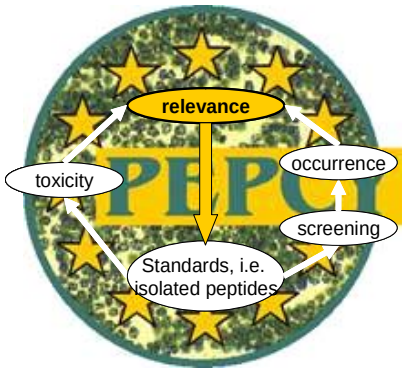


Fig. 1: Interaction in PEPCY to assess the relevance of specific cyanopeptides for risk management.

Fig. 2: Structure of PEPCY, interaction between PEPCY scientific Workpackages (shaded boxes), and outcomes (double-framed boxes)

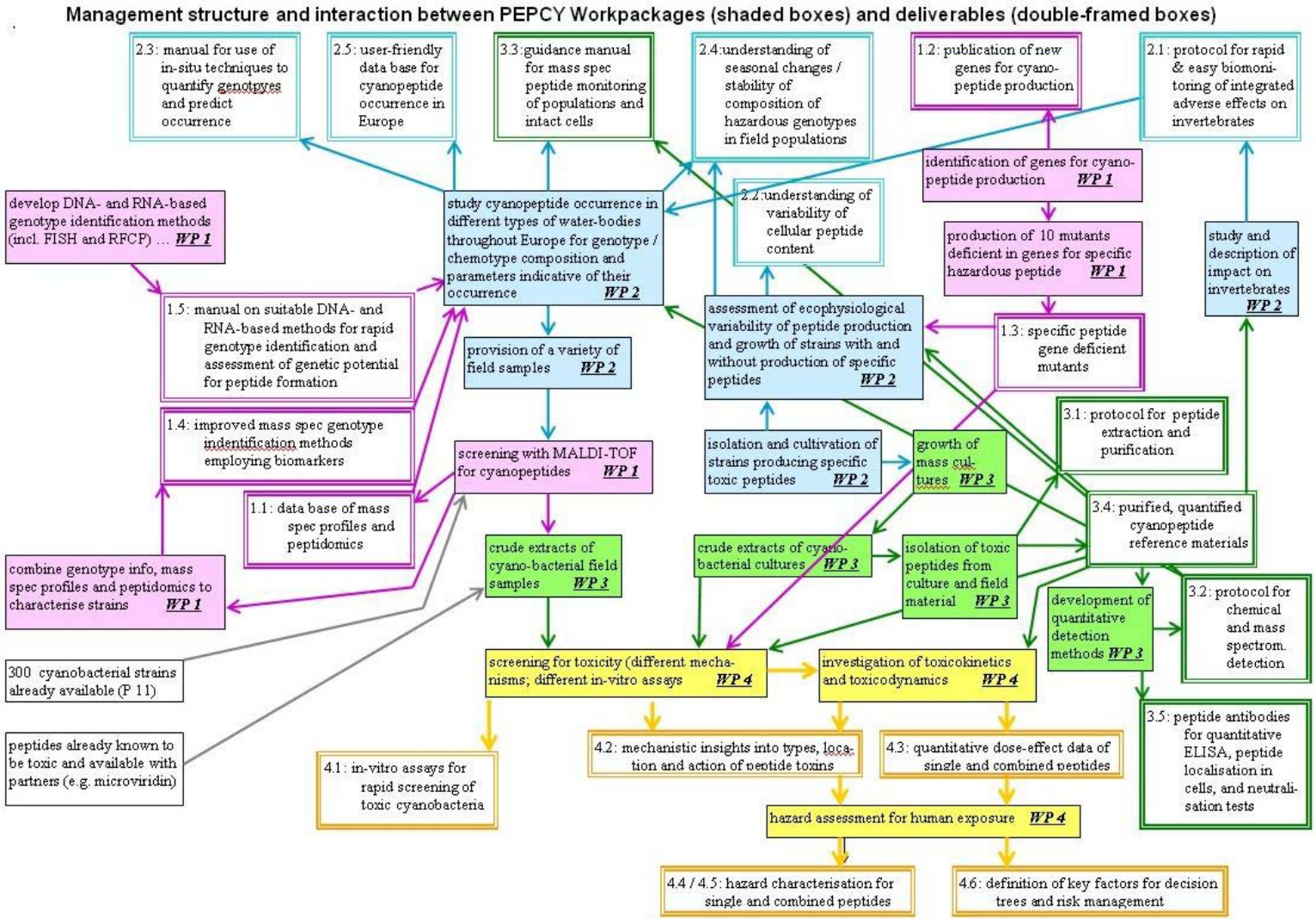


Table 1: PEPCY partner institutes, acronyms, working-group leaders, work package leaders, team members

Partner acr	Partner Institution	Group leader	Team members	Involved in Workpa PEPCY
UBA	Federal Environmental Agency (Umweltbundesamt), Berl Department for Drinking-water Hygiene	Dr. Ingrid Chorus , coordin leader of WP 5	Dr. Verena Niesel, Dr. Jutta Fastner, Grummt, Dr. Rita Heinze, Ana-Helen Ferreira, Trine Warming-Svendsen, I Gabriele Wessel	WP2, WP3, WP4,
UHel	University of Helsinki, Department of Applied Chemistry a Microbiology, Finland	Prof. Kaarina Sivonen	Dr. Leo Rouhiainen, Dr. David Fewer Rantala, Matti Wahlsten	WP 1. WP2, WP3,
UnivDun	University of Dundee, United Kingdom	Prof. Geoffrey A. Codd, lea	Dr. Louise Morrison	WP2, WP3, WP4,
UBer	Humboldt University Berlin, Germany	Prof. Thomas Börner, leader of WP 1	Dr. Elke Dittmann, Yvonne Claussne	WP 1, 2, 5
TUB	Technical University Berlin, Germany	Dr. Hans v. Döhren	Dr. Martin Welker	WP1, WP2, WP3,
UoC	University of Copenhagen, Denmark	Prof. Kirsten Christofferser	Dr. Thomas Rohrlack; Trine Warming	WP2, 5
AAS	Austrian Academy of Sciences	Dr. Rainer Kurmayer, leader of WP 2	Eva Schober, Johanna Schmidt, Ger Roidmayr	WP 2, 5
NIPH	Norwegian Institute of Public Health, Oslo	Dr. Hans Utkilen	Dr. Thomas Rohrlack, Dr. Sanne Lyc	WP1, 2, 5
UvA	University of Amsterdam, Aquatic Microbiology – Institute Biodiversity and Ecosystem Dynamics, Faculty of Science Netherlands	Dr. Petra Visser	Linda Tonk	WP 2, 5
Ukon	University of Konstanz, Department of Environmental Tox Germany	Prof. Daniel Dietrich, leader of WP 4	Dr. Stefan Hoeger	WP3, WP4, WP5
IPas	Institut Pasteur, Paris, France	Dr. Nicole Tandeau de Ma	Dr. Isabel Iteman, Sabrina Cadel	WP1, WP2, WP3,

2 Material, methods and approaches

2.1 Molecular genetic methods

2.1.1 Strain selection and culturing for elucidating peptide synthetase genes and producing peptide-deficient mutants (HUB, UHel, IPas, AAS)

Strain selection and culturing for elucidating peptide synthetase genes and producing peptide-deficient mutants (HUB, IPas, AAS)

HUB: Two *Microcystis* strains and one *Planktothrix* strain were selected as model strains for the search for biosynthetic gene clusters responsible for peptide formation, namely *Microcystis aeruginosa* PCC7806, *Microcystis* Nies 98 and *Planktothrix* Cya126. Strains were cultured at 23°C and were continuously illuminated with 40 µE $m^{-2}s^{-1}$.

UHel: Strains *Anabaena* 90 and *Nostoc* sp. 152 were selected for their known production of diverse cyanopeptides and cultivated as described previously (Rouhiainen et al. 2000; Sivonen et al. 1992b).

IPas: Axenic cultures of *Microcystis* were grown at 25°C in 50 ml of $BG11_0$ (Rippka et al. 1979) supplemented with 2mM $NaNO_3$ and 10 mM $NaHCO_3$ under a continuous photosynthetic photon flux density of 30 µmol $m^{-2}s^{-1}$ (Osram Universal White fluorescent tubes) as measured with a LI-190SB quantum sensor. Cells in exponential-growth phase (OD_{750} = 0.4-0.6) were collected by centrifugation (10,000 ×g, 10 min, 25°C) and lyophilised. *Escherichia coli* strain JM 109 was used as a host for DNA cloning and sequencing. *E. coli* was grown at 37°C overnight in Luria-Bertani medium with ampicillin as a final concentration of 200 µg/ml.

AAS: All strains were grown at 15°C and continuous low light (5-10 µmol $m^{-2}s^{-1}$). The isolates were the basis for testing the specificity of gene probes and primers for genotype quantification.

2.1.2 Identification and sequencing of peptide synthetases genes (HUB, UHel, IPas)

HUB: Peptide synthetases gene fragments were identified using a degenerate PCR approach as described previously (Neilan et al., 1999). The resulting PCR fragments were cloned and sequenced. Positive phagemide clones were identified by hybridization according to standard procedures and subsequently sequenced. Partial sequence gaps were closed using PCR and inverse PCR, respectively (Sambrook et al., 1989). Peptide synthetase sequences of *Microcystis* PCC7806 were obtained from the nonpublic genome database of the Pasteur institute (http://genopole.pasteur.fr/maeru/).

UHel: Preparation of genomic library of *Anabaena* sp. strain 90 has been described in Rouhiainen *et al*. (2000). Primers based on the two conserved sequences found in peptide synthetase adenylation domains were designed and used to amplify *Anabaena* 90 DNA by PCR. Fragments were cloned in pBluescriptSK(–) vector and were used as probes to find anabaenopeptin synthetase genes from the gene library by colony hybridization and sequenced. To produce *Nostoc* sp. 152 library DNA was sheared and a 40 kb fraction was gel purified, end repaired and directly ligated to the pCC1FOS vector (Epicentre Technologies, Madison, WI) following manufacturer instructions. The titer from this method was very low, and the entire packaging extract was used to generate a 2000-clone library. The library was screened and two pairs of overlapping fosmids containing the microcystin and nostophycin synthetase gene clusters were identified from the fosmid library. These fosmids were shotgun sequenced.

IPas: Genomic DNA was extracted from lyophilised cells following DNeasy Plant Mini Kit instructions (QIAGEN) with the following modifications: 5 mg of lyophilised cells were added to 450 µl of 50 mM Tris-HCl pH 8, 10 mM EDTA and 4 µl of RNase 100 mg/ml. The solution was applied in Lising Matrix A tubes (Qbiogene) and two 30 second runs at a speed set at 4.0 in the FastPrep® Instrument were performed to optimise cell lysis. The mixture was incubated for 10 min at 65°C and processed as described by the manufacturer. The regions of interests were amplified from total DNA of *Microcystis* strains using primers designed according to the nucleotide sequence of the aeruginosin and cyanopeptolin gene clusters available in the non-public database containing the partial genome sequence of *M. aeruginosa* PCC 7806 (provided by C. Bouchier and collaborators, Institut Pasteur - Paris). PCR amplifications of ITS (Internal Transcribed Spacer) were performed with the set of primers described in Iteman et al. (2000). Several amplified fragments were gel purified and cloned into pGEM-Tto create a clone library. The obtained PCR products were sequenced (Genome Express, Meylan, France).

2.1.3 In silico analyses of DNA and protein sequences (HUB, IPas and UHel)

HUB: The partial amino acid sequence encoded by each individual gene fragment was analyzed using the NRPS-PKS software (www.nii.res.in/nrps-pks.html) in order to predict the amino acid specificity of the corresponding enzymes. These data were used for a pre-assignment of the gene fragments to individual cyanopeptides. In order to obtain sequence information about complete gene clusters libraries were constructed using the Lambda ZAP®-CMV XR Library Construction Kit (Stratagene, La Jolla USA).

UHel: Analysis and comparisons of sequences were performed with EMBOSS (European Molecular Biology Open Software Suite). Sequence similarity searches in databases were done with Blast (Altschul *et al*. 1997). Searches for conserved domains and motifs were accomplished with the CD-Search program (http://www.ncbi.nlm.nih.gov/Structure/ cdd/ cdd.shtml) and with the Motif Scan program (http://hits.isb-sib.ch/cgi-bin/PFSCAN?).

IPas: Amino acid sequences of the cyanobacterial and bacterial putative *halogenase* genes were aligned utilising the ClustalX program in Bioedit software. ESPript and the Pfam programs were used to identify potential conserved domains. Phylogenetic trees were built in using three methods complemented in the PAUP 4.0b10 software: maximum-parsimony (MP), maximum-likelihood (ML), and neighbour-joining (NJ). For MP analysis, the tree bisection-reconnection (TBR) heuristic algorithm for searching through tree space was used. The evolutionary model for the ML and NJ methods was evaluated with MODELTEST 3.0. Statistical confidence levels for all topologies were evaluated by the nonparametric bootstrap method (100 replicates). To improve phylogenetic information, a combined analysis of the amino acid sequences with the PHYML program using different substitution models was performed. ITS sequences of the same *Microcystis* strains as those used for the study on halogenases were aligned and phylogenetic trees were constructed as described above.

2.1.4 Knock-out plasmid construction and mutagenesis (HUB)

Selected peptide synthetase gene fragments of 3-5 kb length were cloned into commercial PCR cloning vectors (e.g. pDrive, Qiagen, Holden Germany). The resulting plasmids were linearized by restriction enzymes in the peptide synthetases coding regions. Alternatively, parts of the coding regions were deleted. Subsequently the ends of the plasmids were blunted, if necessary, and dephosphorylated. A 1.4 kb BsaA1 fragment of the vector pACYC184 served as chloramphenicol (Cm) resistance gene cassette and was inserted into the prepared peptide synthetases knock-out

vectors. *Microcystis* and *Planktothrix* were transformed using the natural competence of *Microcystis* or alternatively electroporation as described previously (Dittmann et al., 1997, Christiansen et al., 2003). 10 µg of plasmid were applied. *Planktothrix* constructs were separated into single strands by denaturation prior to transformation. Selection of positive *Microcystis* clones was performed on plates containing up to 5 µg/ml Cm. Selection of *Planktothrix* mutants was performed in liquid medium containing up to 3 µg/ml Cm.

2.1.5 Design and testing of primers (UHel and HUB)

UHel: Primers were designed based on the alignment of *mcy*E gene sequences from 30 MC- or nodularin-producing *Anabaena*, *Microcystis*, *Nostoc*, *Planktothrix,* and *Nodularia* strains (Rantala et al. 2004) for use with the *mcyE*-general forward primer, mcyE-F2 (Vaitomaa et al. 2003). The specificity of the resulting primer pairs were tested with over 60 MC- or nodularin-producing and non-producing *Anabaena*, *Aphanizomenon*, *Hapalosiphon*, *Limnothrix*, *Microcystis*, *Nodularia*, *Nostoc*, *Phormidium*, *Planktothrix,* and *Synechococcus* strains maintained in the culture collection of this partner.

Similarly primers were designed and tested to amplify a part of the gene encoding the Ahp subunit from the cyanopeptolin synthetase gene cluster and a region of the gene encoding the D-lysine subunit from the anabaenopeptin synthetase gene cluster. Many of the strains (total of 91) tested from genera *Anabaena* (30/16), *Aphanizomenon* (1/1), *Nodularia* (18/14), *Microcystis* (21/13), *Nostoc* (7/1), *Planktothrix* (12/10) and *Scytonema* (2/1) gave a PCR product (with right substrate specificity signature sequence). The primers were specific according to the known producers. The PCR products were sequenced from representative strains. Sequences were used for design of primers for real-time PCR

Further primers were designed to amplify a region of the gene encoding the D-lysine subunit from the anabaenopeptin synthetase gene cluster. Many of the strains (total of 91) tested from genera *Anabaena* (30/13), *Aphanizomenon* (1/1), *Nodularia* (18/4), *Nostoc* (7/4) and *Scytonema* (2/1) gave a PCR product (correct signature sequence was found) and the primers were specific according to the known producers. *Microcystis* (21/-) and *Planktothrix* (12/-) gave no products even though the strains tested were known to produce anabaenopeptins. The PCR products from representative strains were sequenced (for possible design and testing real-time PCR primers).

HUB: The sequence information of genes for microcystin, cyanopeptolin and aeruginosin biosynthesis was used to design primers for the simultaneous detection of the three gene clusters in field situations. This approach could help to assess the abundance of these genes in the field and to correlate it to the quantities of peptides that are produced. Three primers pairs were designed amplifying PCR products of 100, 200 and 300 bp in length, respectively.

2.1.6 PCR for identifying peptide genotypes in lake water samples (UHel and AAS)

To quantify specific toxic cyanopeptide (microcystin) genotypes in lake water the application of real-time PCR was developed (Kurmayer & Kutzenberger 2003, Vaitomaa et al. 2003).

TaqMan probes via Taq Nuclease Assays (TNA) were applied by AAS, as this technique was found superior to the application of SYBR green staining and subsequent melting curve analysis. To quantify genotype calibration curves for C_t values (= cycle of threshold value, the PCR cycle number at which fluorescence passes a set threshold value) from PCR in relation to cell counts (DAPI) were established and successfully validated against background DNA. For calculating the numbers of genotypes (in cell equivalents ml^{-1}), calibration curves were established by counting cells and performing real-time PCR-analyses from the same cultures. These showed highly significant

correlations between PCR cycles and the cell numbers determined in the microscope. The proportions of cyanopeptide genotypes were calculated by dividing their cell concentrations by the corresponding cell concentrations of phycocyanin genotypes. TNAs were developed for *Planktothrix* spp. including four genotypes, i.e. phycocyanin (PC), *mcy*B, aeruginosin, anabaenopeptin. Only those fluorescence signals were considered that were within the central range of the calibration curves (40 – 40,000 cells in the template for PCR reaction). In total 83 samples (integrated phytoplankton net samples) were analysed.

UHel: To identify potential MCYST producers in lake samples, PCR was performed with four primer pairs designed to amplify regions of the *mcyE* gene. In all reactions, the same forward primer, mcyE-F2 was used (Vaitomaa et al., 2003). All potential MC-producing genera were targeted with the use of a general reverse primer, mcyE-R4 (Rantala et al. 2004), and MCYST-producing *Anabaena*, *Microcystis*, and *Planktothrix* with genus-specific reverse primers, mcyE-12R, mcyE-R8 (Vaitomaa et al., 2003), and mcyE-plaR3, respectively (Rantala et al. 2006).

PCR results were compared to cyanopeptide analyses and correlated to environmental variables Regression analyses were performed to determine whether variation in the response variables, the presence-absence of the three genus-specific *mcyE* genes, and MC concentrations (log transformed) of the concentrated water samples, could be explained by environmental variables.

HUB and IPas: Based on the sequences of the aeruginosin, cyanopeptolin, and microcystin synthetase gene clusters of *Microcystis* PCC 7806 primers were developed for PCR detection of respective genes in *Microcystis*. The primers were designed to allow the simultaneaous detection of all three gene clusters by a single multiplex-PCR reaction. The PCR results were validated by the comparision of peptide production by individual strains as studied by MALDI-TOF MS, HPLC/MALDI, and LC-MS/MS analyses. Multiplex-PCR was then applied to single *Microcystis* colonies sampled in lakes near Berlin in 2005. Data analysis and experiments are still ongoing.

2.2 Methods for peptide detection and quantification

2.2.1 MALDI-TOF MS for identifying peptides (TUB)

Cyanobacterial field samples, strains, single colonies and filaments were analyzed for their peptide content by MALDI-TOF-MS (Erhard et al. 1997, Welker et al. 2006, Czarnecki et al. 2006). The performance of the mass spectral analyses were markedly improved by optimization of the sample preparation procedures. This included the use of particular templates and marix solutions for the analysis of certain types of samples. For example, HPLC-fractions were generally prepared on 2x96 well teflon coated plates with a solution of 20 mg DHB to guarantee optimal sample concentration and crystallization.

A crucial part of the peptide identification was the collation of data on known peptides as well as the *in-silico* generation of theoretical peptides based on residues that have been observed in individual positions. By doing so a large number (>1000) of peptide were generated that were likely to be produced in-vivo (for more details see Welker & von Döhren (2006) and Welker et al. (2006). This database was used as a starting point when peptide masses were encountered that could not be attributed to known peptides.

For identification and elucidation of new peptides in PEPCY mainly mass spectral methods were employed. The rapidly increasing database of reference fragment spectra together with the calculation of theoretical fragment steadily improved the process of structure elucidation and peptide identification process. Structures of peptides were deduced essentially from MALDI-TOF MS PSD/CID data and

compiled in a mass/fragment database to be used in the identification of peptides in various samples (Sejnohova et al. 2006; Saker et al. 2006; Vasas et al. 2006).

2.2.2 MALDI-TOF MS for identifying peptide chemotypes in lake water samples (TUB)

MALDI-TOF MS was employed throughout the project for the identification of cyanpopeptides in environmental samples as well as to assist in the identification of particular peptides in fractionated samples (mentioned above). Furthermore, MALDI-TOF MS was used to characterize peptide chemotypes in field samples based on analyses of single colonies and filaments. The high through-put potential allowed the analyses of samples in sufficiently high numbers for further analyses of the communities studied by applying statistical approaches.

To evaluate the reproducibility and accuracy of peptide analysis of single filaments using sensitive MALDI-TOF MS, 11 clonal strains of *Planktothrix* (isolated from alpine lakes) were analyzed (i) by isolating single filaments and subsequent MALDI-TOF MS analyses and (ii) by fractionating peptide extracts from aliquoted cells using HPLC-PDA and subsequent MALDI-TOF MS analysis of the fractions. From each strain 5 filaments were isolated. In parallel from the same culture 1 mg of dry weight was extracted in 50% MeOH (v/v) on ice, the peptide extract was injected into HPLC-PDA, all visible peaks were collected at 210 nm and analysed using MALDI-TOF MS.

Single *Plankothrix* filaments (n=1233) and single colonies were analysed using automated data acquisition. Each sampling spot was scanned by 300 laser pulses, the resulting signals of which were accumulated in a result mass spectrum. Filaments yielding weak mass signals (n=250) and high background noise were analysed manually for a second time. Samples with mass spectrograms lacking chlorophyll-*a* derivatives (identified from the typical isotopic distribution) and lacking mass signals of peptides frequently produced by *Planktothrix*, i.e. anabaenopeptin B ([M+H 837], Kurmayer et al. 2004) were not considered in further analysis. Mass signals were compared to the results available for *Planktothrix* and other cyanobacteria from earlier studies (Fastner et al. 2001, Welker et al. 2004, and unpublished data). Only mono-isotopic masses were extracted from mass signals with signal-to-noise ratios >5. Mass signals occurring only once and mass signals that could not be linked to any known peptide in *Planktothrix* or other pelagic cyanobacteria were excluded from the data set. The most intense mass signals (n=30) were used for PSD analysis.

Microcystis colonies were washed and placed directly on stainless steel templates (Czech samples) or divided and one half used for mass spectral analysis and the other half for PCR studies. All colonies were photographed and measured before analysed. Extraction for peptide analysis was performed directly on the templates as described in Welker et al. 2006.

Mass spectra were screened for known and theoretical peptide masses and intense mass signals were analysed further by PSD/CID-fragmentation. Data on all identified peptides were collected as presence/absence data for each individual colony.

2.3 Producing peptide standards for chemical analyses and for toxicity testing (UnivDun, UHel, UBA)

Choice of strains and peptides: Due to the large number of cyanopeptide families and isoforms known, and in light of the current exponential phase of cyanopeptide discovery (in part due to the early findings of PEPCY) a selection of key cyanopeptides needed to be chosen as the focus of production and purification. The criteria for the selection of peptides were based on the key needs of the PEPCY Workpackages, combined to give a relevant selection (Fig. 3).

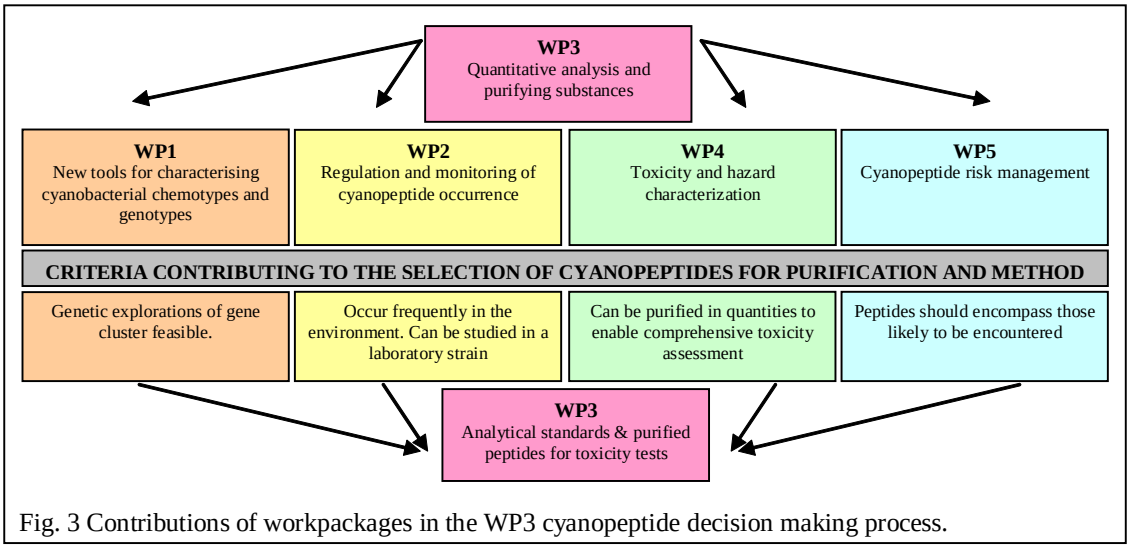


Fig. 3 Contributions of workpackages in the WP3 cyanopeptide decision making process.

Criteria for the molecular genetic work (WP1) were amenability of a peptide-producing strain to genetic manipulations and existence of background knowledge of the structure of gene clusters. Criteria for the choice of peptides for environmental research on occurrence and regulation (WP2) included frequent occurrence in the environment (as inferred from MALDI-TOF MS data) and existence of clonal peptide-producing laboratory cultures. For the toxicological testing (WP4), strains were chosen which produce the respective peptide in sufficient quantities for large-scale purifications and within a peptide spectrum that allows effective separation to ensure that no cross contamination resulted in the purified material. To meet these criteria, the strains chosen for large-scale peptide purification were largely different onese than those used for batch culture experiments. For risk assessment (WP5) selected peptides were to represent those likely to be encountered most frequently in the environment, and to include at least one example of every peptide class. This was particularly relevant as, until toxicity testing could be carried out and peptide potency determined, those peptides not detected frequently in the environment may yet present a hazard to human health. Finally, peptide yield and purity were criteria of practical relevance when choosing strains for peptide production.

Strains cultured for peptide production therefore included some previously known for their ability to produce a specific peptide, such as *Nostoc* CYA 152 and also strains known to yield high quantities of a particular peptide, but which are mainly free of other peptides, or produce other peptides in substantially smaller quantities, such as *Planktothrix* 2. In some cases, initial screening of prospective peptide sources was carried out by intact-cell MALDI-TOF MS, followed by analysis of fractionated samples and isolated peaks. All together, 11 cyanobacterial strains were grown in mass culture for peptide production. Additionally, where a particular peptide was not available from a laboratory culture, not produced in sufficient quantities in a laboratory culture, or where the presence of additional compounds inhibited the isolation of a pure compound in a laboratory culture, environmental bloom material was used as the biomass source for isolations and purification. Over 300g of lyophilized biomass was produced in mass cultures and more than 40g of environmental bloom material collected for the extraction and purification of bioactive cyanopeptides.

Peptide extraction and purification: Small-scale extraction protocols were previously developed and in use for microviridin J (Rohrlack et al., 2004). However, for use in the extraction of samples for batch culture experiments, protocols were optimized for cyanopeptolins and microcystins from *Microcystis*

PCC 7806 (UoA) and for Nostophycin from Nostoc CYA 152 (AAS). Method development strategies were devised and initiated for high-performance liquid chromatography of small, peptide-containing samples in a set of guidelines (see PPGs in the annex). On this basis, specific adaptations with respect to e.g. the quantity of cells to be extracted, solvent type, solvent strength, extraction time, extraction temperature and mechanical agitation, were undertaken by laboratories in the PEPCY consortium to optimise procedures for their respective strains, peptides of interest and laboratory conditions.

For large-scale extraction and purification of cyanopeptides for the provision of materials for analytical standards and larger quantities for toxicity testing less emphasis was placed on the exact quantification of peptides, but preferentially upon obtaining a high yield, increasing purity of peptide of interest, or maintaining presence of more than one peptide for multi-peptide samples. This included avoiding the need to handle large amounts of methanol. For example, microviridin J had previously been purified using 50% methanol extraction (Rohrlack et al., 2003). Large volumes of methanol are a concern for work-place safety. Their concentration in solid phase extraction would require large dilution, which can hardly be subsequently concentrated by rotary evaporation (which would require a second lyophilization step). Therefore, yields using the established 50% methanol extraction method were compared to extraction using 5% acetic acid (Fig 4.2), as is used for hydrophilic microcystins.

Solid phase extraction (SPE) was used to concentrate the peptide content of a large sample volume and also as purification step, both through its selective binding of matrix components, and through fractionation during the elution procedure. The concentration step proved important for the large-scale purification of peptides to substantially reduce the time required, resulting in a more efficient and convenient method. Peptides were purified using HPLC. For some of the peptides required in large quantities for toxicity testing and immunoconjugate production, optimization of large-scale extraction procedures was required to produce fast efficient methods where yields could be maximized.

Production of gravimetric peptide standards: Analytical gravimetric standards were produced by accurate weighing using a micrgogram microbalance (e.g. Sartorius) and specialist weighing tools. Substances were weighed until at least three consecutive constant weights were obtained, the cyanopeptide was then reconstituted and aliquoted using a calibrated micropipette. Statistical analysis (e.g. ANOVA) was carried out using at least 4 randomly selected replicates which were reconstituted and measured using, for example, HPLC. The procedure to prepare, weight and aliquot cyanopeptides for use as analytical standards is described in detail in PPG_UnivDun002 (see annex).

Table 2 shows an example of the rolling record with which peptide production was managed between the partners of the PEPCY consortium. Tab. 3 summarised the purified peptide reference materials produced.

Tab. 2. Example (extract) of working-spreadsheet of cyanobacterial mass culture and purification of peptides

Peptide sourc	Peptides	Lab providi	Mass cul	Batch cu	Grams d	mg (approx partially pu	mg (appr peptide	mg gravime peptide sta
A. Laboratory strains								
Anabaena OTU11		Uhel		UoA				
	Unknown pepetide (685)							
Anabaena 90		UHel	UHel	UoA	71 g			
	Anabaenopeptilide 90A					Finalised en		
	Anabaenopeptilide 90B							
	Anabaenopeptin A							
	Anabaenopeptin B							
	Ananbaenopeptin C							
	[Asp3]MC-RR							
	MC-RR							
	[Asp3]MC-LR							
	MC-LR							
Anabaena NRC 525-17		Carmichel	UnivDun		10 g			
	Anabaenopeptin A					2 mg	2 mg	30 x 0,01 mg
	Anabaenopeptin B					2 mg	see Plank	see Planktot
Aphanizomenon X23		Welker	TUB		23 g			
	Anabaenopeptin I							
	Anabaenopeptin J						8 mg	
	New peptide 642 Da							
	unknown peptide (M+H=(						3 mg	
	unknown peptide (M+H=5						10 mg	
Aphanizomenon X08a		Welker	UBA					1 mg in 0,01
	Anabaenopeptin F						8 mg	
	unknown peptide (M+H=5						11 mg	
Microcystis PCC7806		lPas	lPas	UoA				
			UoC	UoC	ca. 40 g			
	[Asp3]MC-LR							
	MC-LR							
	Cyanopeptolin A							
	Cyanopeptolin C							
	Cyanopeptolin 971							
Microcystis CBS		UoC	UnivDun		34 g			
			UKon		>25 g (6 g to UnivDun)			

Tab. 3: Summary of purified peptide reference materials produced in PEPCY

Legend: [a]:an additional 25 mg of anabaenopeptin B was required for immunoconjugate preparation. [b] in addition to that material purified on a large-scale, smaller quantities (0.25-1mg) was required for the preparation of gravimetric standards.

Peptide	Institution	Large quantities pu	Gravimetric standard pr
Anabaenopeptin A	University of Dunde	2 mg (semi-pure)	
		10 mg	Yes
Anabaenopeptin B	University of Dunde	2 mg semi-pure	
		10 mg	Yes
		25 mg[a]	(see above)
Anabaenopeptin D	University of Dunde	4 mg	Yes
Anabaenopeptin F	Umweltbundesamt	8 mg	Yes
Anabaenopeptin I	Umweltbundesamt	?	No
Anabaenopeptin J	Umweltbundesamt	8 mg	No
Oscillamide Y	University of Dunde	8 mg	Yes
Nostocyclin	University of Dunde	1 mg	No
Cl Cyanopeptolin W	Umweltbundesamt	-	Yes
Oscillarin	Umweltbundesamt	6 mg	Yes
Nostophycin	University of Helsin	110 mg	Yes
Cyanostatin A	University of Dunde	10 mg	No
Microviridin J	University of Dunde	5 mg	Yes
Aeruginosamide	Umweltbundesamt	-	Yes
Oscillatorin	Umweltbundesamt	3.4 mg (semi-pure)	No
Unkown [M+H=645]	Umweltbundesamt	3 mg	No
Unkown [M+H=592]	Umweltbundesamt	10 mg	No
Unkown [M+H=562]	Umweltbundesamt	11 mg	No
Unkown [M+H=1094,	Umweltbundesamt	11.3 mg (semi-pure	No

2.3.1 HPLC for quantifying peptides

While identification of cyanopeptides by HPLC is generally hampered by the lack of distinct and unique UV spectral characteristics (as are found with microcystins), HPLC-PDF was applied to identify and quantify cyanostatins A and B, anabaenopeptin A together with microcystins from environmental bloom samples HPLC-PDA in samples from an archive of environmental bloom samples collected from a single waterbody. Cyanopeptides in these samples were identified on the basis of subtle differences in UV spectral characteristics in combination with retention characteristics and spiking samples with the analytical standards produced as described above. The procedure is described in detail in PPG_UnivDun001 and was tested using a 21-year archive of cyanobacterial bloom samples (Morrison et al., submitted).

2.3.2 LC-MS for identifying and quantifying peptides

Samples from the intensively investigated lakes were analysed for peptide concentrations using LC-MS techniques (NIPH) or LC-MS-MS techniques (UBA).

NIPH focused on the waterbodies dominated by *Planktothrix rubescens*, i.e. three lakes in the Austrian Alps (Mondsee, Wörthersee, Irrsee) and Lake Steinsfjorden in Norway. The peptide concentrations were determined using microcystin-LR as internal standard and were expressed as equivalents of microcystin-LR. This was possible because natural occurrence of microcystin-LR could be excluded on the basis of prior knowledge of the peptide spectrum produced in these lakes.

UBA analysed the remaining samples, usually with mixed populations of cyanobacteria or dominated by *Microcystis*. LC-MS-MS was optimized for simultaneous quantification of six peptides (aeruginosamide, anabaenopeptines A, B, F, Cl-cyanopeptolin W and oscillamide Y), using the gravimetric standards produced within PEPCY. A quantitative LC-MS/MS analysis method was established for six peptides for which environmentally relevant analytical standards were produced as described in section 2.2.5. A crude extract was separated on a C18 column with a gradient of 0.1 % acetic acid and acetonitrile and the peptides detected and quantified in the multiple reaction monitoring (MRM) mode. The MRM mode allows a very sensitive and selective analysis as it is based not only on the detection of the quasi-molecular ion, but in addition on the detection of characteristic fragments from the analyte. Temporary quantification of other peptides in these samples by relation to an internal peptide standard was not pursued for these samples because of their high diversity of peptides which did not allow excluding any from potential natural occurrence.

2.3.3 Elisa for quantifying microcystins (UHel and UniDun)

UHel: For MC measurements by ELISA, unconcentrated 5-ml water samples were sonicated (Labsonic U, Braun) for 2 min with 0.5 sec repeating duty cycle, and filtered through 0.2 µm polyethersulfone membrane (Whatman Puradisc 25 AS). MCs were detected with an EnviroGard Microcystins Plate kit (Strategic Diagnostics Inc.) according to the manufacturer's instructions. The detection limit of MCs was 0.1 µg/l. Absorbances were measured with an iEMS Reader MF (Labsystems) at wavelengths of 450 nm and 620 nm.

2.4 The approach to culture experiments for studying physiological regulation of peptide production (AAS, UOC, NIPH, UBA, UvA, UHel)

The approach to culture methods was harmonized between the 6 partner institutes involved with regard to the experimental design and environmental factors studied, the nutrient media used, and the parameters to be determined (see PPG 3.1.6). Environmental conditions addressed by experiments include nutrient limitation (nitrate and phosphate), light and temperature. While the conditions addressed were agreed across partners, each laboratory following local criteria defined the specific regimes studied, as this was sufficient for capturing ranges of variation. Experiments were conducted with eleven cyanobacterial strains and one knock-out mutant to test for continuity or discontinuity of the production of bioactive cyanopeptides. Each strain tested contained a range of cyanopeptides. Experimental designs were as follows:

Microcystis RST9501, *Microcystis* NPRG-2, *Microcystis* BM 10, *Aphanizomenon* X008a (UBA): The strains were cultured in batch cultures with ASM-1 medium and samples were taken during the exponential growth phase.

Planktothrix agardhii PT2, *Planktothrix rubescens* NIVA-CYA 406 (NIPH): Batch cultures of these strains growing with O2 medium (VanLiere & Mur 1978) were used with a low start density and a limited supply of a given resource (light, N, or P) and were sampled on a daily basis. The increase in culture density led to a gradual decrease in light, nitrogen or phosphorus available to the individual *Planktothrix* cells.

Microcystis aeruginosa MRC and MRS (UOC): Strains were raised in batch culture in O2 medium with irradiance decreasing from 125 µmol m^{-2} s^{-1} down to 2 µmol m^{-2} s^{-1}.

Nostoc 152 (AAS): The strains were cultured in batch cultures with O2 medium and samples were taken during the exponential growth phase. A 2^k levels factorial design was used, where k is the number of four factors that have been tested: P-PO_4 reduction (0.14 µM P-PO_4 vs. 144 µM), N-NO_3 free conditions (5.88 mM N-NO_3 vs. N free, light reduction (50 µmol m^{-2} s^{-1}, 1 µmol), temperature reduction (20°C and 12°C).

Microcystis PCC7806 and *Anabaena* 90 (UvA): Semi-continuous cultures were grown to test the impact of high vs. low light intensity, with photon flux densities chosen according to the optima of the species (i.e. (*Microcystis* PCC7806: 150 µmol m^{-2} s^{-1}; *Anabaena* 90: $I_{average}$ = 50 µmol m^{-2} s^{-1}, I_{in} = ± 75 µmol m^{-2} s^{-1}) and low light (PCC7806: 40 µmol m^{-2} s^{-1}; *Anabaena* 90: $I_{average}$ = 20-24 µmol m^{-2} s^{-1}, I_{in} = ± 30 µmol m^{-2} s^{-1}). Continuous cultures were used for nutrient limitation: *Microcystis* PCC7806 was grown at growth rates of 0.2 d^{-1}, 0.27 d^{-1} and 0.35 d^{-1} under phosphate limiting conditions with P-limiting O2 medium containing 6 µM P. Additionally, the culture was grown at µ = 0.18 day^{-1} on nitrogen limiting O2 medium (200µM N). In these experiments on nutrient limitation the average photon irradiance was 100 ± 15 µmol m^{-2} s^{-1}. *Anabaena* 90 was grown at two different growth rates (µ = 0.21 d^{-1} and µ = 0.26 d^{-1}) under phosphate limiting conditions with P-limiting O2 medium containing 6 µM P, and with an average photon irradiance of 22.5 ± 3 µmol m^{-2} s^{-1}. Batch cultures were used for temperature experiments (20 and 30˚C). In all cultures O2 medium was used.

Anabaena 90 wild type and its anabaenopeptilide (cyanopeptilide) minus mutant (UHel): Two batch culture experiments were conducted at 22°C in temperature controlled water baths in 250 ml Erlenmeyer flasks with 100 ml of liquid medium under continuous cool white fluorescent light (Daylight DeLuxe tubes; AIRAM, Helsinki, Finland). the growth medium was Z8 minus nitrogen, where the concentration of PO_4–phosphourus was reduced by prplacing K_2HPO_4 with KCl, so that the concentration of potassium remained unchanged. the nine light (from 7 to 87 µmol m^{-2} s^{-2}) and phosphate levels from 5 to 2600 µg L^{-1}) were determined by central composite design to minimize the number of replicates and to allow estimation of quadratic effects and interactsions between factors (details see Repka et al., 2004).

Quantification of changes of cyanopeptide content in cultures: In all experiments cells were counted under the microscope or using an electronic particle counter and cyanopeptide content was related either to cells or biovolume (depending on species). For peptide quantification, samples were extracted for peptides using 50% MeOH (v/v) after optimisation of extraction procedures as given in PPG UnivDun_004. The extracts were analysed by LC-MS (*Planktothrix*) or HPLC (others). As standards were available only for some of the cyanopeptides, i.e. microcystin LR, microviridin J, anabaenopeptin B, nostophycin, absolute cell quotas could not be calculated for the others. For these, relative changes in peptide per unit biomass or cell are used to assess the impact of environmental factors.

2.5 Field samples

2.5.1 Sampling of water bodies

The sampling program had two components: (i) intensively sampled lakes (7 lakes sampled several times per season) and (ii) occasionally studied lakes (30 lakes) during the years 2003, 2004, and 2005. Sampling was organized by 5 partners (AAS, UOC, UHe, UvA, NIPH) and included some external collaboration. Guidelines developed at the beginning of the project standardized data acquisition, i.e. methods of sampling, estimation of key environmental parameters (see below.), microscopical counting of phytoplankton, and the preparation of samples for genotype analysis and peptide analysis (see PPG 3.1.6 and Annex). Net samples were collected from the water column with a plankton net (30 µm in mesh size) by vertical tows and used for peptide analysis. These samples formed the basis for the internal database on phytoplankton occurrence and peptide occurrence (see Tab. 4).

Tab. 4: Waterbodies sampled either intensively (bold letters) or occasionally; Au = Austria, D = Germany, DK= Denmark, NL = Netherlands, NOR = Norway

Country	Lake	Surface Ar	Max. de	Altitude [m	Latitude	Longitude	Trophic state
AU	Alte Donau	1,5	6,8	157	48°12' N	16°20' E	mesotrophic
AU	Benda-teich	?		157	48°12' N	16°20' E	
AU	Grüner See			776	47°54' N	15°05' E	
AU	Heustadl-wa	4,7	3,3	154	48°12' N	16°20' E	eutrophic
AU	Holzöster-se	0,1	4,7	460	48°03' N	12°54' E	eutrophic
AU	Irrsee	3,5	32	533	47°55' N	13°18' E	oligo-mesotrop
AU	Mondsee	13,8	68,3	481	10°23' N	47°48' E	mesotrophic
AU	Neue Donau	3,5	6	157	48°12' N	16°20' E	mesotrophic
AU	Offensee	0,6	38	649	47°45' N	13°50' E	oligotrophic
AU	Ottensteiner	4,5	69	490	48°35' N	15°19' E	eutrophic
AU	Schwarzens	0,5	54	716	47°45' N	13°30' E	oligotrophic
AU	Wolfgang-se	12,8	113	538	47°45' N	13°25' E	oligotrophic
AU	Wörthers-ee	19,4	85,2	439	46°37' N	14°07' E	mesotrophic
D	Wannsee	2,7	10	32	52°25' N	13°10' E	eutrophic
DK	Arreskov sø	3,2	3,7	32	55°09' N	10°18' E	eutrophic
DK	Arresø	39,8	5,9	3,9	55 99' N	12°11 E	eutrophic
DK	Bryrup Lang	0,4	9	8	56 01' N	09°31' E	eutrophic
DK	Esrum sø	17,3	22,5	5	55 58' N	12°22' E	oligotrophic
DK	Farum Sø	0,12	14,7	20	55 48' N	12°22' E	eutrophic
DK	Frederiksbor	0,2	9	27	55 56' N	12°18' E	hypertrophic
DK	Halle sø	0,3	3,8	8	55 59' N	09°28' E	eutrophic
DK	Kimmers-lev	0,4	6,8	30,8	68°88' N	?	mesotrophic
DK	Langesø	0,2	4,5	25	55°26' N	10°11' E	eutrophic
I	Arancio	2,2	26	170	37°38' N	13°04' E	hypertrophic
NL	Klingen-berg	0,3	35	0	52°4' N	05,02' E	mesotrophic
NL	Slotermeer	12,4	6	0	52,9' N	05,63' E	eutrophic
NL	t Joppe	0,9	42	13	52°2' N	04°50' E	eutrophic
NL	Tjeuke-meer	20	5	0	52°5' N	05°50' E	eutrophic
NL	Zegerplas	0,7	34	0	52°13' N	04°67' E	eutrophic
NOR	Steins-fjorde	13,9	24	63	60°05' N	10°19' E	mesotrophic

Sampling further included individual filaments of *Planktothrix* and individual colonies of *Microcystis*, directly selected from fresh samples collected by plankton net tows. For *Planktothrix agardhii* and *P. rubescens* populations, samples were taken from 12 European lakes (see Tab. 5), and individual

filaments were isolated and identified as described in Kurmayer et al. (2004) for single-filament PCR and single-filament MALDI-TOF MS. For *Microcystis* spp., individual colonies were selected from pelagic and sediment samples from Brno Reservoir at three occasions during 2004 as well as from a pond near Trebon (both Czech Republic), and from 3 sites along the Havel River System, including the Lakes Wannsee and Schlachtensee, in Berlin (Germany).

Tab. 5: : Waterbodies sampled for single-filament analysis, the rightmost column gives the number of filaments taken.

lake name	species	latitude	longitude	altitude [	surface area [km²	maximum depth [m]	filaments analysed
Ammersee (DE)	P. rub.	47°59' N	11°07' E	533	47	81	101
Lac du Bourget	P. rub.	45°44' N	05°51' E	231	42	145	113
Hallwiler See (C	P. rub.	47°17 N	08°12' E	449	10	48	108
Havel (DE)	P. ag.	52°45' N	12°10' E	23	river	4	50
Lago Maggiore (	P. rub.	45°28' N	08°40' E	193	212	372	101
Mondsee (AT)	P. rub.	47°49' N	13°22' E	481	14	68	106
Jezioro iedwie (I	P. rub.	53°34' N	14°53' E	14	35	44	109
Sapanca (TU)	P. rub.	40°44' N	30°15 E	30	47	55	110
Släensø (DK)	P. ag.	56°7´ N	9° 37´ E	24	0.182	11.5	103
Talsperre Weida	P. rub.	50°42' N	11°58' E				101
Wörthersee (AT	P. rub.	46°37' N	14°09' E	439	19	85	108
Zürichsee (CH)	P. rub.	47°16' N	08°53' E	406	87	143	150
						sum	1260

2.5.2 Environmental parameters and phytoplankton biomass

Temperature (°C), total phosphorus (TP; µg/l), PO_4-P (DIP; µg/l), total nitrogen (TN; µg/l), NH_4-N (µg/l), NO_3-N + NO_2-N (µg/l), water color (mg/l Pt), pH, chlorophyll-*a* (chl-*a*; µg/l), and Secchi depth (m) were determined using standard methods established in the partner laboratories. Dissolved nitrogen (DIN) was calculated as a sum of NH_4-N and NO_3-N + NO_2-N. Phytoplankton and cyanobacterial biomasses (mg/l) and species composition were analyzed by microscopy (at UHel using a Nordic variant of the Utermöhl technique) by trained investigators. Cell counts were converted to biovolumes, either from average cell volumes determined for each species in the specific sample from measuring cell dimensions or from standard cell volumes for the taxa, e.g. from the phytoplankton database of the Finnish Environment Institute (www.environment.fi).

2.5.3 DNA extraction and purification from environmental samples (AAS and UHel)

AAS: DNA extraction from filters was optimized following standard phenol-chloroform procedures and compared with commercially available DNA extraction kits (Kurmayer & Kutzenberger 2003, Schober & Kurmayer 2006).

UHel*:* DNA was extracted from filters with a modified hot phenol method (Rantala et al. 2006).

2.6 Cyanopeptide toxicity testing

In order to clarify the possible toxicity of some of these new peptides, basic toxicity tests were carried out: cytotoxicity test with HepG2 cells and CaCo2 cells exposed to purified and semipurified peptides or to cyanobacterial extracts from several field samples or laboratory culture strains. The apoptotic and necrotic potential of purified and semipurified peptides was investigated with the JURKAT cell line. An Ames test was employed to determine the genotoxicity of single peptides. As toxicokinetics in general have been neglected so far with regard to dose-response relationships of cyanobacterial toxins, organic anion transporting polypeptide transfected HEK293 cells were used to investigate whether active transport of these purified cyanopeptides has any influence on observable cytotoxicity. Microcystin-LR was used as toxicity standard and positive control for all of the transport and cytotoxicity tests. Extraction and crude fractionation of extracts from lyophilized cyanobacterial cells for bioassays followed the procedure detailed in the Pepcy Practical Guideline (see PPG_UnivKon003 and section 2.2.3 above. Briefly, dried cyanobacterial samples were extracted successively with 5% acetic acid, 100% and MeOH, using C18 cadridges for clean-up of the MeOH extract. The resulting aqueous and methanolic extract consisted primarily of a hydrophilic, moderately hydrophilic and hydrophobic components of each cyanobacterial sample. The purified peptides, lab cultures and field samples tested are given in Table 6.

Table 6: Overview of the peptides, semipurified peptides and extracts tested

Peptides / Samples	Cyanobacterial extracts (3 fractions each)		
	Code	source	Genus/species
Aeruginosamide	PEX3	Lab culture	P. agardhii
Anabaenopeptin A	PEX11	Lab culture	Aphanizomenon
Anabaenopeptin B	PEX13	Lab culture	Microcystis
Anabaenopeptin D (putative)	PEX16	Lab culture	Anabaena
Cyanostatin - A	PEX18	Lab culture	Phormidium
Microginin (putative)	PEX19	Lab culture	P. agardhii
Microviridin J	PEX20	Lab culture	Microcystis
Nostocyclin	PEX26	Field sample	P. rubescens
Nostophycin	PEX27	Lab culture	Nostoc sp.
Oscillarin	PEX29	Lab culture	P. agardhii MC-Knock-out
Oscillatorin	PEX30	Lab culture	P. agardhii Aeruginosin-knock-out
Semipurified peptide	PEX31	Lab culture	P. agardhii Anabaenopeptin-knock-out
F4/5 = Oscillatorin (putative)	PEX32	Lab culture	P. agardhii
F1 = UP1	PEX33	Lab culture	P. rubescens
Aphanizomenon X0023 = UP2(59	PEX35A	Field sample	mix
Aphanizomenon X008a X8D1.1 =	PEX35B	Field sample	mix
X8P2 = UP4	PEX36A	Field sample	mix
Mixed taxa in field sample	PEX36B	Field sample	mix
Mixed taxa in field sample	PEX37	Field sample	mix
	PEX38	Field sample	P. rubescens
Mixed taxa in field sample	PEX39	Field sample	mix

Cytotoxicity and Mutagenicity: The assays for cytotoxicity were performed with intestinal and/ or hepatic cell lines as detailed in PPG_UnivKon001 using MTT (dimethylthiazol-diphenyl-tetrazolium bromide) and Lactate dehydrogenase (LDH) leakage as endpoints. With this test system the metabolic activity of the cells (MTT) and the integrity of the cell membrane (LDH leackage) can be tested within one approach. The Ames test (Ames et al. 1973) was carried out according to the instructions of the manufacturer (Xenometrix by Endotell GmbH, Allschwil, Switzerland). The Ames test is based on the detection of reversely mutated histidine auxotroph strains (they need histidine for growth – histidine auxotroph) of the bacterium *Salmonella typhimurium*. When a substance is mutagenic, some of the histidine auxotroph bacteria undergo reverse-mutations and thus are capable to grow without histidine addition to the growth medium, whereas wild-type (histidine auxotroph) will not survive.

Apoptosis: During apoptosis phosphatidylserine switches from the inner membrane to the outer membrane. The phospholipid annexin V possesses a high affinity for phosphatidylserine. Annexin V does not pass intact cell membranes and therefore only binds to the phosphatidylserine at the outer membrane of apoptotic cells. Via conjugation of annexin V with fluorescin isothiocyanate, apoptotic cells can be identified and quantified using fluorescence activated cell sorting (facs).

Necrosis: Propidiumjodide does not pass intact cell membranes. If the cell membrane is damaged by a necrotic process, propidiumjodide can enter the cell and stain the nucleus.

For the apoptosis/necrosis test a commercially available test kit was used (BD Biosciences, Heidelberg). This test kit contains FITC conjugated annexin V and propidiumjodide. For FACS analysis the “calibur” system was used (BD Biosciences, Heidelberg).

The test was carried out using JURKAT cells, a human t–cell derived cell line (DSMZ). The tests were carried out in 24 well plates in duplicate. Cells were incubated for 2h with the test sample. Solvent control was methanol in test relevant concentrations, unexposed cells served as the negative control, while apoptosis or necrosis inducing disinfection side products were used as positive controls. The percentage of apoptotic and necrotic cells were determined relative to the total number of cells analysed.

Mouse assay: Lyophilised cells of *Planktothrix agardhii* CYA126 wildtype (Pex27) and the McyB deficient mutant (Pex29) were supplied by UKon. Cell suspensions were prepared in deionised water at a concentration of 100 mg/ml, ultrasonicated and administered in 1 ml intraperitoneally to 25 gm mice. Bioassays were run for up to 2 hours, behavioural abnormalities and mortalities being noted. After termination, livers were excised, examined for gross pathological changes, weighed and hepatosomatic indices determined.

2.7 Cyanopeptide biomonitoring

The bio-monitoring test systems used proteases (trypsin) extracted from zoopankton (*Daphnia*) gut material and subsequent trypsin activity assays by supplementing substrates (caseine) in vitro. Dose-response curves were established and the IC_{50} values calculated by interpolation from enzyme inhibition tested with different concentrations of purified peptides, aquaeous methanolic extracts (50/50, v/v) from lyophylised cyanobacterial strains and field samples (see PPG 3.1.6 and Rohrlack et al. 2005). Using the database, a given sample’s toxicity to an invertebrate can be compared with its cyanopeptide content and any further parameters of interest registered in the database. In addition symptoms of toxification, caused by cyanopeptides, i.e. microviridin J, were studied for three genera of

herbivorous zooplankton (*Daphnia*, *Chydorus* and *Brachionus*) (Rohrlack et al. 2003, 2004 and in prep.).

2.8 Cyanopeptide risk assessment and risk management

Alert levels frameworks as proposed by Bartram et al. (1999) have been successfully used in cyanotoxin risk assessment and risk management. They typically provide decision trees for specific management interventions to be taken at specific levels of cyanobacterial cell density and/or cyanotoxin concentrations. While the action levels and specific interventions they suggest are not *a priori* prescriptive, they do tend to be used rather schematically.

PEPCY therefore followed the new approach of the World Health Organisation's Guidelines for Drinking-water Quality (WHO 2004), in which priorities and targets are not generic, but are defined specifically for a given setting or drinking-water supply system. The overall target is to improve public health most effectively. This requires assigning top priority to controlling the hazards that have the strongest negative impact on public health. For example, in settings, in which infant mortality rates are high due to pathogens in drinking-water, or cancer rates are high due to arsenic in deep wells, these hazards are likely to take priority over cyanotoxins, and a local government may choose not yet to implement the provisional WHO Guideline value for Microcystin-LR in Drinking-water or to permit higher cyanaotoxin concentrations until the priority hazards are under better control.

In this overall "**Water Safety Framework**" it is the responsibility of the public health authorities to set the public health targets, to translate them into water quality targets for specific hazardous agents and to conduct surveillance as to whether these targets are being met. It is usually the responsibility of the drinking-water supply operators to develop a "**Water Safety Plan**" (WSP) to anazlyse the hazards in the specific setting, the risk they pose and the supply systems capapbility to keep these risks unter control. This includes ensuring that all processes which are critically important to render water safe to drink – from catchment to consumer – are under sufficiently tight control so that the targets can be met. Figure 4 summarises this approach in very simple terms, and Figure 5 details the questions to address and/or steps to take when developing a Water Safety Plan.

The Water Safety Plan concept explicitly requires compiling a competent interdisciplinary team for developing the plan. The core team is composed of different levels of operators of a given water supply that know the supply in detail (i.e. from senior management to technical staff) and are thus able to analyse the potential hazards and to assess the risk they might pose. This team may be expanded to include further experts, e.g. limnologists understanding causes for cyanobacterial development in a reservoir or toxicologists to help improve hazard assessment. Thus, developing a WSP for a given supply system explicitly calls for thinking about the expertise needed for risk assessment to be well-founded and such a plan to be effective.

An important aspect of the Water Safety Plan concept is that it explicitly calls for a continuous process, i.e. periodic review asking the question whether the WSP is still valid. This addresses the specific information about the water supply as well as changes in the state of knowledge about drinking-water safety, i.e. the scientific and technical background information. Review would ask questions such as: Has catchment use changed? Have conditions in the water resource changed? Is the treatment plant still operating with the same efficacy (or are repairs needed)? Is new information available about hazards – e.g. cyanobacterial toxins. Review includes filling information gaps recognised when the WSP was first developed or during the previous review. For example, where previously only scum scouting information was available indicating toxic cyanobacterial occurrence in a given reservoir, the first version of a WSP may have identified a need for cell counting and

cyanotoxin analyses, and this information would help focus the second edition of a WSP more sharply on situations posing a health risk.

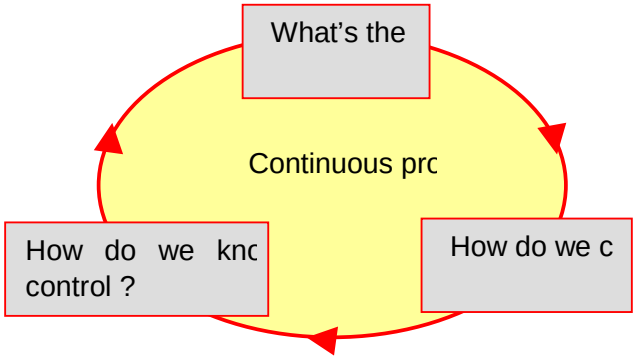


Figure 4: Summary of the Water Safety Plan Concept (after Davison et al. 2003)

Besides hazard analysis and risk assessment, the core of a Water Safety Plan is the identification of "control measures". These are management and/or technical measures by which crucial processes are kept under control to ensure they meet the targets set for the system. Control measures can be in catchment protection (e.g. setting a maximum for permitted stock density, requiring a minimum of forest cover or riparian buffer zones), in reservoir management (e.g. requiring a minimum water exchange rate, or defining criteria for artificial mixing), in drinking-water treatment (e.g. defining the required efficacy of filtration or ozonation) and in distribution (e.g. preventing stagnation in the mains or ingress of contaminants through leaks). A WSP will define "an operational monitoring system" for each control measure. Here, "monitoring" does not mean water quality monitoring, but rather process monitoring. For example, for the control measure examples in the catchment mentioned above this could be regular head counts for stock density or regular land use surveys for forest cover and for integrity of riparian buffer strips. For the example of filtration in drinking-water treatment, the operational monitoring system could be (and in fact often is) continuous turbidity monitoring at the outlet of each filter. Such process monitoring schemes incude "critical levels" that provide an alert as soon as monitoring indicates the system to perform poorly, i.e. the data generated by monitoring are "out of bounds" (e.g. stock density above the critical limit agreed in the management plan, riparian buffer zone showing gaps, forest cover reduced below the critical level, extended water retention times rendering cyanobacterial blooms more likely, turbidity above the critical limit indicating poor filtration performance in the treatment plant). The Water Safety Plan will also define "corrective action" to be taken when monitoring indicates critical limits to be exceeced. Importantly, none of these control measures or monitoring systems and critical limits are generic. Rather, the challenge is to tailor their combination to optimally fit the specifics of a given water supply system.

Further important steps in developing and reviewing a Water Safety Plan are proper documentation of the plan itself, the rationale behind each of its steps, the data from monitoring control measure performance and from water quality monitoring, as well as any events of exceedances and how the system was brought back under control.

While discussed here for drinking-water supply, the approach can also be applied to other systems exposing humans to hazardous agents, e.g. recreation, occupational safety, food production. (In fact, it originates from the HACCP-system used in food safety.)

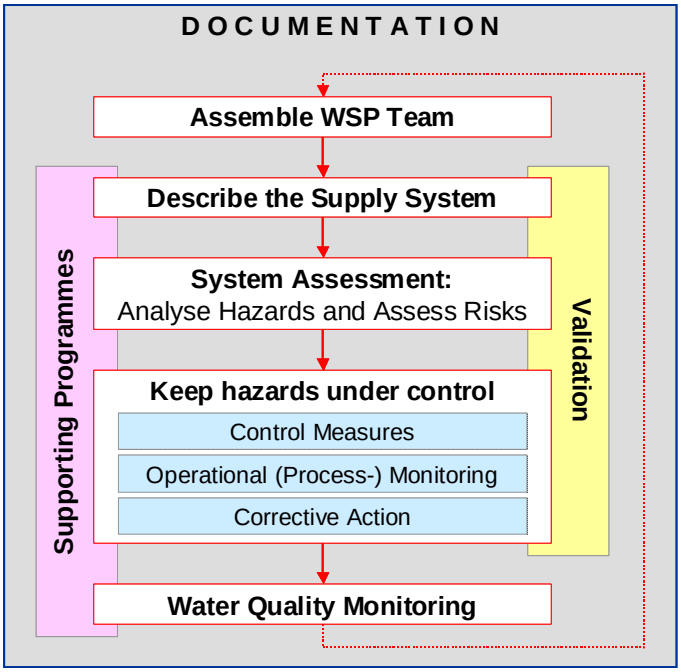


Figure 5: Steps and Components of a Water Safety Plan

The methodological approach taken in PEPCY for the objective of developing decision trees and a risk management approach was to investigate how toxic cyanobacteria could be included in developing a Water Safety Plan. Rather than providing schematic decision trees, this implied structuring a scheme of questions to guide practitioners thorugh the steps outlined in Fig. 5. This was interlinked with background documents to build a web-based decision support tool. The draft version of this tool was then distributed through the PEPCY consortium's network to practitioners for testing, and valuable feed-back was integrated into the final version.

3 Results

3.1 Methods developed in PEPCY

3.1.1 Genetic tools developed for rapid screening and detection of toxin-producing cyanobacterial genotypes

This part of the project focused on the development of PCR techniques based on primers, which could be deduced from the gene sequences identified mainly in the frame of PEPCY. In this way, new primer pairs could be designed for application in **'conventional' PCR** to detect genes for the biosynthesis of several bioactive cyanopeptides (see Methods).

The applicability and usefulness of these primer pairs for field studies was demonstrated with DNA samples from 70 lakes from Finland collected in July 2002 (before the apparent bloom forming season) and analysed by conventional PCR based on *mcy*E. The general primers found 84% of the lakes positive in PCR and containing potential microcystin producers. With genus specific primers 70% of the samples were positive for *Microcystis*, 63% for *Planktothrix* and 37% for *Anabaena*. . Interestingly, the eutrophic and hypereutrophic lakes contained highest frequencies of simultaneous occurrence of different microcystin producers (Fig. 6). In correlation analysis, the presence of multiple MC-producing genera was associated with higher cyanobacterial and phytoplankton biomass, pH, chlorophyll-*a*, total nitrogen, and MCYST concentrations. Total nitrogen, pH, and the surface area of the lake predicted the prevalence probability of *mcyE* genes, whereas total phosphorus alone accounted for MCYST concentrations in the samples by logistic and linear regression analyses, respectively. Only 20% of the nonconcentrated samples contained measurable amounts of microcystins by ELISA (Rantala et al., manuscript in preparation).

Additionally, more advanced PCR techniques were established. Three primer pairs were successfully tested fur their use in **multiplex PCR** for the simultaneous detection of microcystin, cyanopeptolin and aeruginosin biosynthesis genes in field colonies of *Microcystis* at HUB in collaboration with IPas. The technique was applied to axenic strains and to field samples in co-operation with UBA and TU Berlin. However, as with conventional PCR, there are still discrepancies between PCR data and peptide composition as determined by MALDI–TOF-MS and/or HPLC of some stains.

Quantitative Real-Time PCR assays were developed at AAS and UHel to quantify microcystin-producing *Anabaena*, *Microcystis* and *Planktothrix* strains using the TaqMan probe approach as well as SYBR green staining. The TaqMan assay was optimised and standard curves were prepared (GeneAmp5700, ABI Prism Gene Sequence Detection System). The quantitative estimates of toxic genotypes between three instruments (GeneAmp 5700, ABI 7300, ABI 7500) employed by three research groups showed good correspondence. Cell numbers of phycocxyanin (PC) and *mcy*B genotypes showed significant linear correlations between instruments obtained for field samples taken monthly from Lake Wannsee (Berlin, DE) during July 1999 until October 2000 (Fig. 7 A).

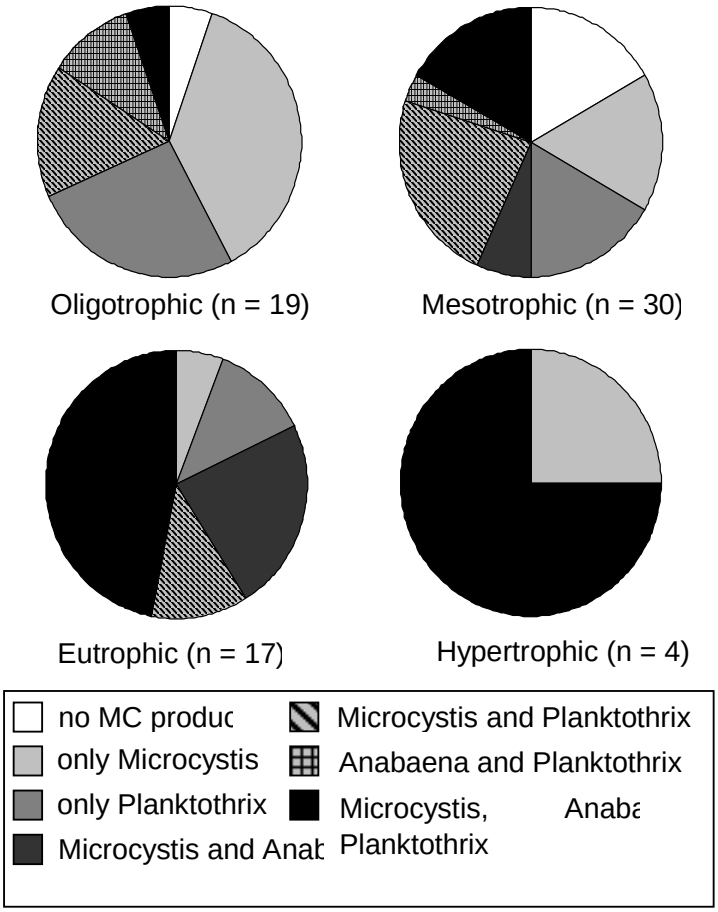


Figure 6: Proportion of lakes with different combinations of potential microcystin (MC) producers, Anabaena, Microcystis, and Planktothrix, based on the presence of genus-specific mcyE genes in oligotrophic (TP < 10 µg/l), mesotrophic (TP: 10 – 34 µg/l), eutrophic (TP: 35 – 100 µg/l), and hypertrophic (TP > 100 µg/l) lakes.

The minimum-average-maximum proportion of *mcy*B genotypes was 4-10-31% (GeneAmp 5700), 5-13-32% (ABI 7300), 7-20-47% (ABI 7500), respectively (Fig 7 B). Although the mcyB proportions were found to be higher using the ABI7500 system. The proportions of *mcy*B genotypes were found to correlate significantly. During the study period the number of *mcy*B genotypes were on average linearly related to the number of PC genotypes. It is concluded that the average proportion of cyanopeptide genotypes is reproducible between research groups. The highest occasional deviations occurred in winter at low cell density.

Compared to the quantification of population density from field samples in terms of cell numbers, quantifying the proportion of specific genotypes using real-time PCR is more sophisticated: while the seasonal or geographic range of variation of cell densities in field populations reaches a factor of more than a million, studying the relative share of a genotype in a sample provides results between 1 and 100. This implies that real-time PCR is more sensitive, and inaccuracies in cell number estimation

caused by systematic errors may have a much larger influence, particularly in the range of low cell densities of a given genotype.

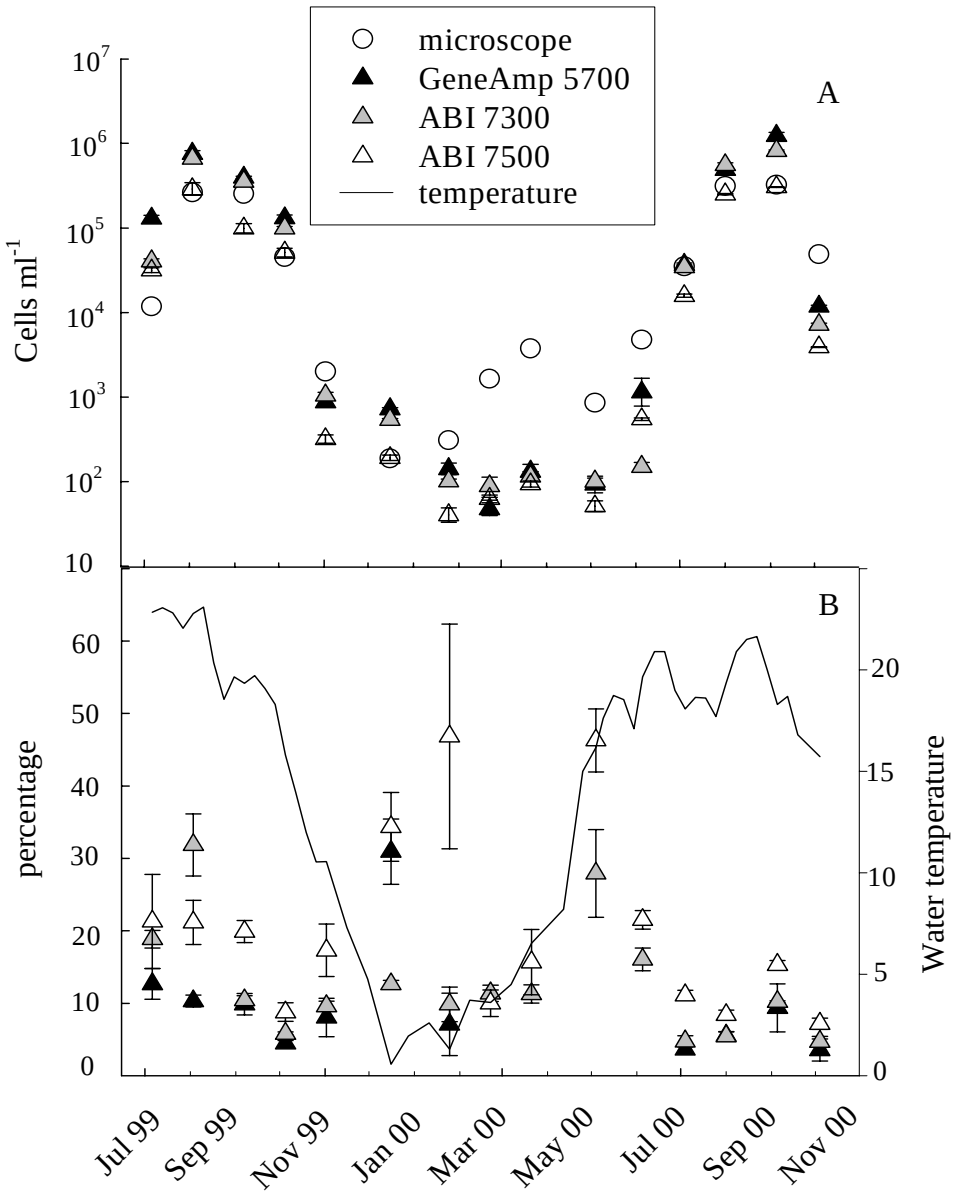


Fig. 7: (A) *Microcystis* sp. cell numbers in Wannsee from July 1999 to October 2000 determined in the microscope and via TNA (PC) using three instruments (GeneAmp 5700, ABI 7300, and ABI 7500), mean (± 1SE). (B) Proportion of *mcy*B genotypes during the same period determined using three instruments (GenAmp 5700, ABI 7300, ABI 7500), mean (± 1SE), and water temperature.

3.1.2 Quantification of cyanopeptide genotypes by real-time PCR

Real-time PCR is the only option for quantifying the share of specific genotypes that constitute field populations of a given species. and PEPCY attempted this for the first time. The results demonstrate the applicability of the real-time PCR technique in the field by quantifying *mcy* genotypes in lake water via standard curves between cell numbers and the amplification products obtained by the Taq Nuclease Assay (TNA) using two gene regions: (1) the intergenic spacer region within the phycocyanin (PC) operon to quantify the total population and (2) the *mcy*B gene which is indicative of microcystin synthesis (Kurmayer & Kutzenberger 2003). Taq Nuclease Assays developed for *Planktothrix* spp. included four genotypes, i.e. phycocyanin, *mcy*B, aeruginosin, anabaenopeptin. Gene knock out experiments and subsequent peptide analysis in knock out mutants in comparison to the wild-type demonstrated the involvement of the target genes in synthesis of the corresponding peptides (Christiansen et al. 2003, Ishida et al. in prep., see also 3.3). The PCR primers were checked for specificity among 71 *Planktothrix* strains isolated from Austria and northern European countries (Kurmayer et al. 2004).

For all populations of *Planktothrix* spp. highly significant linear relationships between the number of phycocyanin genotypes (in cell equivalents ml^{-1}) and the number of microcystin genotypes, aeruginosin genotypes and anabaenopeptin genotypes were found (Fig. 8). Using least square approximation linear curves were calculated according to y=y0 + ax, where y is the log cell number ml^{-1} calculated via Taq Nuclease Assays (TNAs) for mcyB or aeruginosin or anabaenopetin and x is the log cell number ml^{-1} calculated via TNA for phycocyanin to quantify the total population. The number of phycocyanin genotypes linearly predicted the number of microcystin, aeruginosin and anabaenopeptin genotypes through a range of 10^2 - 10^6 cells ml^{-1}. In all cases more than 50% of the variability found in the concentration of microcystin, aeruginosin and anabaenopetin genotypes could be explained by the increase or decrease of the total population. For populations of *P. rubescens*, the net samples revealed much higher concentrations of genotypes, however otherwise identical results when compared to the integrated samples.

For the microcystin genotypes, the regression curves differed significantly in the intercept between populations from *P. rubescens* (Mondsee, Irrsee, Wörthersee, AT) and P. agardhii (Wannsee, DE) and *P. agardhii* (Frederiksborg, Slottso). On average the concentration of microcystin genotypes was one to one (a=1) linearly related to the phycocyanin genotypes in populations of P. rubescens while it increased linearly by a factor of a= 0.75 for the population in Frederiksborg Slottso. In contrast the regression curves did not differ in slope and intercept between Planktothris spp. for aeruginosin and anabaenopeptin genotypes.

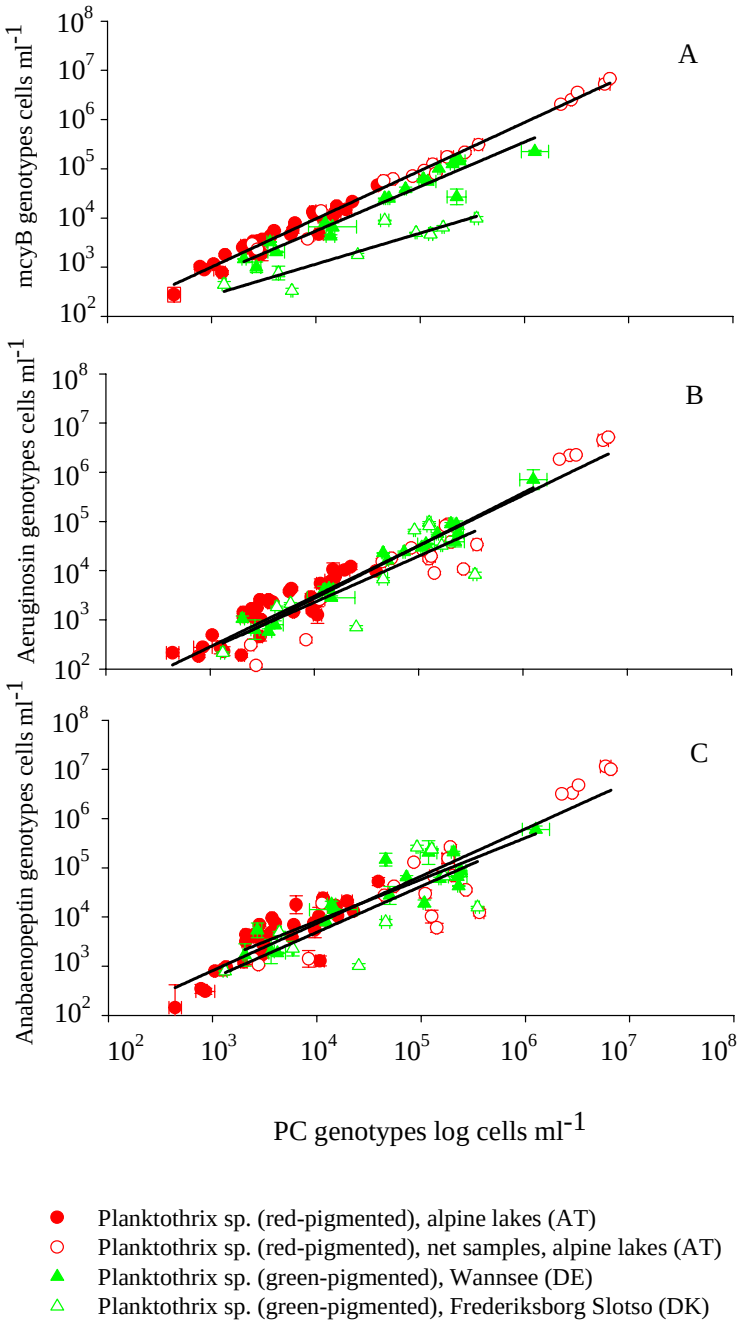


Fig. 8: Relationship between the number of (A) *mcy*B, (B) aeruginosin, (C) anabaenopeptin genotypes and the number of phycocyanin genotypes (in cells ml^{-1}) as determined via the Taq nuclease assay in red-pigmented and green-pigmented populations of *Planktothrix* spp.

Linear relationships were calculated following least square approximation according to y=y0 + ax, where y is the log cell number ml^{-1} calculated via TNAs for *mcy*B or aeruginosin or anabaenopetin and x is the log cell number ml^{-1} calculated via TNA for phycocyanin to quantify the total population. All linear regression curves were found to be highly significant ($R^2 > 0.5$, $p < 0.001$).

3.1.3 Evaluation and improvement of the HPLC/ MALDI-TOF mass spectrometry method:

In total, 34 peptides were characterized: 5 aeruginosins, 4 anabaenopeptins, 3 cyanopeptolins, and 3 microcystins. Further peptides could not be classified or elucidated based on their fragment sprectra and were considered as entirely new peptides. Peptides that were identified in HPLC-fractions were generally also detected in single-filament mass spectra, especially when producing high peak areas in chromatograms. The relationship proved also to be quantitative. For nine strains a highly significant linear relationship (R^2 ranging from 0.74 to 0.99) between relative peak areas in HPLC and relative peak heights in MALDI-TOF MS (averaged for five filaments) was observed. For the two remaining strains a significant rank correlation was observed. In analogy, linear relationships were found for individual peptides that were produced by multiple strains.

These results imply that relative peak heights in mass spectra of individual filaments depend directly on the relative amount in which these peptides are contained in individual strains. For example, if a strain contains primarily anabaenopeptin B and [Asp3]Mcyst-RR in high cell quota, it will be these two peptides that produce the highest peaks in mass spectra of single filaments. On the other hand, the study showed that minor peptides can be undetectable – in both HPLC and MALDI-TOF MS – and the characterization of the complete peptide inventory of a strain requires multiple fractionation steps. With regard to field studies, however, the results clearly demonstrated that peptide analysis in single filaments is reliable to identify chemotypes and to study chemotype diversity and dynamics in field populations. Moreover, the results show that a quantification by MALDI-TOF MS is in principle possible for most peptides.

The optimised sample preparation procedure allowed to obtain PSD-fragment spectra directly from very small samples as single colonies and filaments to idenify individual peptides. In the case of *Microcystis* colonies for many peptides even a structure elucidation was possible: a tremendous advantage compared to classical isolation procedures that involve laborious cultivation and chromatographic isolation of compounds.

Peptide identification and elucidation of new structures was improved by systematic studies of fragmentation patterns of similar structural congeners. For some peptide classes single fragments are indicative, e.g. m/z = 140 Da for the Choi-immonium ion in aeruginosins, while for others series of fragments are necessary for a classification, namely in cyanopeptolins. With a rapidly increasing number of reference fragment spectra respective series of fragments typical for particular peptide types could be established and used for the identification and elucidation of new peptides in all kinds of samples. The power of MALDI-TOF MS PSD/CID fragmentation as analytical tool was demonstrated, for example, by studying single *Microcystis* colonies where 18 new (flat) structures could be proposed (Welker et al. 2006).

The database for mass spectral analysis of peptides was expanded with MALDI-TOF MS. Guidelines to the identification of peptide mass signals in complex mixtures of metabolites were developed (Welker et al., 2006) as identification is often hampered by the co-occurrence of adduct peaks, fragment peaks, and pseudo-molecular peaks. Empirical data were collected on the relationship of structural properties and intensity of related mass peaks and fragmentation. For cyanopeptolin type peptides the data indicate, for example, a pronounced shift from the protonated peak to a water-abstracted fragment in congeners with a Lys in position 2 of the cyclic part or a shift in peak intensity to the M+Na with congeners possessing a Tyr in this position (Czarnecki et al. 1006).

From hundreds of post source decay-fragment spectra of peptides a summarizing guideline to identify peptides or types of peptides was developed (Welker et al., 2006) The main objective was to characterize fragments and series of fragments indicative of certain peptides and to develop a 'key' to

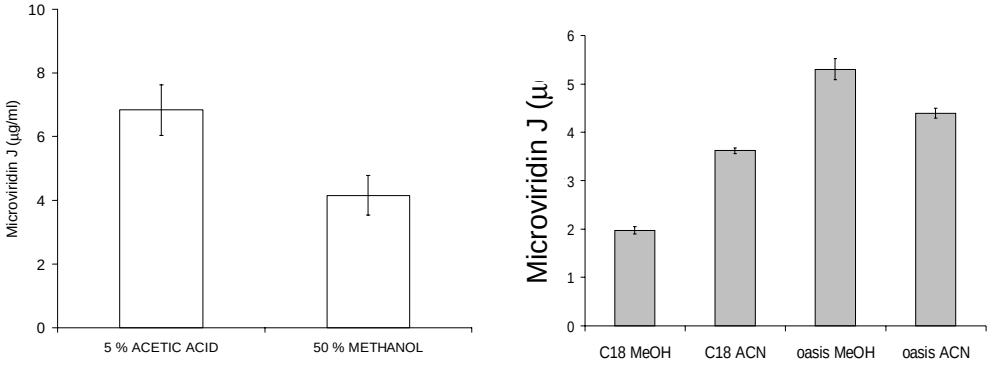
the analysis of fragment spectra. A comprehensive review of cyanopeptides is given by Welker and von Dohren (2006), likely helpful to reduce the confusion caused by unregulated peptide naming.

3.1.4 Cyanopeptide extraction

Comprehensive extraction protocols were developed for several peptides (see PPG's UnivDun003-005 and PPG_UvA001 in the annex). The extraction and laboratory handling of anabaenopeptins A and B was investigated and recommendations made regarding the most suitable methods. In this case, it was found that variation of the strength of methanol as an extraction solvent resulted in no clear optimal solvent concentration and maximal concentrations could be extracted as long as repeat extractions were employed (Morrison et al., 2006). Similar results were found in the optimization of anabaenopeptin extraction from a variety of other sources, including environmental samples.

Optimization of large-scale extraction procedures for fast efficient methods with maximal yields could be maximized. Testing yields of extraction with 5% acetic acid against those of the established 50% methanol extraction method (as is used for hydrophilic microcystins) demonstrated higher yields with the benefit of avoiding methanol (which also has the draw-back of potential methylation of microviridin J at room temperature). This also avoids the need to dilute methanol concentration to ensure retention by solid phase extraction (SPE).

Optimisation of SPE methods for microviridin J extraction showed poor retention on C18 cartridges and although retention using graphite carbon cartridges was good, only a very small quantity of peptide could be eluted. Comparison was therefore made with a polysorbant cartridge (Waters Oasis



HLB), which was found to be most suitable for microviridin J retention and elution (Fig 9).

Fig 9: Optimization of microviridin J extraction for purification. Right-hand panel: total yields obtained on extraction with 5% acetic acid and 50% aqueous methanol. Left-hand panel: total yields obtained after SPE of extract with a C18 cartridge or polymeric 'Waters Oasis' cartridge eluted with methanol (MeOH) or acetonitrile (ACN).

For the large-scale extraction and purification protocols for several cyanopeptides developed in PEPCY, see also Morrison et al.(2006), Young et al. (2006) and Morrison et al.(submitted).

3.1.5 Cyanopeptide biomonitoring

Using invertebrate trypsin and porcine trypsin, assays were run for 123 strains of *Planktothrix* spp., (originating from Norwegian and Austrian collections), approx. 150 field samples of lakes from Austria,

The Netherlands, Denmark and Norway as well as 10 purified peptides isolated by UoC, NIPH, UBA; IPas UoK and UnivDun.

Daphnia crude enzyme extracts (trypsin-like) proved to show substantial reactions both to laboratory strains and to field samples dominated by cyanobacteria. In most cases the data output followed the expectation of a linear relationship between the concen-tration of the cyanobacterial material and the inhibition of the enzyme activity (Fig. 10).

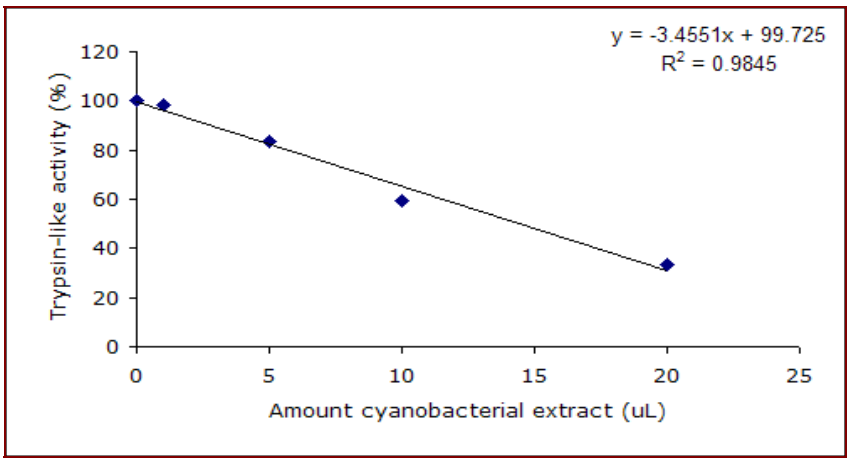


Fig. 10: The relationship between the amount of extracted cyanobacterial material and the inhibition of Daphnia and porcine trypsin.

Of the 10 purified cyanobacterial peptides tested it was found that microcystin, microvirdin, anabaenopeptin B, cyanopeptolin 984, and nostocyclin inhibit Daphnia trypsin while anabaenopeptin A, anabaenopeptin D, oscillatorin, cyanostatin A, cyanostatin B, nostocphycin and oscillamide did not have any detectable inhibitory effect. The evaluation of the occurrence of those peptides which inhibit Daphnia trypsin indicates at least one of them to occur in 61 % of all samples, and work in progress is currently comparing this result to the inhibition caused by the samples themselves.

The results document that peptide induced protease inhibition is a frequent phenomenon in *Planktothrix* populations and field samples dominated by *Microcystis*. Thus, it reflects the presence of some cyanopeptides, and these are likely to impact on the health of Daphnia populations. The Daphnia crude enzyme extract assay thus proved to be an inexpensive tool to monitor aquatic toxicity caused by cyanobacteria.

3.1.6 Pepcy Practical Guidelines (PPG)

Further to the methods for extraction, purification and analysis already published during PEPCY, a protocol format was established to allow methods to e easily accessible and improve documentation. These PEPCY Practical Guidelines (PPGs) aim to ensure standardized and traceable methods for use throughout-, and for life after-, PEPCY and are seen as a substantial output. PPG titles available and under review to date are shown in Tab. 4.3. (see also Annex 4.2).

Tab. 6: PEPCY Practical Guidelines (see the annex for the full text of these PPGs).

No.	Reference	Author	Title
1	PPG_UnivKon001	SJ Hoeger, DR Dietri	Cytotoxicity test with intestinal and/ or hepatic
2	PPG_UnivKon003	S Hoeger, M Welker, Morrison	Extraction and crude fractionation of extracts f lyophlized cyanobacterial cells for bioassays.
3	PPG_UnivDun001	LF Morrison, GA Coc	Analysis of anabaenopeptin B by high perform chromatography with photodiode array detecti
4	PPG_UnivDun002	LF Morrison, GA Coc	Preparation of gravimetric cyanopeptide stand
5	PPG_UnivDun003	LF Morrison, S McInr	Extraction and purification of anabaenopeptin
6	PPG_UnivDun004	LF Morrison, GA Coc	Routine small-scale extraction of anabaenope lyophilised cyanobacterial biomass.
7	PPG_UnivDun005	LF Morrison, GA Coc	Routine small-scale extraction of cyanostatins from lyophilised cyanobacterial biomass.
8	PPG_UoA001	L Tonk, LF Morrison	Routine extraction of microcystin-LR, cyanope cyanopeptolin C and cyanopeptolin 970 from l cyanobacterial biomass
9	PPG_TUB1	M Welker	Optimising extraction and HPLC analysis of pe cyanobacterial strains

3.2 New cyanopeptide genes and gene clusters

Identifying a range of genes encoding for cyanopeptide synthesis was a major objective of PEPCY, as this understanding is the prerequisite both for developing genetic tools to identify peptide producers and to generate peptide-deficient mutants. Genes were elucidated by (1) screening selected strains of *Nostoc, Planktothrix* and *Microcystis,* thereby focussing on those bioactive cyanopeptides that are most common in fresh water, (2) by mining the partial genome sequences of *Microcystis* PCC 7806 and *Anabaena* 90 for peptide and polyketide synthetase genes.

The following genes clusters could be characterised: The **cyanopeptolin** synthetase gene cluster of PCC 7806 encoding cyanopeptolins A, B and 963A includes four genes (*apd* genes; 27 kb). The gene *apdE* encodes an ABC transporter of the FAE family (Pearson et al. 2004), and the three other genes, *apdA-B* and *apdD*, encode NRPS with two, four and one CAT module, respectively. The *apdB* gene contains a N-methylation domain in its last module and *apdD* terminates with a thioesterase domain. The gene responsible for the formation/incorporation of hexanoic acid may be one of the not yet identified PKS clusters found in the genome.

A further *Microcystis* cluster was assigned to **microginin** biosynthesis. Each of the enzymatic reactions that can be predicted from the sequence is well in agreement with the structure of the linear peptide. The microginin cluster shows close similarity to a gene cluster that was previously sequenced from another *Microcystis* strain (D. Kramer and T. Börner, unpublished). This cluster was also implicated in microginin synthesis. The sequences from the two *Microcystis* strains show a number of differences in individual domains and proteins. Differences in the gene clusters correspond to the differences in the structures of microginin isoforms produced by the two strains.

Another gene cluster was sequenced encoding an NRPS module, polyketide synthase (PKS) modules and a putative prenyl transferase. Although sequencing of the cluster is not finished yet, this cluster could be assigned to **aeruginoguanidin** (AGD) biosynthesis since the enzymatic reactions deduced from the sequence correspond exactly to the unusual structure of AGD.

Biosynthetic genes putatively coding for the biosynthesis of the hexapeptides **anabaenopeptins** A, B, and C in *Anabaena* 90 form a cluster of five peptide synthetase genes (28.5 kb). The specific feature of this gene cluster is that it contains two genes presumably coding for the biosynthesis of the dipeptides, L-arg/L-lys-D-lys and L-tyr-D-lys, which occur alternatively in anabaenopeptins. Usually peptide synthetases show relaxed substrate specificity leading to the various structures of nonribosomal peptides.

A gene cluster of 44 kilobase pairs (kb) from *Nostoc* 152 was sequenced and according to in silico analysis this cluster codes for the biosynthesis of **nostophycin**. The gene order is co-linear with the order and the structure of the components in the nostophycin: the polyketide building block and the hexapeptide part. The specific elements in the nostophycin synthetase are two internal epimerization domains, not frequently found in bacterial nonribosomal peptide synthetases. Nostophycin bears a structural resemblance to microcystins, and the synthetases share many functional similarities, but there is only a low homology between the biosynthetic genes.

Nostoc 152 is one of the few *Nostoc* strains known to produce microcystins. All the putative genes (56 kb) for the biosynthesis of **microcystins** in *Nostoc* 152 were sequenced. The sequence analysis showed that the microcystin synthetase genes of *Nostoc* 152 are arranged in the same way as in *Anabaena* 90. There are, however, two exceptions: the O-methyltransferase gene (*mcy*J) is missing in *Nostoc* 152 and there is an unknown gene instead. This gene may code for the acetylation of Adda found in the microcystin of *Nostoc* 152 instead of methylation, which is common in microcystins. Like all the sequenced *Anabaena* 90 peptide synthetase gene clusters, also the *mcy* cluster of *Nostoc* 152 includes a gene of the ABC transporter family.

The **aeruginosin** synthetase gene cluster of *Planktothrix* (Figure 11) and of two *Microcystis* strains have completely been sequenced. They share homologous genes coding for peptide synthetases, polyketide synthases and genes that are likely to have tailoring functions and to be involved in the formation of Choi (supported by additional biochemical studies and mutation of the genes), a characteristic component of aeruginosins. One gene codes for an ABC transporter of the FAE family (Pearson et al. 2004) and another for a putative halogenase. Interestingly, individual enzymatic steps seem to differ in the strains. These differences are expected to be responsible for creating the different isoforms of the peptide.

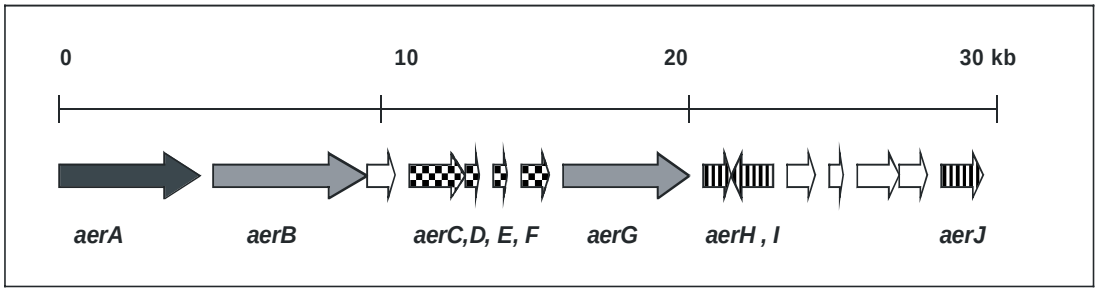


Figure 11: Aeruginosin biosynthesis gene cluster of Planktothrix. NRPS or NRPS/PKS genes are indicated in light and dark grey, respectively. Chequered arrows are suggested to encode genes to be involved in Choi biosynthesis. Further tailoring genes are highlighted with stripes. Genes (ORFs) that could not be associated with aeruginoside biosynthesis are shown in white (Ishida et al. unpubl.).

As was found for the cyanopeptolin synthetase gene cluster, the **halogenase** gene in the aeruginosin gene cluster of *Microcystis* PCC 7806 is bordered by direct repeats that likely result from the

duplication of the target sequence upon insertion of the halogenase gene in the chromosome. Comparison of the amino acid sequences deduced from nucleotide sequences of the two types of halogenases identified in the cyanopeptolin and aeruginosin synthetase gene clusters show that they both belong to the FADH2-dependent halogenase family. As expected, these putative halogenases possess a FAD binding site with the conserved motif GGGxxG located in the N-terminal part of the amino acid sequence. Phylogenetic analyses showed that the halogenases of the *Microcystis* strains and of *Anabaena* 90 (that synthesizes anabaenopeptilide, a cyanopeptolin-like oligopeptide) (Rouhiainen et al. 2000) form a monophyletic group subdivided into two sub-groups, one including cyanopeptolin (anabaenopeptilide) synthetases, and the other aeruginosin synthetases. A parallel analysis based on *Microcystis* ITS sequences was conducted. The data together suggest an ancient acquisition of cyanobacterial halogenases from Proteobacteria, followed by functional differentiation of the enzymes generating different specificity for the two substrates involved, tyrosine (for cyanopeptolins and anabaenopeptilide) and hydroxyphenyl-lactic acid (for aeruginosins). As already shown for microcystins, no relationships between ITS groups, the presence/absence of chlorinated/non-chlorinated cyanopeptolins or aeruginosins, and the geographical origin of the strains were observed.

Results from gene cluster sequences and mass spectral anaylses raised the question why in some strains peptides are chlorinated while in others they are not. Analysis of the presence and absence of halogenase genes in aeruginosin and cyanopeptolin synthetase clusters of *Microcystis* strains of PCC and NIES collection showed a good correlation between the presence of of the halogenase gene in gene clusters and the production of respective chlorinated aeruginosins and cyanopeptolins as studied by mass spectral analysis of the strains. Interestingly, halogenase genes were found either in the aeruginosin or the cyanopeptolin synthetase gene cluster but never in both clusters of individual strains.

3.3 Cyanopeptide-deficient mutants

The generation of **mutants** was essential to provide evidence for the proposed function of identified gene clusters and to have unambiguous material as controls for toxicitiy tests (see section 3.9.4. Suitable strains for genetic engineering were *Microcystis* PCC 7806, *Planktothrix* CYA 126 and (with more difficulties) *Anabaena* 90. All attempts to manipulate further strains, i.e. to knock out genes involved in the biosynthesis of peptides, failed. The nature of the barrier against exogenous DNA is not known. There are, however, many indications that *Microcystis* and *Anabaena* possess different types of exo- and endonucleases that probably impede successful integration of foreign, unprotected DNA (E. Dittmann and L. Rouhiainen, pers. comm.; Lyra et al. 1989). The following mutants were generated:

- mutants lacking microcystin: three *Microcystis*, one *Planktothrix*
- mutants producing modified microcystin: one *Planktothrix*;
- mutants lacking anabaenopeptilide: one *Anabaena*
- mutants lacking anabaenopeptin: one *Planktothrix*
- mutants lacking aeruginosin, one *Microcystis*, three *Planktothrix*.

Further mutants continue to be under investigation after the termination of PEPCY. For the purpose of studying potential adverse effects of cyanobacterial lipopolysaccharids (LPS) several *Microcystis* mutants were generated. One of them was characterized and showed an altered LPS composition.

3.4 Physiological regulation of cyanopeptide production

Planktothrix strain 2 (PT2), which produces large amounts of anabaenopeptins B and F (potential carboxypeptidase inhibitors) along with microviridin I (protease inhibitor) grew exponentially as long as the light intensity in the middle of the culture vessels was higher than 3 µmol m^{-2} s^{-1} (i.e. up to approximately the 8th day of culture growth; see Fig. 12, left-hand panel). Irrespectively of light supply, the net-production of anabaenopeptins B and F as well as that of microviridin I was closely correlated to the culture´s growth rate ($p<0.001$). As a result, the amount of anabaenopeptins and microviridin I per biovolume of *Planktothrix* remained almost constant, i.e. the changes observed were not significant (Fig 9, right-hand panel).

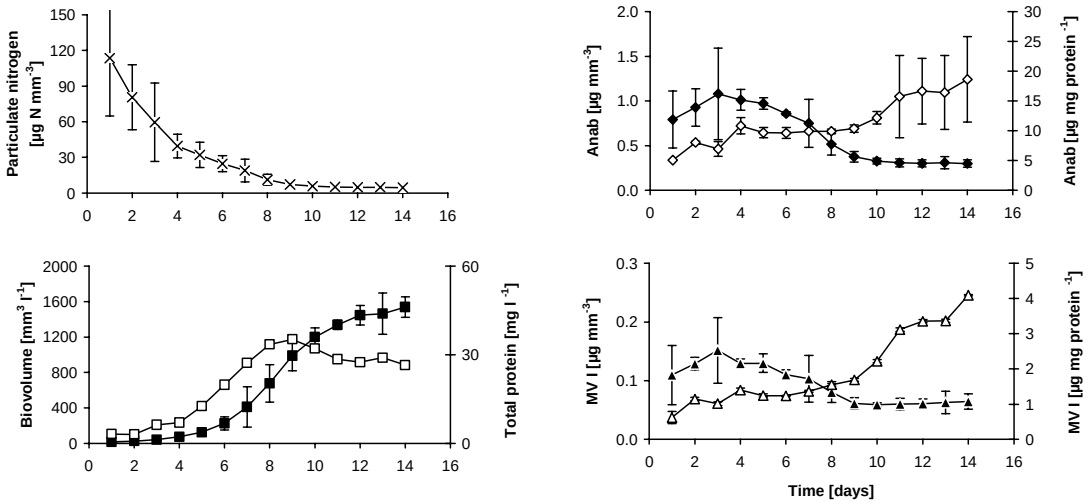


Fig. 12: Time courses of nitrogen, biovolume and peptide content in batch cultures of *Planktothrix* PT2 (Rohrlack & Utkilen, subm.)

In this experiment nitrogen was the limiting factor and this limitation was assessed by measuring particulate nitrogen. Legend: crosses: particulate nitrogen; solid black rectangles: biovolume; open rectangles: protein; solid diamonds: anabaenopeptin per biovolume; open diamonds: anabaenopeptin per protein; black rectangles: microviridin I per biovolume; open rectangles: microviridin I per protein

Results for nostophycin and microcystin in Nostoc strain 152 also showed continuous production of both peptides at all investigated environmental conditions. Cellular peptide content was generally increased under low light conditions (1 µmol m^{-2} s^{-1}) irrespective of temperature. Low P concentrations (0.14 µM) had an additional increasing effect on both peptide contents, with contents being higher by a factor of about 3-4 (63-174 fg nostophycin $cell^{-1}$, 71-135 fg microcystin $cell^{-1}$). The increasing effect of low P concentration on nostophycin content was more pronounced than on microcystin content. However, significant linear regression curves between the growth rate and the peptide production rate were found independently of the type of environmental limitation (Fig. 13), thus confirming the hypothesis proposed for microcystin by Orr & Jones (1998; see also section 1.3) of peptide production being constitutive and closely linked to growth.

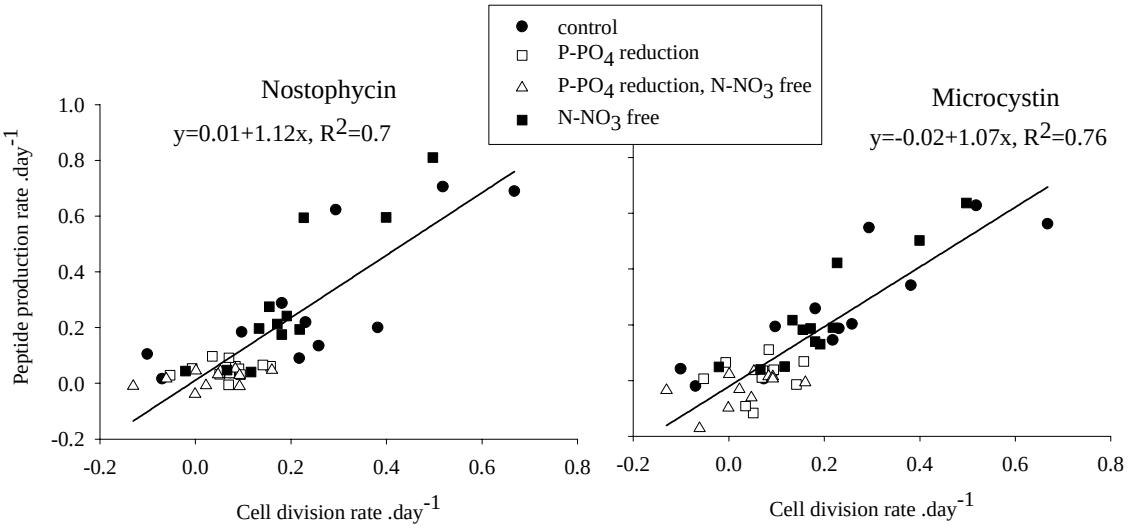


Fig. 13: Nostophycin (NC) net production rate versus growth rate in *Nostoc* strain 152 (Kurmayer et al. in prep.).

Taking the data from all treatments together peptide net production rates were calculated using the equation: prod. rate = (lnX2 – lnX1)/(t2-t1), where X is the concentration in cells .ml^{-1} or dissolved and particular peptide concentrations (in µg ml^{-1}) at two consecutive sampling dates (t1, t2). Taking all data together the intracellular nostophycin content and microcystin content varied from 11-174 fg cell^{-1} and 31-213 fg cell^{-1}, each.

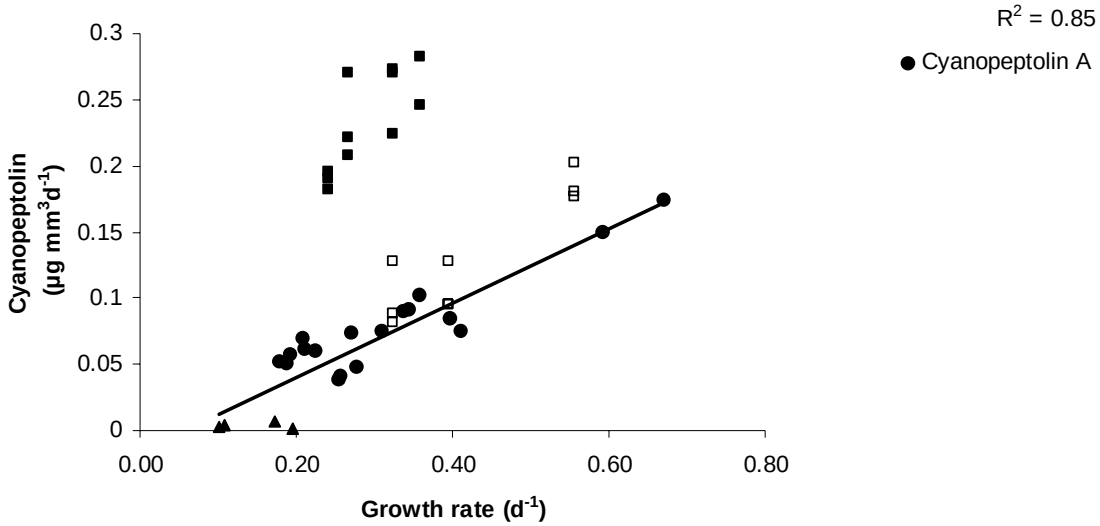


Fig. 14: Cyanopeptolin A production rate versus growth rate in *Microcystis* PCC7806.

Dark circles indicate cyanopeptolin production under phosphate limitation, dark triangles under nitrate limitation, white squares under low light intensity and dark squares under high light intensity. The data on the cyanopeptolin production rate under high light conditions were not included in the regression line.

Significant correlations between growth rate and production were also found for most peptides in *Microcystis* PCC7806 and *Anabaena* 90. As demonstrated by one example of these studies (cyanopeptolin production in *Microcystis,* Fig. 14), a correlation was found under P and N limitation, while different light intensities resulted in a much higher or lower production than could be expected from this correlation. This was also found for other peptides.

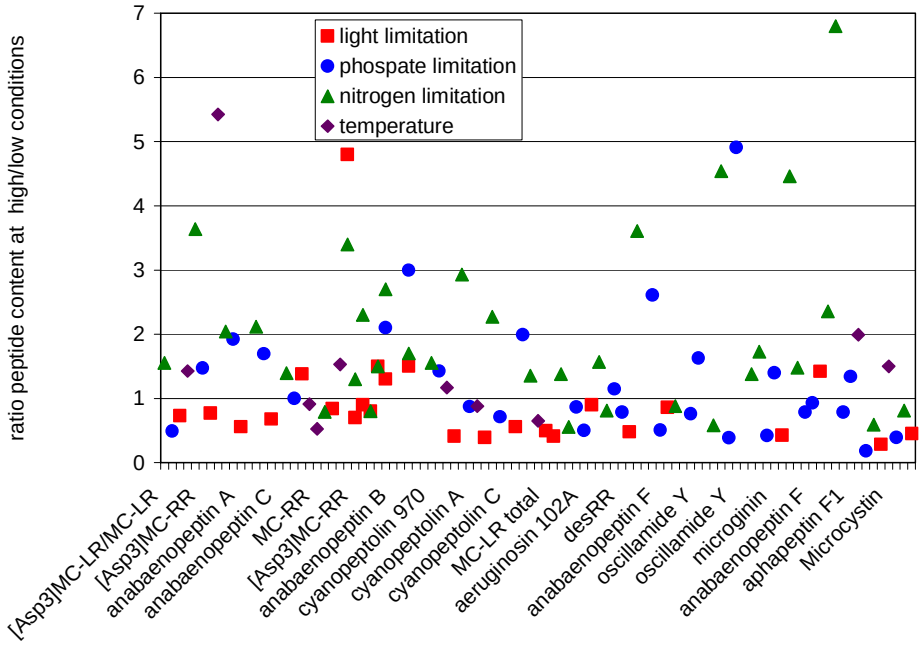


Fig 15: The ratios of peptide contents of all investigated strains under contrasting high and low light conditions, contrasting phosphate and nitrate concentrations and temperature conditions.

Taking all results together a clear result is emerging that although some ecophysiological regulation is induced by the environment, cyanopeptide production is not switched on and off for time periods sufficiently long to have a major impact on cellular content. In most cases peptide contents vary with a factor 0.4 to 4 (Fig. 15). For a few peptides, e.g. Nostophycin, this range was higher, up 7-8 fold under P-reduced conditions *vs*. control conditions, however even such higher rates of peptide variation did not change the general linear relationship between cell growth and peptide production.

Wildtype vs. anabaenopeptilide deficient mutant of Anabaena 90: These results were further confirmed by the experiment with the wildtype and the anabaenopeptilide deficient mutant of *Anabaena.* Both showed similar growth under the conditions tested, including light limitation and phosphate limitation. The absence of anabaenopeptilides had only minor measurable effects on the growth of the cells of the mutant. Interestingly, however, the mutant contained, in gravimetric units, approximately twice as much anabaenopeptins as the wildtype.

3.5 Occurrence of cyanopeptides in cyanobacterial strains – screening results

The screening of several hundred strains – both cultured isolates and single colonies and filaments from field samples – with the advanced HPLC/ MALDI-TOF mass spectrometry method revealed an extreme diversity of cyanopeptides. A large number of entirely new peptides and new congeners of known peptide classes could be detected (Table 7; see also Welker et al. 2006, Czarnecki et al. 2006, Boaru et al. 2006). For the former ones generally no full structure elucidation could be performed due to the lack of reference fragment spectra. For the new congeners the increasing number of available reference spectra enabled a rapid structure elucidation of new congeners of known peptides. Analyses were especially focused on cyanopeptolins and aeruginosins to establish a reliable database for primer design and testing for specific peptide synthetase genes. However, a number of new microginin congeners were also identified. A compilation of the data on cyanobacterial peptides can be found in Welker and von Döhren (2006).

Table 7: Number of new peptide variants identified and characterized by MALDI-TOF MS PSD/CID during PEPCY. For each peptide class the number of new congeners is given for which a complete flat structure could be proposed and the number of congeners for which only a partial structure could be obtained. See Welker and von Döhren (2006) for details.

peptide class	flat structure	partial structure
aeruginosins	15	11
microginins	9	5
anabaenopeptins	2	1
cyanopeptolins	38	12
microcystins	2	-
microviridins	-	12
others	2	-
new peptides	-	> 50

Most of the new congeners showed only one or two modification compared to known peptides, like a chlorination or an exchange of a single amino acid. Most of these modification have been observed in one or the other congener before and hence new congeners were not surprisingly encountered but rather fill gaps. In aeruginosins, for example, the Hpla moiety can be unchlorinated, singly chlorinated or doubly chlorinated and therefore a respective chlorination is expectable to occur with any possible amino acid sequence backbone (Welker et al. 2006). Thus, the number of potential congeners can be calculated by a permutation considering all moieties that have been found in specific positions (Welker & von Döhren 2006). For aeruginosins this results in more than 500 congeners.

3.6 Occurrence of peptide chemotypes in field samples

3.6.1 The PEPCY internal data base

As a result of intensive field sampling during 2003 and 2004 the internal data base of PEPCY contains a total of 270 samples from 37 lakes from three eco regions (defined according to the WFD: 3 from Italy (Sicily), 9 from Central highlands (Alps) and 14 from Central Plains), covering six countries (Austria, Denmark, Germany, Italy, Netherlands, Norway). This includes data from deep-stratified and

mesotrophic water-bodies dominated by *Planktothrix rubescens* as well as shallow and more eutrophic lakes dominated by *Planktothrix* agardhii and/or *Microcystis* and/or *Anabaena*. Phytoplankton composition (217 samples), environmental conditions (235 samples) and peptide composition (210 samples) have been sampled and analyzed by 6 partners (AAS, UOC, NIPH, UBA, UAM, TUB). The parameters available in the database are: Cyanobacteria composition (mm^3/l), total phytoplankton biovolume concentration (mm^3/l), environmental conditions (nutrient concentrations, water temperature, Secchi Depth, pH), peptide composition (measured by MALDI-TOF MS) and peptide concentrations for microcystins and for those 6 peptides which could be quantified with the help of the gravimetric standards produced in the project. Out of the 270 samples, 210 samples contain both data on cyanobacteria composition and data on the peptide composition. A total of 67 different peptides were recorded and identified using PSD characterisation. The structure of eleven peptides is unknown. This data base has a high value for ongoing and and considerable potential for future research of partners in the PEPCY consortium, and future use will be based on agreements between data providers within the consortium and any further users to which it would be disseminated.

3.6.2 Peptide chemotypes in field populations

The same peptide classes occurred across all field samples, with no clear relationship to their dominant cyanobacterial taxon, i.e. *Microcystis*, *Planktothrix* and *Anabaena*, consequently field samples showed no clear relationship between the occurrence of a peptide class and the occurrence of a genus. Notably microcystins and anabaenopeptins were found in all combinations of the cyanobacteria genera. However, samples dominated by *Microcystis* (11 samples) as well as those dominated by *Planktothrix* (56 samples) tended to contain several peptide groups, mostly microcystins, anabaenopeptins and cyanopeptolins. One sample dominated by *Aphanizomenon* contained microcystins. In contrast, samples dominated by *Anabaena* (5 samples) mostly showed microcystins, anabaenopeptin and microginin. Some peptides occurred much less frequently, i.e. aeruginoguanidin, kasumigamide and the microginins.

However, within a given peptide class, some differentiation of variants across taxa was observed: While anabaenopeptins B and F occurred in many samples without a correlation to any particluar dominant taxa, some structural variants of the aeruginosins, such as Aeruginosin A and Cl-Aeruginoside 126B, were only found in samples dominated by *Planktothrix* spp.. On the other hand, many structural variants of cyanopeptolins, e.g., cyanopeptolin W and Cl-cyanopeptolin W were only found in samples dominated by *Microcystis*.

Tab. 8: Relative frequency of occurrence of peptide classes in phytoplankton dominated by different cyanobacteria

(dominance defined as > 90% of the biovolume): MIC = Microcystis, APH = Aphanizomenon, AN = Anabaena, Pl = Planktothrix, Ly = Lyngbia. Note that the number of lakes and samples that were investigated differed between the different phytoplankton communities. All samples also contained peptides with unknown structure.

Phytoplankton composition	n samples	n lakes	Aeroginoguanidin	Aeruginosin	Anabaenopeptin	Cyanopeptolin	Kasumigamid	Microcystin	Micorginin
MIC	11	6	9	18	77	18	11	100	9
APH	1	1	0	0	100	0	0	100	0
PL	56	5	0	60	100	21	0	96	5
AN	5	3	0	0	20	20	0	80	60
MIC LY	1	1	0	100	100	100	0	100	0
MIC APH	4	2	0	0	25	0	25	100	0
MIC PL	7	2	0	0	100	57	86	86	14
MIC APH PL	1	1	0	0	100	0	0	100	100
MIC AN	10	5	0	10	90	40	10	80	20
MIC APH AN	9	5	0	11	78	78	33	88	22
MIC PL AN	7	3	0	28	56	43	0	86	0
MIC APH PL AN	12	5	8	8	92	58	42	75	8
MIC APH PL AN LY	2	1	0	0	50	50	0	100	0
PL LY	7	3	0	86	100	71	0	100	0
PL AN	7	1	0	58	100	28	14	100	0
PL AN LY	1	1	0	0	100	0	0	100	0
APH PL AN	2	1	50	50	100	50	0	100	0

3.6.3 Peptide chemotypes in individual filaments and colonies from field populations

Peptide composition in 1000 individually analysed *P. rubescens* filaments from 10 lakes in 7 European countries (Austria, Germany, France, Italy, Poland, Switzerland, Turkey) revealed 90 mass signals of putative peptides that were obtained from mass spectra, 24 of which could be assigned to known peptides, 33 were new peptides well characterized from strains and HPLC-fractions, while the remaining 35 mass signals represent completely unknown metabolites. A few sampling sites with *P. agardhii* (Havel River in Germany and Lake Sloenso in Denmark) showed very high drop out rates (i.e. no pigment signal was found, indicating that transfer of the filament to the template for analysis was not successful) and were therefore not considered in quantitative statistical analysis. For all other samples drop-out rates were <35% and for six lakes more than 90% of filaments gave positive results.

For the statistical analysis (still ongoing) a total of 37 peptides was considered in 964 *Planktothrix* filaments (from ten waterbodies, except of the Havel River and Lake Sloenso) with a frequency >1% including 20 known peptides and 17 peptides that have been identified in HPLC fractions analysed from *Planktothrix* strains (see materials and methods). A few peptides were detected in >80% of all filaments (Table 8; Fig. 16, left-hand panel): anabaenopeptins B and F, [Asp3]*Mcy*st-RR. Other

frequently occurring peptides included oscillarin (in 30% of all filaments), aeruginoside 126B (42%), Cl-aeruginoside 126B (30%), anabaenopeptin A (64%), oscillamide Y (27%), anabaenopeptin G (27%), [Asp3]*Mcy*st-LR (47%), and [Asp3,MSer7]*Mcy*st-RR (30%).

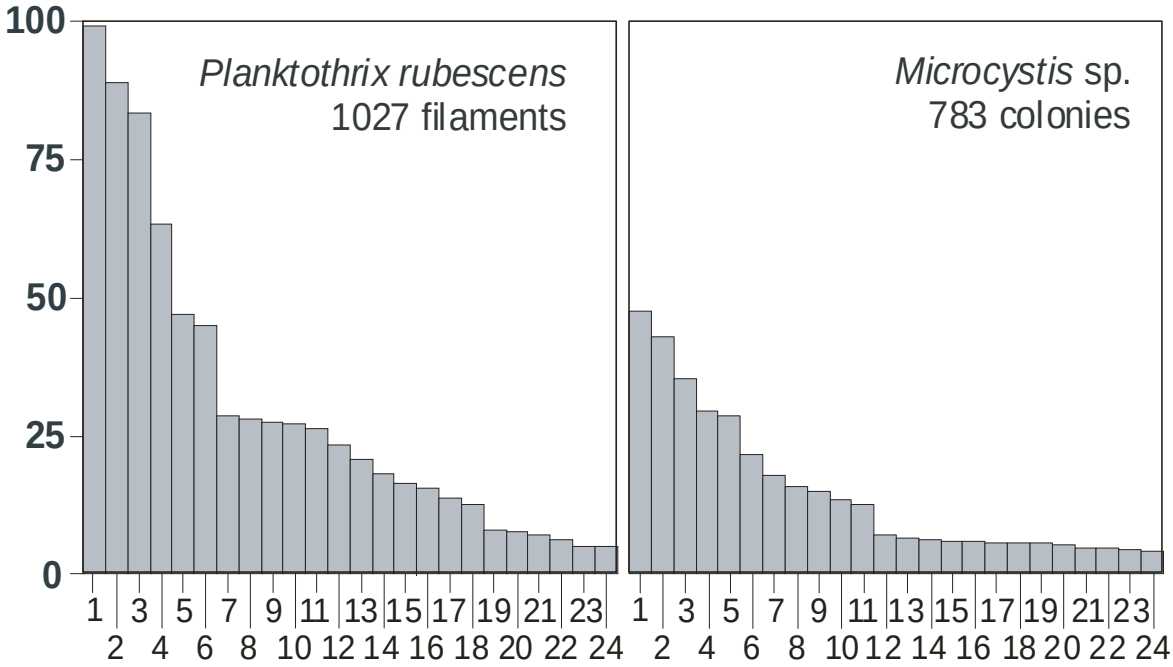


Fig. 16: Frequency of individual peptides in two genera detected in single filaments and colonies. *Planktothrix* filaments were isolated from European 12 lakes. *Microcystis* colonies were isolated from Brno reservoir. Numbers refer to peptides listed in Table 9

Table 9: Most frequently detected peptides in single filaments of P. rubescens and single colonies of Microcystis as shown in Fig. 13 (see above).

	P. rubescens
1	Anabaenopeptin B
2	[Asp^3]*Mcy*st-RR
3	Anabaenopeptin F
4	Anabaenopeptin A
5	[Asp^3]*Mcy*st-LR
6	Aeruginoside 126B
7	Aeruginosin A
8	[Asp^3,MSer^7]*Mcy*st-RR
9	Cl-Aeruginoside 126B
10	Anabaenopeptin G
11	Oscillamide Y
12	peptide 816
13	Planktopeptin BL 1125
14	peptide 800
15	peptide 995
16	Aeruginosin 592
17	Anabaenopeptin K
18	Oscillapeptin
19	cyanopeptolin 1126
20	[Asp^3]*Mcy*st-HtyR
21	peptide 967
22	cyanopeptolin 1040
23	peptide 1008
24	peptide 994

	Microcystis
1	Aeruginosin 602
2	*Mcy*st-LR
3	*Mcy*st-YR
4	*Mcy*st-RR
5	cyanopeptolin 1063
6	Aeruginosin 89
7	Cl-Aeruginosin 89
8	cyanopeptolin 920
9	[Dha^7]*Mcy*st-LR
10	[Asp^3]*Mcy*st-RR
11	peptide
12	Microcystilide A
13	cyanopeptolin 1034A
14	Aeruginosin 102
15	Anabaenopeptin F
16	cyanopeptolin 1000A
17	cyanopeptolin 972C
18	Anabaenopeptin B
19	cyanopeptolin 1006D
20	microginin FR3
21	Cl-Aeruginosin 102
22	microviridin 1777
23	Aeruginosin 98B
24	Cyanopeptolin 972A

For P. *rubescens,* a few peptides differed between filaments obtained from different populations and countries. For example, anabaenopeptin A occurred in all lakes except for the Weida Reservoir (Tab. 10). Anabaenopeptin G was detected in all filaments from lake Miedwie, in 87% of filaments from Hallwiler See, and in 45% of filaments in Zürichsee but not in Ammersee, Mondsee, Sapanca, Weida, and Wörthersee. Aeruginosin 126B and Cl-aeruginosin were more frequent in Miedwie (99%) and Hallwiler See (84%), less frequent in Sapanca (77%), Zürichsee (44%), and Lago Maggiore (36%) but never found in filaments isolated from the Weida Reservoir.

Table 10: Frequency of detection of specific peptides in single filaments of *Planktothrix rubescens* by MALDI-TOF MS in percent.

Capitalised peptide names refer to known peptides while lower-case names refer either to provisional names when the structure has been elucidated by PSD-fragmentation or to the peptide class. When no such information is given, only the peptidic nature of the compound could be revealed unambiguously. Frequencies >50% are shaded. Comments: 1) published peptide structure; 2) peptide characterized by MALDI-TOF PSD-fragmentation from Planktothrix rubescens filaments; 3) as 2 but from Planktothrix sp. filaments; 4) as 2 but from cyanobacterial samples other than Planktothrix; 5) at least one PSD spectrum obtained directly from filaments.

m/z	name	comment	total	Ammersee, DE	Lac du Bourget, FR	Hallwiler See, CH	Lago Maggiore, IT	Mondsee, AT	Miedwie, PL	Sapanca, T	Talsperre Weida, DE	Wörthersee, AT	Zürcher See, CH
593.3	aerugnosin	2,5	16	23	10	14	9	22	71		8	1	8
617.3	Oscillarin	1,2,3,5	30	32	13	18	14	35	82		97	5	29
691.4	Aeruginosin 690	2,5	1		1			1	10	1			
715.4	Aerugnosin 126B	1,2,3,5	42	17	10	84	36	23	99	77		10	44
725.4	Aeruginosin 724A	2,3	2	1	4			5				8	
749.3	Cl-Aeruginosin 126B	2,5	30	53	18	5	47	38	7	94		23	12
801.4	peptide	2	19	10	45	51		16	1	1	29	19	15
817.4	peptide	2	25	6	71	46		41	1	1	17	34	18
837.5	Anabaenopeptin B	1,2,3,4,5	99	100	100	100	100	100	91	100	97	100	100
844.4	Anabaenopeptin A	1,2,3,4	64	88	75	66	71	75	78	65	3	42	62
851.5	Anabaenopeptin F	1,2,3,4,5	87	81	94	67	90	98	59	100	98	95	90
858.5	Oscillamide Y	1,2,3,4	27	29	40	23	20	36	52	21	11	15	22
867.4	Ferintoic acid A	1,2,3,4	1		4		9						
881.5	Ferintoic acid B	1,2,3,4	3		4	4	9				11	1	2
905.5	peptide	3,4	1	13				3					
909.5	Anabaenopeptin G	1,2,3,5	27		1	87	9		100				45
916.5	Anabaenopeptin K	1,2,3	13			18			77				25
968.5	peptide	3	8	1	1		7	14			25	10	21
981.5	[Asp3,Dhb7]-*Mcyst*-LR$	1,2,3,5	47	22	36	91	33	11	79	68	63	26	35
990.6	cyanopeptolin	3	3	13	2		26	1				1	1
995.6	cyanopeptolin	2,3	5	10	2	1	9		22				6
996.5	peptide	2	17	10	3	5	7	3	35	74	17	9	5
999.6	[Asp3,Thr7]*Mcyst*-LR$	1,2,3	3		2	11	4	4	1			1	2
1009.6	peptide	2,5	5		6	3	1	7	3		2	17	10
1024.6	[Asp3,Dhb7]-*Mcyst*-RR$	1,2,3,5	88	62	81	95	67	90	100	100	100	80	92
1029.5	[Asp3,Dhb7]*Mcyst*-HphR$	1,3	<1				6						
1031.5	[Asp3,Dhb7]-*Mcyst*-YR$	1,2,3	4		22	1			4			2	2
1041.5	cyanopeptolin	2	7	9	2	4	1	4	2		51	5	5
1042.6	[Asp3,Thr7]*Mcyst*-RR$	1,2,3,5	30	3	28	12	3	71	61	56	12	34	5
1045.5	[Asp3,Dhb7]-MC-HtyR$	1,2,3,5	8	6	4	28	4	9				14	11
1047.6	peptide	2	2	10	5	3							1
1088.5	Oscillapeptin A - SO_3	1,2	13	21	15	25	10	12	18		2	15	14
1094.6	Oscillapeptin G - H_2O	1,3	4		1	19		2	6		6		2
1108.6	Planktopeptin BL1125 - H_2O	1,2	22	15	22	45	4	21	27		63	15	20
1109.6	cyanopeptolin	2	9	23	16	2		5	1		20	10	12
1240.7	Oscillatorin	1,2,3	2		19								
1275.7	peptide	3	4		19	1	1	4				2	4
	No. of peptides		N	25	34	29	26	28	26	13	20	27	31

In 800 *Microcystis* colonies from Brno reservoir (Czech Republic) a total of 92 peptides were identified or characterized, some of which were only encountered in a few colonies (Welker et al. 2006). In contrast to *Planktothrix rubescens, Microcystis* spp. apparently generally shows a significantly more diverse chemotype composition (Fig. 16, right-hand panel). All colonies were classified into 37 distinct peptide chemotypes by cluster analysis (K-measns clustering). One chemotype not producing any (detectable) peptide could be microscopically identified as *M. wesenbergii* while for other chemotypes no such clear relationship between morphospecies and peptide production was found. One reason may be that the number of morphospecies (6-8 in temperate waters) is far lower than the number of chemotypes encountered in a single water body. Therefore an approach is needed that does not rely on species determination but on combinations of morphological features allowing the characterization of a multitude of morphotypes. A multivariate statistical appoach is currently in progress to reveal the relationship of sets of particular morphological features and peptide fingerprints (Sejnohova et al. in prep).

During the season a pronounced shift in the pelagic *Microcystis* chemotype composition was observed: from a very diverse community at the onset of the bloom, a steady decline in diversity indices was observed leading to a dominance of two chemotypes in November that accounted for more than 80 % of the colonies (Sejnohova et al. 2006). The benthic community also showed a shift in chemotype composition though less pronounced. Interestingly the benthic and pelagic samples at the onset of the bloom differed substantially, thus suggesting that inoculation from the sediment only plays a minor role in the bloom development in the water column.

A further important finding was the highly significant correlation of seston MCYST-content to the relative share of MCYST-producing colonies. The highest seston MCYST-content was found at the onset of the bloom, then steadily declining to levels below detection limits. In parallel, the number of colonies with detectable MCYST declined steadily, and at the end of the season the community was dominanted by only two non-producing chemotypes . This demonatrates that the chemotype composition is the major determinant of the (MCYST-)toxicity of a bloom (Sejnohova et al. 2006).

The colonies sampled from lakes around Berlin showed also a high chemotype diversity. The data analysis and PCR experiments are ongoing, but preliminary results indicate that, again, new peptides could be identified and characterized. In particular, colonies from the Havel River contained chlorinated microginins that have only rarely been found in other samples. Chemotype diversity was lowest in Lake Schlachtensee, with the same few dominant chemotypes that had been found dominant in this lake in 2000. In Lake Wannsee, one chemotype was found in low numbers that had exactly the same peptide pattern as colonies sampled in Brno Reservoir, underlining the potentially wide distribution of particular chemotypes or clones.

3.7 Occurrence of peptide genotypes in field samples

Quantitative real-time PCR methodology as described in sections 3.1.1 and 3.1.2 was applied to field samples using the four TaqMan Nuclease Assays detecting four gene loci in *Planktothrix* spp., i.e. using the phycocyanin signal as quantitative indicator for the genus and the others for differentiating between genotypes containing microcystin, aeruginosin, and anabaenopeptin. This is the first time that a population could be further differentiated this far by peptide genotypes.

Phycocyanin genotypes indicative of *Planktothrix* spp. occurred in >90 % of all field samples, and peptide genotypes for the three cyanopeptides, for which real-time PCR was developed (i.e. microcystin, aeruginosin, anabaenopeptin) were also frequently detected via quantitative real-time PCR (Taq nuclease assay) throughout the study period.

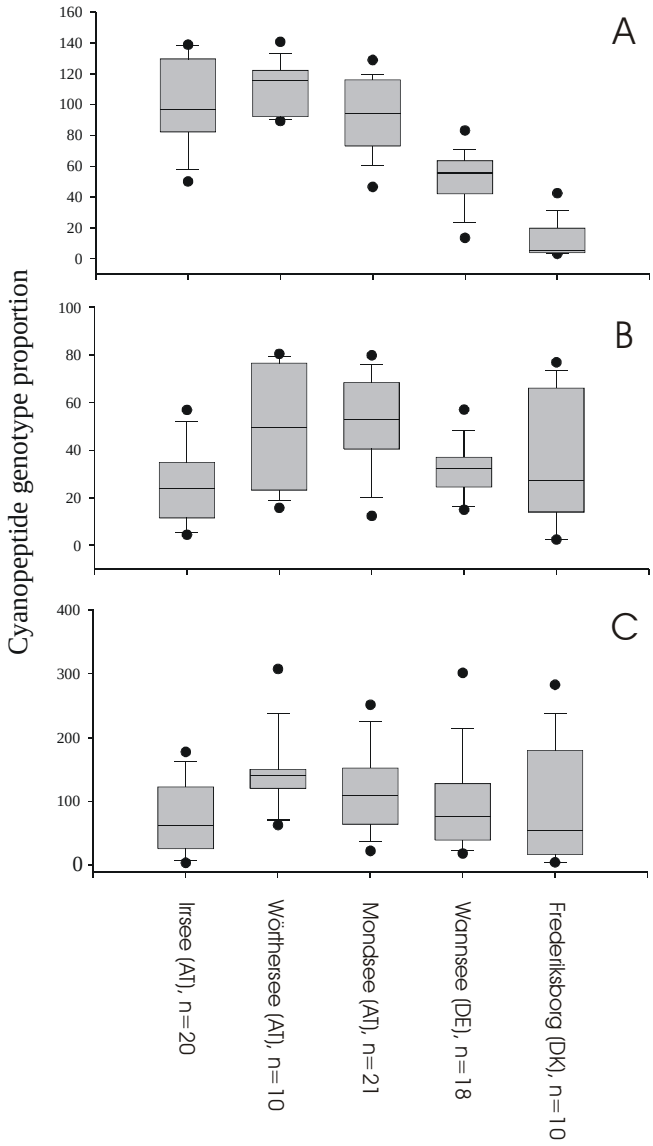


Fig. 17 Proportion of (A) *mcy*B, (B) aeruginosin, (C) anabaenopeptin genotypes in red-pigmented and green-pigmented populations of *Planktothrix* spp. as determined by Taq Nuclease Assays.

Proportions >100% are due to inaccuracies in cell number estimates due to the semi-logarithmic back calculation from cell based calibration series.

Averaged over the whole study period the populations of *Planktothrix* spp. differed significantly in the proportion of microcystin genotypes, i.e. P. *rubescens* populations in lakes in the Alps (Mondsee, Irrsee, Wörthersee) showed the highest proportions – close to one hundred percent throughout the year (Fig. 17). In contrast the two populations of *P. agardhii* (Wannsee, DE, Frederiksborg Slottso, DK) showed significantly lower percentages of microcystin genotypes. Frederiksborg Slottso showed

the lowest proportion of microcystin producers throughout the study period. The aeruginoside genotypes never constituted one hundred percent of the total population and their proportions did not differ between populations of P. *rubescens* and *P. agardhii*. Anabaenopeptin genotypes showed highest proportions in all investigated *Planktothrix* samples irrespective of the geographic origin.

3.8 Peptide concentrations in water-bodies

Among the intensively investigated lakes: three lakes in the Austrian Alps (Mondsee, Wörthersee, Irrsee) and Lake Steinsfjorden in Norway were dominated by *Planktothrix rubescens*. As discussed in section 2.2.5, these were analysed for peptide concentrations with LC-MS techniques using microcystin-LR as internal standard. Results are therefore expressed as equivalents of microcystin-LR. *Planktothrix* samples from Lake Steinsfjorden in Norway and the lakes in the Alps both showed high concentrations of desmethyl-microcystin-RR (m/z 1024) and anabaenopeptin B (m/z 837). However the lakes differed in the concentration of aeruginosins and cyanopeptolins (Fig. 18). This result is in accordance with the divergence in peptide occurrence among specific populations of *Planktothrix rubescens* revealed by single filament MALDI-TOF MS (see above, Table 10).

Planktothrix samples from Lake Steinsfjorden in Norway and the lakes in the Alps both showed high concentrations of desmethyl-microcystin-RR (m/z 1024) and anabaenopeptin B (m/z 837). However the lakes differed in the production of aeruginosins and cyanopeptolins (Fig. 18). This result is in accordance with the divergence in peptide occurrence among specific populations of *Planktothrix rubescens* revealed by single filament MALDI-TOF MS (see above, Table 10).

Taking all data together significant linear relationships were observed between *Planktothrix* biovolume and concentrations of desmethyl-microcystin-RR (m/z 1024) and anabaenopeptin B (m/z 837) (Fig. 19). Within the alpine lakes significant linear relationships were also observed for concentrations of aeruginosin A and oscillapeptin 1126. Within Lake Steinsfjorden the concentration of aeruginosin 583 was found linearly related to *Planktothrix* biovolume, however, the concentrations of oscillaginin A and oscillapeptin G varied independently of *Planktothrix* biovolume.

In summary the *Planktothrix* biovolume was a strong predicting variable for the concentrations of dominant peptides, such as microcystins, anabaenopeptin and aeruginosin. A few less abundant peptides occurred irregularly during the study period and this variation may be induced by shifts in peptide genotype composition.

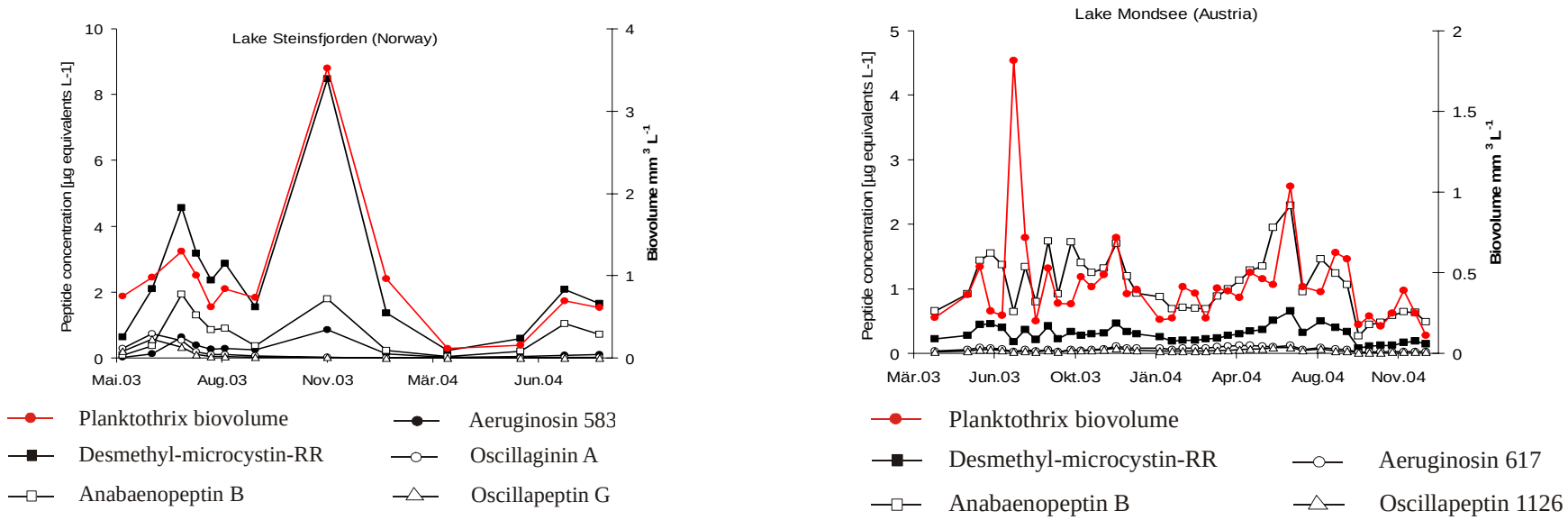


Fig. 18 Selected peptide concentrations in Lake Steinsfjorden (Norway) and Lake Mondsee (Austria) dominated by *Planktothrix rubescens* in the years 2003, 2004.

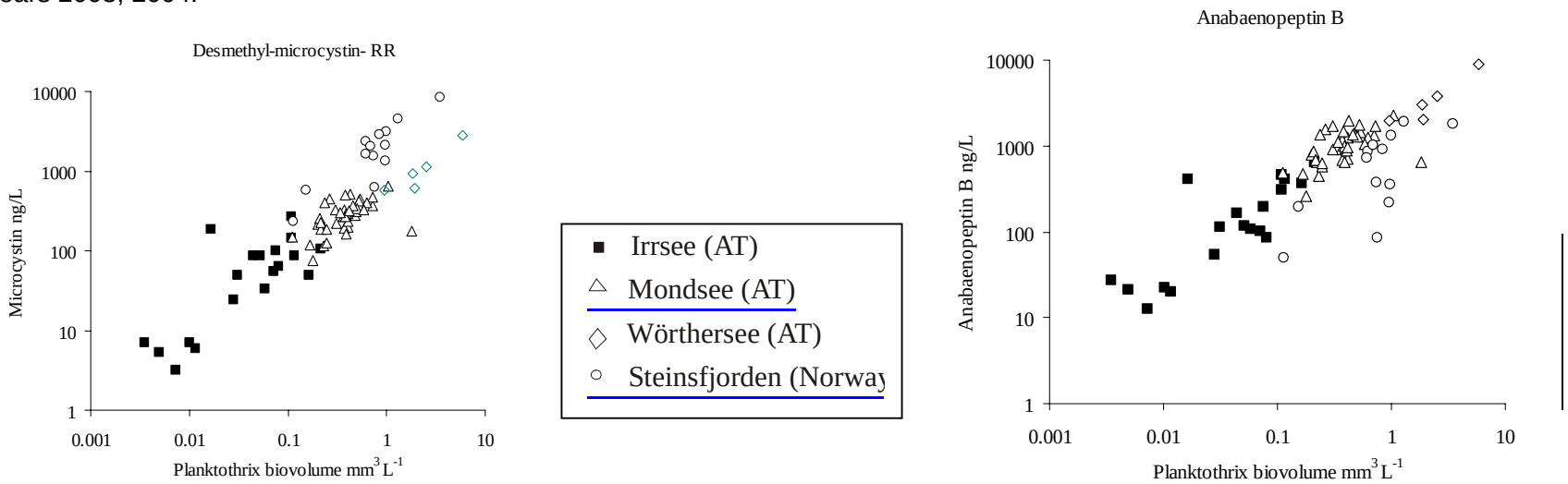


Fig. 19: Relationships between *Planktothrix* biovolume and concentratioms of desmethyl-microcystin-RR and anabaenopeptin B in three lakes in the Alps and one lake in Norway dominated by *Planktothrix rubescens* during the years 2003, 2004.

In 82 samples from Danish, Dutch and one German lake the following 6 cyanopeptides were quantified l LC-MS/MS: aeruginosamide, anabaenopeptines A, B, F, Cl-cyanopeptolin W and oscillamide Y (se section 2.2.5). Furthermore, microcystins in these samples were analysed by HPLC-PDA; individu microcystin variants, however, are not addressed here and only the sum of microcystins is given.

Cyanopeptides and microcystins frequently occurred in the lakes investigated. While aeruginosamide ar anabaenopeptines were found in all samples, Cl-cyanopeptolin W and microcystins were slightly le frequent (90 and 87 %).

The data show that in all lakes the sum of the other cyanopeptides was higher than that of microcystii (Fig. 20, Table 11). On average microcystins comprised only 10% of the total peptide concentration. Tl highest total peptide concentration was 82 µg/l in Wannsee (08/21/2003), and generally this lake showe higher concentrations for all of the peptides than lakes from Denmark and the Netherlands (Fig. 21, Tab 11).

Table 11: Median concentrations of peptides (µg/L) for lakes from Denmark (Arreskov sø, Arresø, Bryru Langsø, Esrum sø, Frederiksborg Castle Lake, Halle sø, Kimmerslev sø, Langesø), Tl Netherlands (t Joppe, Zegerplas) and Germany (Wannsee)

Peptide	Danish lakes	Dutch lakes	German lake
Aeruginosamide	0.14	0.09	0.52
Anabaenopeptin A	0.41	0.28	1.00
Anabaenopeptin B	0.27	0.22	0.77
Anabaenopeptin F	0.99	0.98	3.87
Cl Cyanopeptolin W	0.15	1.57	4.78
OscillamideY	0.49	0.77	2.09
Microcystins	0.56	0.23	1.15

The phytoplankton of the lakes was dominated by cyanobacteria from different genera. Samples fro Denmark were either solely dominated by *Microcystis*, *Planktothrix agardhii*, *Anabaena* *Aphanizomenon*, or by varying combinations of these genera. The German lake showed a similar pictu with blooms of *Microcystis* and *Aphanizomenon* in summer followed by *Planktothrix agardhii* in autumn. contrast, Dutch lakes were largely dominated by *Microcystis* and only occasionally accompanied l *Planktothrix agardhii* or *Anabaena*.

Relationships between cyanobacterial species or genera and peptide concentration, as shown f *Planktothrix rubescens* above, were not observed. This is likely due to the heterogeneity in phytoplankto composition but also due to the fact that the anabenopeptines analysed (anabaenopeptines A, B, oscillamide Y) can be produced by several genera such as *Microcystis*, *Planktothrix* and *Anabaena*. A allocation of their occurrence to certain taxa thus is not possible for these lakes. Only aeruginosamide ar Cl-cyanopeptolin W have been reported so far only for *Microcystis*. However, while *Microcystis* populatio in the Netherlands and Wannsee contained high amounts of Cl-cyanopeptolin W (see Table 11, Fig. 2 this was not found for the Danish *Microcystis* populations. Qualitative divergence in peptide occurren has been shown earlier and our quantitative results confirm this.

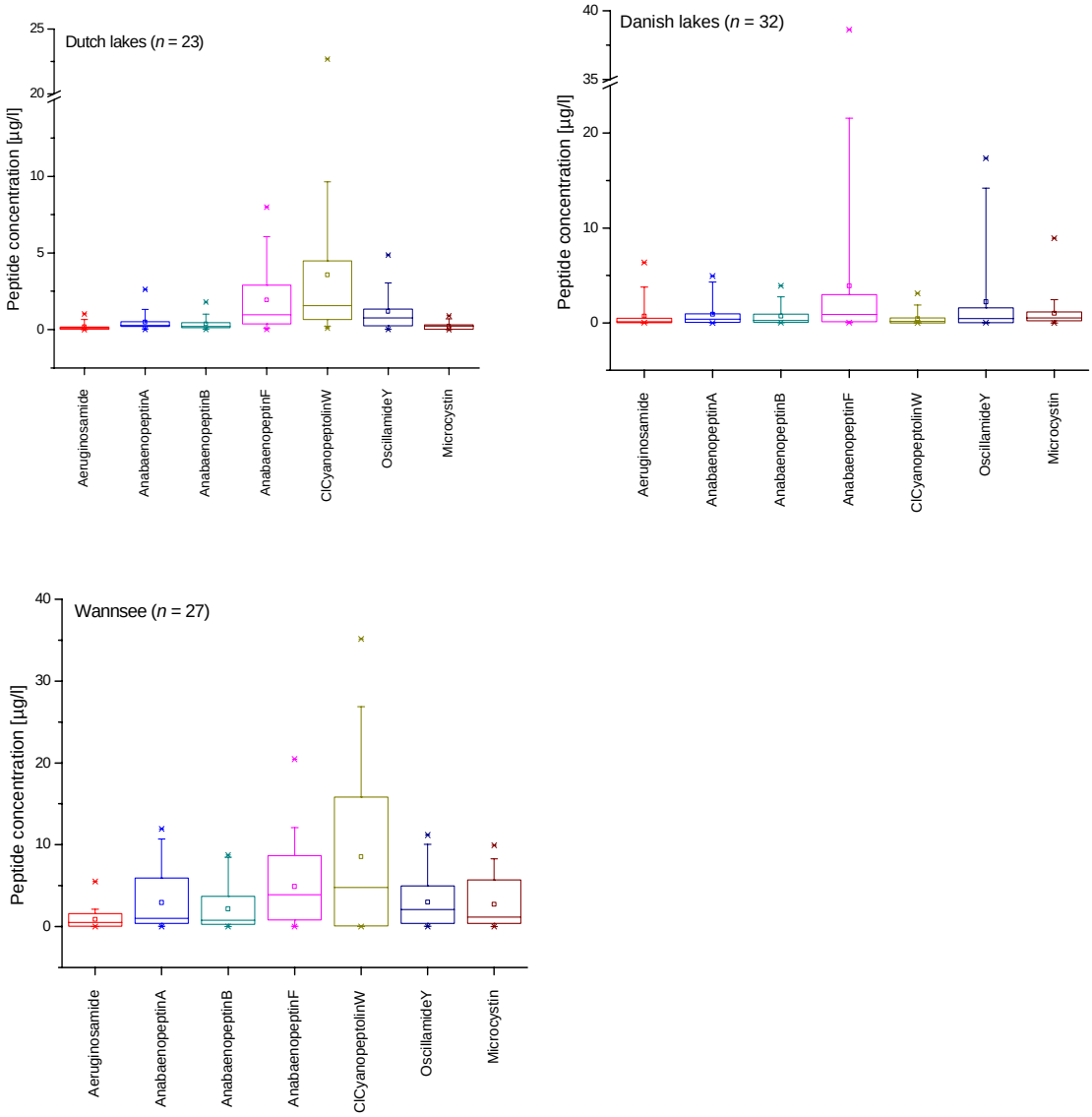


Fig 21: Concentration of different cyanopeptides in lakes from The Netherlands (t Joppe, Zegerplas), Denmark (Arreskov sø, Arresø, Bryrup Langsø, Esrum sø, Frederiksborg Castle Lake, Halle sø, Kimmerslev sø, Langesø), and Germany (Wannsee); "Microcystin" refers to the sum of all variants.[C5] Note different scale for the Dutch Lakes, n = number of samples.

3.9 Cyanopeptide toxicity

3.9.1 Mutagenicity

None of the purified or semipurified peptides tested (see overview in Table 12 demonstrated a mutagenic activity in the AMES test (Table 12).

Table 12: Ames test with various purified and semipurified cyanobacterial peptides showing no mutager potential

n.m.*: not measured; neg co**: negative control; po co: **: positive control; TA 98 ± S9: 4-NQO (Nitrochinolin-N-oxid) + 2-NF (2-Nitrofluoren), (12.5ng/ml + 50ng/ml), TA 100 – S9: 4-NQO (3.3µg/ml), 100 + S9: 2-AA (2-Aminoanthracen) (125µg/ml). For neg co and po co of Anabaenopeptin Cyanostatin-A, Microviridin J, Oscillarin and UP4 see values listed for Aeruginosamide.

Tested peptide/peak	concentration	TA 98		TA 100	
		- S9	+ S9	- S9	+ S9
Aeruginosamide	18µM	7.3 ± 1.2	7.7 ± 1.5	13.7 ± 3.1	5.0 ± 2.0
	neg co**	8.0 ± 2.6	10.7 ± 4.7	15.3 ± 5.0	7.7 ± 2.1
	po co**	31.0 ± 0.0	47.3 ± 1.2	48.0 ± 0.0	47.3 ± 1.2
Anabaenopeptin A (semipurified)	1800µM	1.7 ± 1.2	4.7 ± 1.2	13.0 ± 1.6	n.m.
	36µM	5.0 ± 0.8	5.0 ± 0.8	9.3 ± 1.7	n.m.
	neg co**	3.0 ± 1.2	5.3 ± 2.7	12.1 ± 2.6	n.m.
	po co**	41.3 ± 1.2	48.0 ± 0	41.7 ± 2.6	n.m.
Anabaenopeptin B (semipurified)	1800µM	3.0 ± 2.4	2.7 ± 1.9	12.7 ± 0.9	n.m..
	36µM	4.7 ± 3.1	6.7 ± 1.2	13.0 ± 1.6	n.m.
	neg co**	2.6 ± 1.1	6.8 ± 2.2	10.9 ± 2.6	n.m.
	po co**	40.0 ± 1.6	48.0 ± 0	42.0 ± 1.6	n.m.
Anabaenopeptin D	12.1µM	5.7 ± 1.2	7.7 ± 3.2	9.3 ± 1.2	6.7 ± 2.1
Cyanostatin-A	13.7µM	8.0 ± 3.5	8.0 ± 2.6	9.3 ± 2.1	7.7 ± 0.6
Microviridin J	5.9µM	6.7 ± 1.5	7.0 ± 1.7	8.3 ± 1.5	7.0 ± 2.0
Microginin	900µM	2.0 ± 1.0	9.3 ± 2.6	*n.m.**	n.m.
	18µM	4.5 ± 0.5	5.0 ± 1.6	10.3 ± 2.4	n.m.
	neg co**	3.0 ± 1.9	5.2 ± 0.8	9.6 ± 1.9	n.m.
	po co**	41.5 ± 1.5	48.0 ± 0	38.3 ± 2.4	n.m.
Oscillatorin	911µM	2.7 ± 1.2	6.3 ± 2.9	11.3 ± 1.9	n.m..
	16µM	1.0 ± 1.4	7.0 ± 2.4	12.0 ± 2.2	n.m.
	neg co**	2.8 ± 2.1	5.8 ± 2.1	11.3 ± 2.3	n.m.
	po co**	41.3 ± 1.7	48.0 ± 0	40.3 ± 0.5	n.m.
Nostocyclin	900µM	3.3 ± 0.5	6.3 ± 1.2	*n.m.*	n.m.
	18µM	3.7 ± 1.2	4.7 ± 2.4	11.7 ± 0.9	n.m.
	neg co**	3.0 ± 1.4	5.9 ± 1.4	11.1 ± 2.5	n.m.
	po co**	40.7 ± 1.9	48.0 ± 0	37.7 ± 2.1	n.m.
Nostophycin	900µM	2.7 ± 1.0	4.3 ± 1.2	14.3 ± 2.6	n.m..
	18µM	2.7 ± 0	4.7 ± 1.7	9.7 ± 2.1	n.m.
	neg co**	4.2 ± 2.0	5.2 ± 1.6	9.8 ± 2.7	n.m.
	po co**	40.0 ± 2.0	48.0 ± 0	42.7 ± 3.4	n.m.
UP1	3.8mg/ml	4.0 ± 0.8	5.7 ± 2.5	10.7 ± 2.9	n.m..
	0.08mg/ml	1.0 ± 1.4	5.3 ± 1.2	9.0 ± 1.6	n.m.
	neg co**	3.4 ± 1.0	5.4 ± 2.3	9.2 ± 2.6	n.m.
	po co**	39.7 ± 1.2	48.0 ± 0	34.3 ± 6.0	n.m.
UP2	2.8mg/ml	1.7 ± 1.7	6.7 ± 0.5	14.7 ± 2.6	n.m..
	0.06mg/ml	4.7 ± 1.2	5.7 ± 2.4	8.3 ± 3.3	n.m.
	neg co**	3.7 ± 2.0	5.1 ± 2.3	10.7 ± 2.3	n.m.
	po co**	39.7 ± 2.9	48.0 ± 0	39.0 ± 2.9	n.m.
UP4	10µg/ml	11.7 ± 5.1	6.7 ± 2.1	13.7 ± 2.5	6.3 ± 1.5

3.9.2 Necrosis and apoptosis

None of the purified or semipurified cyanobacterial peptides tested demonstrated any necrotic or apopto potential in concentrations of 50 and 100 µg/ml (Table 13). With the exception of PEX 19.3, whi demonstrated a mild necrotic potential (Table 13), no effects were observable with any of t cyanobacterial extract incubations. In this case necrosis means primary and secondary necrosis, where primary necrosis can be defined as an uncontrolled and pathogenic form of cell death and seconda necrosis as a result of apoptotic cell death, when apoptotic cells fail to be removed by heterophagy.

Table 13: Lack of apoptotic and necrotic effects after incubation of JURKAT cells with various purifi (including MC-LR) and semipurified cyanobacterial peptides

Legend: DBP: disinfection by-products; PJ: propidiumjodide; unexposed cells served negative control, apoptosis or necrosis inducing disinfection by-products were used as positi controls. The percentage of apoptotic and necrotic cells were determined relative to the to number of cells analysed.

Tested peptide/peak	concentration	Apoptosis (%)	Necrosis (%)
		Annexin-FITC pos	Annexin-FITC + PJ pos
Aeruginosamide	89µM	3	5
	178µM	4	3
Anabaenopeptin D	60µM	3	3
	120µM	3	3
Cyanostatin-A	69µM	3	5
	138µM	3	3
MCLR	50µM	2	4
	100µM	2	4
Microviridin J	30µM	3	4
	60µM	3	3
Nostocyclin	45µM	3	4
	90µM	3	4
Oscillarin	50µM	3	3
	100µM	3	4
Oscillatorin	40µM	4	5
	80µM	3	3
UP1	50 µg/ml	3	4
	100 µg/ml	3	4
UP2	50 µg/ml	3	4
	100 µg/ml	3	3
UP4	50 µg/ml	3	5
	100 µg/ml	3	4
MeOH	1%	3	4
	2%	2	3
Negative control		3	4
		2	3
Positive control apo	60 µg/ml	13	9
(DBP V)	125 µg/ml	35	11
	250 µg/ml	46	16
Positive control nec	15 µg/ml	4	9
(DBP XVI)	60 µg/ml	11	31
	250 µg/ml	15	60
	1250 µg/ml	2	91

Table 14: Lack of apoptotic and necrotic effects after incubation of JURKAT cells with variou cyanobacterial extracts

Legend: DBP: disinfection by-products; necrosis: primary and secondary necrosis; P propidiumjodide; cells were incubated for 2h, lower concentrations also up to 24h (as specifie in the table); unexposed cells served as negative control, apoptosis or necrosis inducir disinfection by-products were used as positive controls. The percentage of apoptotic ar necrotic cells were determined relative to the total number of cells analysed.

Tested peptide/peak	concentration	Apoptosis (%)	Necrosis (%)
		Annexin-FITC pos	Annexin-FITC + PJ
PEX 3.1	4 mg/ml	2	5
	0.1 mg/ml	3/5 (24h)	5/9 (24h)
	0.05 mg/ml	5 (24h)	7 (24h)
PEX 11.1	4 mg/ml	3	6
	0.1 mg/ml	3/5 (24h)	6/6 (24h)
	0.05 mg/ml	6 (24h)	7 (24h)
PEX 13.3	4 mg/ml	3	6
	0.1 mg/ml	3/6 (24h)	5/7 (24h)
	0.05 mg/ml	6 (24h)	7 (24h)
PEX 19.2	4 mg/ml	3	5
	0.1 mg/ml	3/4 (24h)	4/7 (24h)
	0.05 mg/ml	5 (24h)	8 (24h)
PEX 19.3	4 mg/ml	3	11
	0.1 mg/ml	2/5 (24h)	4/6 (24h)
	0.05 mg/ml	5 (24h)	7 (24h)
PEX 20.1	4 mg/ml	2	7
	0.1 mg/ml	3/5 (24h)	5/8 (24h)
	0.05 mg/ml	5 (24h)	7 (24h)
PEX 20.3	4 mg/ml	3	4
	0.1 mg/ml	2/5 (24h)	4/9 (24h)
	0.05 mg/ml	5 (24h)	8 (24h)
PEX 26.1	4 mg/ml	3	5
	0.1 mg/ml	3/6 (24h)	4/7 (24h)
	0.05 mg/ml	6 (24h)	8 (24h)
PEX 26.3	4 mg/ml	4	8
	0.1 mg/ml	3/5 (24h)	7/8(24h)
	0.05 mg/ml	7 (24h)	9 (24h)
PEX 27.1	4 mg/ml	3	4
	0.1 mg/ml	3/5 (24h)	5/7(24h)
	0.05 mg/ml	5 (24h)	6 (24h)
Negative control		4/5 (24h)	5/7 (24h)
Positive control apo (DBP V)	250 µg/ml	36/37	9/11
Solvent control (MeOH)	2%	3	5
	1%	4	5
	0.1%	3	6
	0.05%	3/5 (24h)	5/7(24h)
	0.03%	6 (24h)	6 (24h)

3.9.3 Cytotoxicity tested with CaCo2, HepG2 and HEK293 cell lines:

Single peptides/fractions: None of the investigated peptides or fractions was strongly cytotoxic in HepG CaCo2 or HEK 293 (Table 15). Only mild cytotoxic effects could be observed after incubation wi Aeruginosamide, Anabaenopeptin A, Anabaenopeptin B, Oscillarin and UP1 and UP2.

Cyanobacterial Extracts: Crude extracts of both, culture strains and field samples dominated b cyanobacteria, were tested in three fractions of different hydrophilicity / hydrophobicity for cytotoxici (Table 16). Remarkably, all stains and field samples did show cytotoxicity in some fractions, with the mo toxic fraction varying between strains and samples (and effects often being most pronounced in the mo hydrophilic fraction). In fact, only one strain (MCT (CBS) =Pex12) showed almost no effects while for others – and for all field samples – at least one of the 3 fractions had impacts, usually consistently seen both cytotoxicity tests (two different endpoints: MTT test: metabolic activity of the cells; LDH-tes membrane integrity of the cells). However, the extract concentrations producing these mild toxic effec (see those marked with + in Table 16), were rather high. Thus a possible explanation is that the hig number and variety of molecules applied with the extract disturb the metabolic activity of the test cells ar are responsible for the effects observed, rather than these being caused by any specific single tox substance(s) in the sample. The most toxic extracts were those from Planktothrix strains, while extrac from other species /field samples were not as clear.

With the exception of Planktothrix wild type (Pex27) none of the culture strains tested containe microcystins, so the observed cytotoxic effects appear to result from other cyanobacteri peptides/metabolites. In addition, as microcystins are cytotoxic at concentrations above concentrations 50-120 µM, the cytotoxic effects observed with extracts from field samples would most likely not resu from lower concentrations of microcystins if they were present in some of these extracts.

Cell line	HepG2			CaCo 2		HEK 293 (co)	HEK 293 (1B1)	HEK 293 (1B3)
Test system	LDH	MTT	NR	LDH	MTT	MTT/LDH		
Aeruginosamide	EC_{50} ~ 100µM(48h)	EC_{50} ~ 100µM(48h)	nt	EC_{50} 130µM(48h)	Mild toxic at 160µl	≥15µM (48h)	≥15µM (48h)	≥15µM (48h)
Anabaenopeptin A	≥60µM (24h) ≥280µM (48h)	≥280µM(48h)	≥60µM (48h) Effect: 60µM (72h)	≥30µM (48h)	≥30µM (48h)	≥98µM (48h)	≥98µM (48h)	≥98µM (48h)
Anabaenopeptin B	EC_{50} (48h) ~ 100µM	EC_{50} (48h) ~ 100µM	≥60µM (72h)	≥30µM (48h)	≥30µM (48h)	≥99µM (48h)	≥99µM (48h)	≥99µM (48h)
Anabaenopeptin D	≥170µM(48h)	≥170µM(48h)	nt	nt	≤>170µM(48h)	≤>101µM (48h)	≤>101µM (48h)	≤>101µM (48h)
Cyanostatin A	≤>280µM(48h)	≤>280µM(48h)	nt	nt	≤>280µM(48h)	≤>114µM (48h)	≤>114µM (48h)	≤>114µM (48h)
Microginin (unspec)	≤>30µg/ml (48h)	≤>30µg/ml (48h)	≤>30µg/ml (48h)	≤>30µg/ml (48h)	≤>30µg/ml (48h)	nt	nt	nt
Microviridin J	≤>56µM(48h)	≤>56µM(48h)	nt	nt	≤>56µM(48h)	≤>56µM(48h)	≤>56µM(48h)	≤>56µM(48h)
Nostocyclin	≤>30µM (48h)	≤>30µM(48h)	≤>30µM	≤>30µM (48h)	≤>30µM (48h)	nt	nt	nt
Nostophycin	≤>100µM (24h) ≥250µM (48h)	≤>250µM(48h)	≤>300µM (72h)	≤>30µM (48h)	≤>30µM (48h)	≤>94µM (48h)	≤>94µM (48h)	≤>94µM (48h)
Oscillatorin	≤>60µM(48h)	≤>60µM(48h)	nt	nt	≤>60µM(48h)	≤>67µM (48h)	≤>67µM (48h)	≤>67µM (48h)
Oscillarin	≤>50µM (48h)	≤>50µM (48h)	nt	nt	≤>50µM (48h)	Mild effect at 125	Mild effect at 125µ	Mild effect at 125µ
Oscillatorin (semipurifie	≥80µM (48h) ≥31µM (24h)	≤>24µM (48h)	≤>31µg/ml (72h)	≤>24µM (48h)	≤>24µM (48h)	Mild effect at 67µ	Mild effect at 67µl	Mild effect at 67µl
UP1	≤>515µg/ml (48h), ≤:	≤>30µg/ml (48h)	≤>126µg/ml (24h) EC_{50}(48h/72h) ~ 30/1:	≥30µg/ml (48h)	≥30µg/ml (48h)	≥83µg/ml (48h)	≥83µg/ml (48h)	≥83µg/ml (48h)
UP2	≤>242µg/ml (48h) ≥93µg/ml (24h)	≤>242µg/ml (48h)	≤>92 µg/ml (48h) Effect: 92 µg/ml (72h)	≥30µg/ml (48h)	≤>30µg/ml (48h)	≤>83µg/ml (48h)	≤>83µg/ml (48h)	≤>83µg/ml (48h)
UP3	≤>42µg/ml (48h)	≤>42µg/ml (48h)	nt	nt	≤>42µg/ml (48h)	≤>83µg/ml (48h)	≤>83µg/ml (48h)	≤>83µg/ml (48h)
UP4	≥83µg/ml (48h)	≤>83µg/ml (48h)	nt	nt	≤>83µg/ml (48h)	≤>83µg/ml (48h)	≤>83µg/ml (48h)	≤>83µg/ml (48h)

Table 15: Cytotoxicity of various cyanobacterial peptides in HepG2, CaCo2 and HEK293.

The values listed are the peptide (µM) or unknown peptide concentrations (µg/ml), up to which cytotoxicity was tested without detecting effects, i.e. < xy mM: no at concentrations up to this level). Gray shading: peptide concentrations that demonstrated mild toxic effects; nt: not tested; co: control transfected; 1B1: OAT transfected; 1B3: OATP1B3 transfected: UP: unidentified peptide

extracts		Pex3			Pex11			Pex13			Pex16			Pex18			Pex19			Pex20		
fraction-nr.		1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
HepG	MTT	+	+	-	++	+	-	+	+	-	+	-	-	+	-	-	+	++	++	-	-	-
	LDH	-	-	-	-	+	-	-	++	+	-	-	-	+	-	-	+	+	++	-	-	-
CaCo	MTT	+	-	-	++	-	-	+	+	+	+	-	-	+	-	-	+	++	++	-	-	-
	LDH	nt	nt	-	nt	nt	-	+	nt	-	nt	nt	nt	nt	nt	nt	-	++	++	-	-	+
extracts		Pex26			Pex27			Pex29			Pex30			Pex31			Pex32			Pex33		
fraction-nr.		1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
HepG	MTT	++	++	++	++	-	++	+	-	-	+	-	-	-	-	-	-	-	-	+	-	-
	LDH	++	++	+	+	+	++	++	-	-	++	-	-	+	-	-	-	-	-	-	-	-
CaCo	MTT	++	-	+	+	-	-	*	-	-	++	-	-	+	-	-	-	-	++	+	+	-
	LDH	++	+	+	+	-	-	++	-	-	++	-	-	+	-	-	-	-	+	-	+	-
field samples		Pex35A			Pex35B			Pex36A			Pex36B			Pex37			Pex38			Pex39		
fraction-nr.		1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
HepG	MTT	+	-	-	+	-	-	+	-	+	+	-	+	+	-	+	+	-	+	+	-	-
	LDH	-	-	-	+	-	-	-	-	-	+	-	-	-	-	-	-	-	+	-	-	-
CaCo	MTT	+	-	-	+	-	-	+	-	-	+	-	-	+	-	-	+	-	-	+	-	+
	LDH	+	-	-	+	-	-	-	-	-	+	-	-	+	-	-	-	-	-	+	-	+

Table 16: Cytotoxic effects of cyanobacteria extracts

(Pex = Pepcy extract): every sample (field, culture samples) was extracted for 3 fractions (see extraction protocol/WP3): hydrophilic (1), moderately hydrophili hydrophobic (3). The effects were classified into three categories according to the range for the EC_{50} after 48h incubation (mg dry weight/ml final extract): - : no e in the highest concentration tested (appr. 17 mg DW/ml extract)+: EC_{50} values between 2-17 mg DW/ml extract++: EC_{50} values less than 2 mg DW/ml extract

3.9.4 In vivo acute mouse bioassay using a cyanobacterial wildtype and the corresponding Mcy deficient mutant strain:

The largely negative results of the cytotoxicity tests using semi-purified cell extracts showed a need for more comprehensive assay to assess whether the bioactive effects identified in wild type cyanobacter and mutant strains (deficient in the ability to produce particular non-ribosomal peptides) was applicable previously characterised toxins (microcystins) or toxic effects not previously attributed to non-microcyst cyanopeptides. Acute hepatotoxicity to mice was found using P. agardhii CYA 126 and no acu hepatotoxicity was found using the P. agardhii CYA 126 Mcy B deficient mutant. However in thes animals, mild diarrhoea was observed. The absence of acute toxicity in the P. agardhii CYA 126 Mcy deficient mutant indicated that effects identified in in vitro cytotoxicity tests are due to microcystins. Th acute effects were absent in the P. agardhii CYA 126 Mcy B deficient mutant indicating that the presen of non-microcystin cyanopeptides did not induce toxic effects. Had the P. agardhii CYA 126 Mcy deficient mutant demonstrated toxic effects similar to those found in the wildtype strain, an additior bioassay could have been carried out with CYA 126 mutants deficient in the production of eith anabaenopeptin(s) or aeruginosin(s). No further testing of mutant strains was carried out as the acu toxicity associated with this particular wildtype strain was attributable to the microcystin content.

3.9.5 Transport experiments

The results of the transport experiments using OATP transfected HEK 293 cells demonstrated that th differences in the capacity of Oatps/OATPs to transport different MCYST congeners can be one importa reason for the previously noted different LD50 values of the single MCYST congeners (Table 17). Thes two organic anion transporting peptides, among other yet unidentified or unspecified transporters, appe capable for actively transporting microcystins into the cell. Interestingly the capacity of these two OATP transport the more hydrophobic microcystins (MCYST-LF/ MC-LW) was 10-50 times higher whe compared to the “model microcystin” MCYST-LR, which is used as one of the most toxic representative for the microcystin family in risk assessment. In contrast, EC_{50} values for MCYST-RR and nodularin we distinctly higher compared to MCYST-LR. None of the Anabaenopeptides tested showed a stron cytotoxicological effect in any of the exposed OATP or control transfected HEK 293 cells (Tables 15 ar 17).

Table 17: EC_{50} values of microcystin variants and nodularin: test systems HEK 293 cell line (contr transfected, transfected with OATP1B1 and transfected with OATP1B3; for the complete list cyanobacterial peptides and semipurified peptides tested with the HEK 293 cell line system, se Table 15).

EC_{50} (48h)	HEK 293 control (I	HEK 293 1B1 (nI	HEK 293 1B3 (
MCYST-LF	>410	9	2
MCYST-LW	>417	12	8
MCYST-LR	>3200	106	132
MCYST-RR	>2750	>2750	440
Nodularin	>1347	600	>1347
Anabaenopeptin A	≥15000	≥15000	≥15000
Anabaenopeptin B	≥98000	≥98000	≥98000
Anabaenopeptin D	≥99000	≥99000	≥99000

3.10 Cyanopeptide biomonitoring

Of the 123 extracts from strains were tested, most of which were from *Planktothrix spp.,* most extrac showed an inhibitory effect. IC_{50}s were very variable, for example among the 27 *Planktothrix* strains fro

AAS 5 were of lowest activity (IC_{50} >1) while among the other strains the IC_{50} was <1 but differed sever hundred fold. Trypsin inhibition did not correlate with the concentration of microcystin. Notably, all *Planktothrix* strains from NVA causing strong or intermediate inhibitory effects contained cyanopeptoli (Rohrlack et al. 2005), and experiments with HPLC-fractions clearly linked the trypsin inhibition effect cyanopeptides, in particular to cyanopeptolons containing a sulphate group in the side chain (Rohrlac unpublished data).

Field samples from P. *rubescens* typically showed a strong inhibition of invertebrate and porcine tryps derived activity. Most field samples dominated by *Microcystis* spp. contained a higher share of oth cyanobacteria, so effects can be less unambiguously attributed to *Microcystis* spp., and the responses samples from these lakes were found to be more variable. As for the culture strains, no correlation with t microcystin concentration was observed in the field samples. For example, in a scum sample from Lal Esrum Sö showing the highest microcystin content (2.76 mg g^{-1} DW), enzyme inhibition was low (IC_{50} 11.2) whereas a non-microcystin containing sample from a New Zealand lake showed highest trypsi inhibitory activity (IC_{50} = 0.001). This clearly demonstrates that other substances are involved in t enzyme inhibition.

Survival experiments with cyanobacterial cells containing protease inhibitors suggest that thes compounds can have an acute toxic effect on *Daphnia*. This effect can be identified by a unique set symptoms (Rohrlack et al. 2004): The animals were unable to shed the old integument to complete t molting process. Parts of the old integument remained attached to or completely warped the 2nd antenn thus preventing the antennas` brunches from unfolding in a normal way. Other appendages such as t filtering legs suffered the same fate. Additionally, the entire body surface became rapidly covered wi particles originating from the food suspension. This was presumably due to the secretion of some sort body substance. All these effects strongly inhibited *Daphnia's* swimming and feeding abilities. T individuals that had developed the molting disruption died within days or earlier due to starvation.

Table 18: Planktothrix strains tested, their origin and identification (in red and bold) of those inhibiti *Daphnia magna* trypsin

Species/Strain id in NIVA culture collection	Origin (country)	Origin (water)
Planktothrix agardhii		
168	England	Lake Windermere
21	Finland	Gulf of Finland
126	Finland	Lake Långsjön
127	Finland	Lake Vesijärvi
229	Finland	Lake Östra Kyrksundet
302	Germany	Lake Kiessee
68	Netherlands	Lake Veluwemeer
9, **30**	Norway	Lake Frøylandsvatnet
10, **29**, **86**, 117/3, 117/4	Norway	Lake Gjersøen
11	Norway	Lake Akersvatnet
12, 116	Norway	Lake Årungen
15, 34, 61/1	Norway	Lake Kolbotvatnet
56/1, 56/2, 56/3, 137, **278**, **393**, **394**, **405**	Norway	Lake Steinsfjorden
59/1	Norway	Lake Jarenvatnet
64/1, 64/3, 64/4, 64/5, 64/6	Norway	Lake Helgetjernet
65	Norway	Lake Vansjø
133	Norway	Lake Øgderen
263/2	Norway	River Glåma
299	Norway	Lake Kalvsjøtjernet
397	Norway	Lake Stovivatnet
420, 421, 422, 423, 424, 425	Norway	Lake Bergesvatnet
232	Russia	Rybinsk Reservoir
88/1, 88/2, 88/3	Sweden	Lake Ören
91, 429	Sweden	Lake Mälaren
Planktothrix rubescens		
151	Denmark	Lake Almend sø
128	Finland	Lake Vesijärvi
129	Finland	Lake Långsjön
13, 37/1, 37/2, 37/4, 55, 97/1, 97/3, 97/4, **392**, 401, **402**, **406**, 407, 408	Norway	Lake Steinsfjorden
18, 85, 119	Norway	Lake Gjersøen
35, 320	Norway	Lake Kolbotnvatnet
395, 396	Norway	Lake Stovivatnet
24, 87	Sweden	Lake Levrasjön
89/1, 89/3, 89/4, 89/5	Sweden	Lake Ören
90, 108, 427	Sweden	Lake Mälaren
1	Switzerland	Lake Zürich

3.11 Cyanopeptide human health risk assessment

The term “risk assessment” is used widely with different meanings, often synonymously to haza assessment. PEPCY follows the definition used in the World Health Organisation Guidelines for Drinkin water Quality (WHO 2004) of risk being the combination of (i) likelihood of a hazardous event to occur a (ii) the severity of its human health impact. This uses risk assessment not generically, but always speci

to the respective setting. It thus becomes an important basis for decisions on management priorities, e. for upgrading systems to protect and/or treat drinking-water. Risk assessment is tightly linked to syste assessment: while the likelihood for hazards to occur can be assessed individually, hazard by hazard, a assessment meaningful for management will comprehensively analyse the hazards likely to occur in given setting and rank them according to the relative risk they pose to public health.

For cyanobacterial blooms this understanding of risk assessment translates to assessing (i) the likeliho of blooms producing hazardous peptides to occur (in relation to that of other hazards occurring in th setting) and (ii) the expected impact of these peptides on human health (again in relation to that of oth hazards occurring in the setting). PEPCY contributed to the latter with two results, (i) by demonstrating th to date cyanopeptides appear to be little cause for concern, although other cyanobacterial metabolites ma well be (see section 4.8), and (ii) by showing that transport of microcystins across cell membranes may b very effective for microcystin variants for which currently no standards exist (see section 4.7), and th these therefore should be equally included in hazard assessment

The fragmentary scientific knowledge base for assessing the cyanotoxin hazard to human health is ve similar to the situation for most other hazardous agents (i.e. chemicals and pathogens) that may occur water – for these also, the scientific knowledge base is far from comprehensive. However, although bo the likelihood of occurrence of a hazardous event and its impact on human health can usually only b estimated, comparing estimates from different teams of experts often shows rather good agreemer Furthermore, it is often more important to “get started” than to “be perfect”, while documenting th uncertainties of an assessment and making these transparent to the public. For practical purposes, relative ranking of risks for different hazardous agents is more important than an assessment in absolu terms, as it helps identify priorities for those management actions likely to be most important to prote human health in a given setting. In consequence, the PEPCY Decision support tool shows an example how a cyanotoxin risk can be assessed in relation to risks from other hazards in a given setting (Fig. 22).

In this imaginary case a community uses a eutrophic reservoir with some sewage impact as drinking-wat source having only conventional drinking-water treatment (i.e. without ozonation and activated carbo treatment), and recently some short-term failure of the chlorination system has been suspected. Suc settings occur in many parts of Europe. The hazard assessment conducted by the drinking-water suppli in this imaginary setting has identified the hazards shown in Fig. 22: repeated outbreaks of gastroenteri caused by *Cryptosporidium* not sufficiently removed drinking-water treatment have been reporte wastewater contamination of the surface water source and unreliable disinfection causes concern Hepatitis-a virus reaching consumers (though as of yet there is no indication of this actually happening ar causing illness); microcystins occur regularly and for several months in the reservoir at concentratio around 1 µg/L but for periods lasting up to several weeks also at up to 10 µg/L; *Legionella* (with th potential of causing pneumonia) have been found in warm water supplies of several public building (though no outbreaks of illness have been identified to date); consumers complain about iron causi “brown water, and in spring nitrate levels may exceed 50 µg/L (but never 100 µg/L; see Chorus 2006 for more detailed description of the setting). In this example, the public health authority assessed the heal risk due to microcystin as “moderate” in most cases, but as “high” if situations with up to 10 µg/L last f more than a few days, because the treatment plant is not equipped to eliminate dissolved microcystin ar the treatment process is likely to cause some cell lysis and toxin release. At these concentrations, th impact on public health is thought to be potentially severe (chronic liver damage, tumour promotion), b events with several weeks of up to 10 µg/L in finished drinking-water are considered less likely to occur. contrast, as the events of *Cryptosporidia* infections have repeatedly been demonstrated and syste assessment indicated occurrence and break-through of Hepatitis-a virus to be a major concern. Thus, th likelihood of occurrence of such incidents was classified as “certain” or “high” in this setting, and the pub health impact of outbreaks of infectious disease would be major, so remedial measures against thes hazards have top priority. The PEPCY Decision Support Tool emphasises that this is one example onl and the outcomes of risk assessment will be different for each setting analysed.

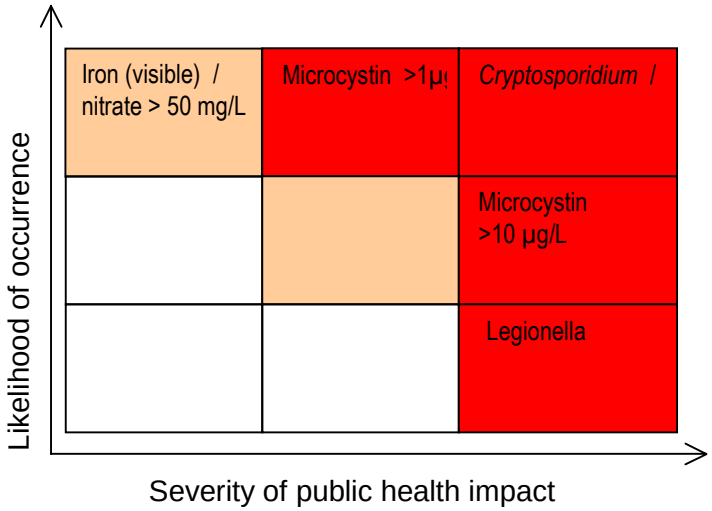


Figure 22: Risk matrix for identifying priorities; red fields denote situations likely to cause high risk to public health

3.12 Cyanopeptide public health risk management

As described in section 2.7, instead of a schematic approach to decision trees and alert levels, PEPC followed the WHO (2004) Water Safety Plan (WSP) approach and developed a web-based tool to gui practitioners through the steps of deriving their own WSP for their specific setting. The screen shot of t starting page of this tool (Fig. 23) shows that it includes some background information both on the WS approach and on cyanotoxins and then takes users through the steps of defining performance targets (Fi 24) for the measures implemented (or to be implemented) to control cyanobacterial proliferantion a cyanotoxin occurrence in their respective setting. The link to “preparaotory steps” outlines the experti likely to be needed in the WSP team. “Assessing risks and the system’s performance in controlling ther takes users through sets of tables addressing the different steps in the supply chain, interlinking background information on how these can be controlled and which operational limits may be appropria for which measures or how to derive them. Table 19 shows examples of these proformas for t catchment and the water-body; similar proformas guide users through assessing their drinking-wat offtake and treatment system. The scientific background for this approach to cyanotoxin risk manageme is given in Chorus (2005) and an introduction into the tools in Chorus (2006).

After finalisation of PEPCY, this decision support tool will be kept available on the PEPCY website and w be further maintained and updated by UBA. This will involve adding new links to further informatio particularly in the context of the revision of WHO guidance on cyanotoxins.

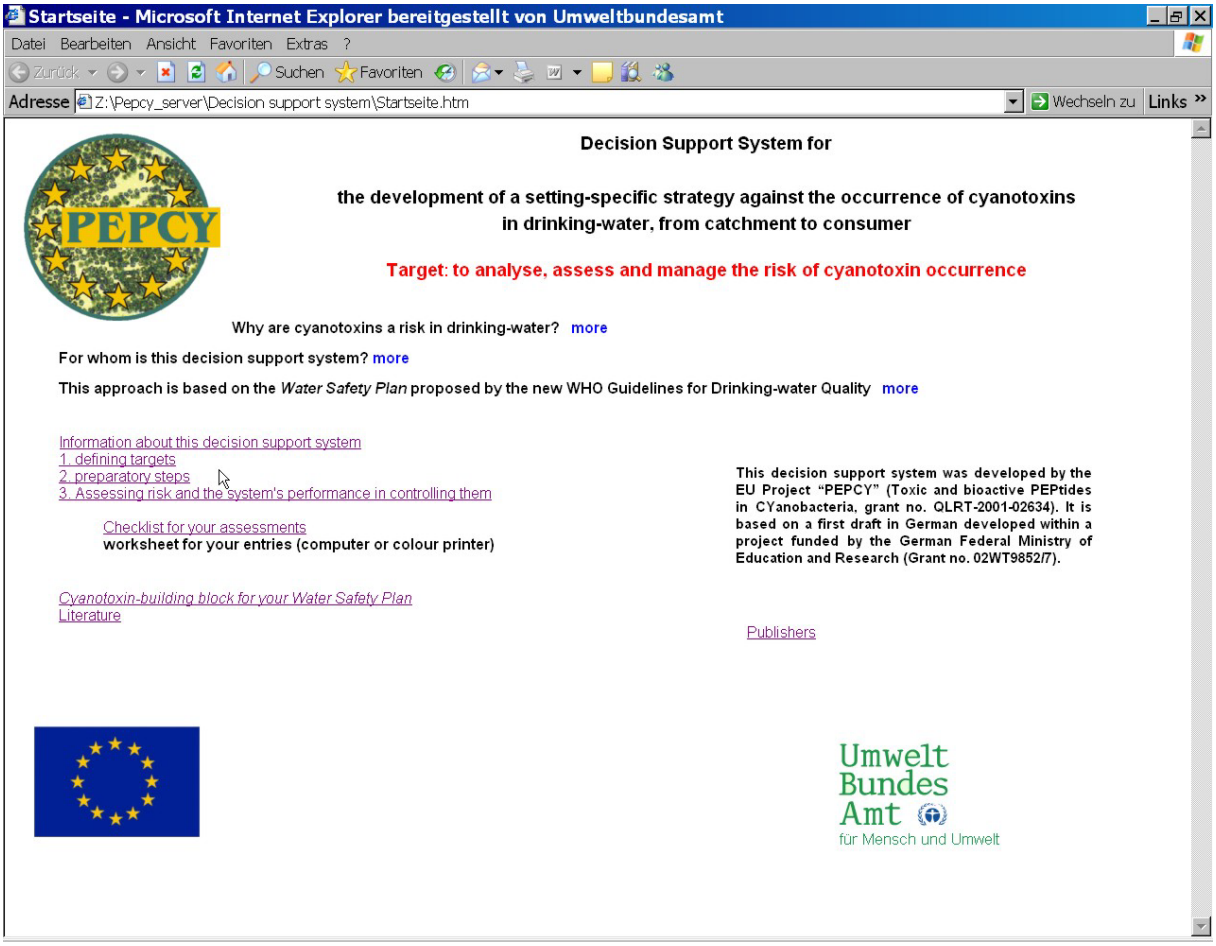


Fig. 23: Screen shot of the PEPCY web-based decision support tool for cyanotoxin risk management

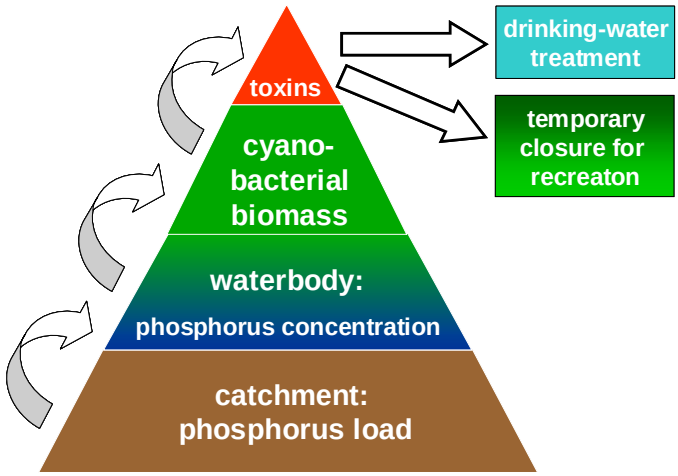


Figure 24: Setting performance targets for measures which control cyanobacterial proliferation and cyanotoxin occurrence

Table 19: Examples of proformas guiding decision tool users through assessing their catchment ar waterbody for the risk of cyanotoxin occurrence

A) Have cyanobacteria, cyanotoxins or indication of toxicity been observed ?

For this assessment, supporting expertise is recommended in the areas of public health, toxicology, and limnology, particularly phytoplankton

Were any of the following indications of cyanotoxin occurrence observed?

Legend: N = not observed; O = observed occasionally; F = observed frequent ? = no information; Text in *blue italics* is the example filled in for Reservoir X

Criteria	N	O	F	?	Your assessm the situation	Uncertainty of this assessm
Humans / animals: Illness indica presence of cyanotoxins				X	*Cyanobacterial biovolur for several weeks; P. ru occasionally observed t surface; MCYST-analys only sporadically but sh levels per unit biomass concentrations of sever*	*Microcystin occurrence is cert illnesses have been reported, basic information from regular monitoring over a period of 7 y data show regular seasonal p thus allow a good estimate an prediction of cyanobacterial p development.*
Cyanotoxins observed at > 1 µg microcystins			X			
Cyanobacteria – visual inspectio blooms, discolouration; conspicu			X			
Cyanobacteria – quantification: 1 mm³/L Biovolume detected by > 1 µg/l Chlorophyll-a, largely ca cyanobacteria			X			

B) Are cyanobacteria to be expected from water-body characteristics?

For this assessment, supporting expertise is recommended in limnology particularly with a focus on phytoplankton ecology

Legend: N = no, does not apply; P = applies partially; Y = yes, applies; ? = no information; Text in *bl italics* is the example filled in for Reservoir Y

Criteria of the		N	P	Y	?	Your assessment of t	Uncertainty of this assessm
Overall condition of water-body	Is it eutrophic; deep with thermal stratification or usually mixed; not acidi retention time > 1 month ?			X		*Slightly eutrophic, max. stably stratified, with lon times; typical habitat for rubescens*	*Fairly certain*
Current situation	Is the water temperatur If the water-body stratifi is it stably stratified now Are TP-concentrations > Is Secchi Disc transpare				X	*Recent data on tempera TP-concentrations and readings not available*	*Improved monitoring urgently*

C) Are current and future nutrient loads under sufficient control in order to meet the target level for this water-body?

For this assessment, supporting expertise is recommended in the areas of limnology or geoecology, particularly for estimating or modelling nutrient budgets in water-bodies

Legend**:** W = well under control; P = partially under control; N = not sufficiently controlled; ? = information ; Text in *blue italics* is the example filled in for Reservoir Z

Criteria	W	P	N	?	Your assessment of the sit	Uncertainty of this assessment
Assessment t inspection		X			*Site inspection confirmed ong farming with poor tillage pract the area, but also that some o the catchment no longer appe operated. About 60% of the area is forested.*	*Overall good confirmation of the data use*
Assessment t modelling loa			X		*Load modelling showed that l twofold above the target level determined for Reservoir X to bacterial proliferation. A small farmland (about 15 %) with th slopes contributes more than load.*	*Weaknesses of the load model are poor d and manure application as well as on eros heavy rainfall events. Overall however, m are well supported by the data available fr loading measurements (see PhD-Thesis b* *Overall information basis is satisfactory; in estimate currently should not be a priority.*

The outcome of having gone through the PEPCY decision support tool is the cyanobacterial element for Water Safety Plan for a given Drinking-water supply. Table 20 shows three different examples “skeleton” Water Safety Plans summarized as models; a “real” assessment would provide more detail.

Lake A (blue shading in Table 20) is an example for a setting with well-known toxic blooms of *Microcys* spp., i.e. the human exposure risk is high and this assessment is quite certain. However, to drinking-wat consumers this risk is substantially reduced by an excellent drinking-water treatment system includi ozone and filtration through granular activated carbon (GAC). Nonetheless, the managers classify t residual risk for drinking-water as “medium” rather than “low”, because they are not sufficiently confide with having only one reliable barrier (i.e. treatment) against cyanotoxins in their finished drinking-wat They have therefore begun to negotiate a fertilisation management system with the 5 farmers in t catchment in order to control phosphorus loading, but until this system is not yet fully in place, the residu risk remains classified as “medium”. The resulting recommendations for action focus on maintaining t good condition of the drinking-water treatment plant, making sure operational monitoring wou immediately indicate if treatment steps were not working properly, and on further developing the fa fertisisation management system. In the medium term this is the only way forward to reduce risks recreational use, while in the short term, the only way to control the risk of recreational exposure is implement an effective system of information and warning for site users.

Case study B (green shading in Table 20) is a lowland river notorious for its olive-greenish discoloratio from July till October. Occasional microscopical investigations have shown this to be caused *Planktothrix agardhii*, a cyanobacterial species very typical for highly eutrophic shallow and slowly-flowi or stagnant water-bodies of the region. Judging from the literature, this species almost certainly contai microcystins, but no local analyses have been conducted. The drinking-water treatment plant does n have an oxidation step or GAC, and toxin breakthrough is likely, though the uncertainty of this assessme is high as no analyses have been performed and effective flocculation/filtration may well be effective retaining these largely cell-bound toxins. Land use upstream is too intensive for control of phosphor loading to have success chances in the medium-term. However, geological information shows t underground to be sandy and very suitable for infiltration. The resulting recommendation is to switch fro direct abstraction of this surface water to wells drilled along the shoreline which would abstract ba

filtrate, i.e. surface water effectively filtered through the sandy underground. As first and simple step improve the information base, phytoplankton analyses are suggested. This will also be important as bas for informing and warning recreational users of the river.

The third example, Reservoir Z (pink shading in Table 20), is a typical prealpine mesotrophic reservo harboring metalimnetic populations of *Planktothrix rubescens.* These can be avoided through varying th offtake depth for drinking-water abstraction, but only as long as the reservour is thermally stratified. during winter mixing the levels of these cyanobacteria remain high, the only barrier available is to ad powdered activated carbon (PAC) during drinking-water treatment. However, it is unclear to which exter cyanotoxin levels are high enough to be a hazard, and only a small fraction of the catchment is farmlan The actions recommended therefore include toxin analyses to improve the understanding of the risk ar catchment management to reduce phosphorus loading in order tackle the source of the potential proble and avoid further eutrophication. Recreational use is no problem as during summer, *Planktothr rubescens* remains in the metalimnion.

For a full Water Safety Plan, these "skeletons" would be fleshed out with more detail documenting th rationale behind the assessments and the management of the control measures. Management plan documenting the operational monitoring methods for each control measure would include the operation limits within which monitoring results should remain, the corrective action to take if they don't, as well a lines of reporting (see WHO 2004 for more detail). They would also include instructions for documentatio as well as contingency plans for emergencies such as, e.g. massive cyanobacterial blooms well beyon the "normal" level for which the system is designed. Periodic validation of the plan would revisit the whc system, including an inspection of the catchment, to check if conditions have changed, as well as ne scientific literature to check if new information necessitates updating of risk assessments, e.g. ne information about the toxicity of cyanobacterial metabolites.

While the Water Safety Plan approach was primarily designed for drinking-water supplies, the system ca similarly be applied to other human exposure scenarios. The PEPCY Decision Support Tool include recreational exposure. However, fewer barriers exist to protect people from exposure: measures to contr cyanobacterial proliferation only encompass the steps of catchment and water-body management. F drinking-water supply treatment is a further barrier, but the only further barrier against recreation exposure is to keep people out of the water when cyanobacterial and/or cyanotoxin levels exce guideline values. The decision support tool therefore interlinks users with the EU Bathing Water Directiv which contains an article on cyanobacterial risks, requiring "*appropriate monitoring*" when "*the bathi water profile indicates a potential for cyanobacterial proliferation*", as well as "*adequate manageme measures … taken immediately to prevent exposure, including information to the public*" whe "*cyanobacterial proliferation occurs and a health risk has been identified or presumed*".

Case stu	Hazardous ever	Hazard	Risk of occurrence; b ment	Uncertainty of assessm	Existing control measu	Residual risk; basis for	Uncertainty of assessmer	control measures to strenghten or imp
Lake A	Microcystis blo	Cyanotoxins; particu drinking-water; recr	High; concentration previous blooms	Low; good understand reservoir from 10 year	1. Drinking-water treat ozone+GAC 2. Farm fertilisation ma	Medium; for DW: gooc MCYST removal, but f management just begi High for recreational u	Low; MCYST was alway effectively; load models f farms are well validated	1. improve management plans for ozo 2. improve control of catchment 3. implement warning system for recr
River B	Proliferation of agardhii	Cyanotoxins; particu drinking-water; recr	High; Secchi disk re greenish discolorati	High; no phytoplankto data	Poor drinking-water tre farming in catchment	High both for drinking- recreational use	High; no phytoplankton c	1. introduce bank fitration 2. introduce regular phytoplankton mo 3. implement warning system for recr
Reserv	*Plankothrix rub* metalimnion or	MCYST possible in	High; literature indic to contain high level	Medium; cell data but available	Variable offtake	Medium; winter mixing biomass is still high; o to remove MCYST	Medium; no own data on occurrence	1. improve catchment management 2. run screening programs on MCYST

Table 20: Three examples for outcomes of assessments of the risk of cyanotoxin occurrence and a drinking-water supply system's performance in controllin them (from: Chorus 2006)

Note: each example is from a different setting; these scenarios would not occur in the same water-body

Abbreviations: GAC: granular activated carbon; PAC: powdered activated carbon; MCYST: microcystins

4 Discussion

4.1 New methods developed in PEPCY

4.1.1 Genetic tools developed for rapid screening and detection of toxin-producing cyanobacteri genotypes

Studies in PEPCY to assess PCR techniques for their applicability showed that real-time-PCR is a usef method to quantify toxic *vs.* non-toxic genotypes in environmental samples. This technique is accurate applied correctly and will find broad application in the field of molecular ecology of cyanobacteri However, it needs special equipment and specific conditions have to be to be elaborated for various ge loci of any cyanopeptide. Multiplex PCR could be shown to be principally suitable for identifying genes f the biosynthesis of several cyanopeptides in one single assay.

The value of PCR and of the primers designed was demonstrated e.g. in the Finnish '70-lake-study According to the genus-specific PCR results over half of these lakes contained at least two microcysti producing genera and nearly a fourth contained all three genera studied. This is important knowledge th could not have been gained by other means (e.g. microscopy) than the molecular method developed in th study. The PCR proved very sensitive and suitable for the detection of potential MCYST-producers environmental samples. Its ability to reveal the toxic potential of the lakes can be utilized as an ea warning method for toxic blooms. This method can identify all the principal MCYST-producers in th sample, and thus can serve to evaluate the phytoplankton community composition of MCYST-producers a waterbody.

Thus far, a majority of the studies had concentrated on the detection of only one producer genus (Dittma & Börner, 2005). If more than one MCYST-producing genus exists in a lake, any one of them has th potential to become dominant or to form blooms in response to changed conditions. The PCR-techniqu developed in PECPY thus provide a means for early detection of inocula that may grow into blooms.

4.1.2 Cyanopeptide extraction and purification

In the extraction of peptides from cyanobacterial biomass, different trends were observed regarding th optimal solvent concentration required. However, in all cases it was determined that where repe extractions were employed, optimal conditions for the extraction of microcystins could be used (e. Fastner et al., 1998). However, specific methods (incorporating a preferred method of mechanic agitation, optimal solvent concentration and temperature) for individual peptides were successfu developed and employed by the particular laboratory investigating that peptide.

Large-scale extraction methods were required for provision of sufficient material to allow WP4 comprehensively assess the bioactivities and toxicities of the cyanopeptides under investigation. A small quantity of purified material was also required for the production of analytical standards. Development these methods and steps taken to improve existing methods resulted in fast, efficient, reliable protocols.

4.1.3 Cyanopeptide detection and quantification

Mass spectral screening of cyanopeptides in PEPCY has initiated an exponential phase of peptid discovery and has greatly advanced reliable, optimized and easy-to-use methods for the identification cyanopeptides by mass spectrometry. Several years of intensive mass spectral analysis of cyanobacteri strains have resulted in a large peptide inventory which, however, is far from being complete, not even f the most common freshwater cyanobacteria and most investigated genera like *Microcystis*. Nonetheles the large inventory of strain-specific peptide production established at TUB together with the PCR-data genotypes producing these peptides will be very useful as reference for future work. Within this data se the data obtained from the PCC strains are particularly valuable because they have advantage

originating from axenic strains, thus unambiguously linking the peptide to the cyanobacterial strain, a these strains continue to be readily available from the Pasteur Culture Collection.

For peptides other than microcystins and nodularin, may be identified by their unique UV absorpti spectra, cyanopeptide quantification remains dependent on the availability of purified gravimet standards.

4.2 Occurrence of cyanopeptides

The PEPCY field data base containing 270 European lake samples is unique worldwide. It clea demonstrates that mass developments of cyanobacteria composed by at least one of the gene investigated in this project are highly likely to contain bioactive peptides. Some, e.g. aeruginosins a cyanopeptolins, proved to occur more frequently and in higher concentrations than microcystins. So peptides were even found with a hundred percent frequency while others occurred less regularly.

Patterns of occurrence showed differences between genera. Most information is available for *Microcys* sp. and *Planktothrix rubescens*, which were contained most frequently in the samples of the PEPCY da base, while the sample number containing *Anabaena* sp. and *Planktothrix agardhii* was lower. Notab samples dominated by *P. rubescens* showed a much higher similarity in peptide composition wh compared with samples dominated by *Microcystis* sp. Filaments of P. *rubescens* contained a number peptides in frequencies of almost 100% while in colonies of *Microcystis* sp. the most frequent peptid were detected in at maximum 50% of all colonies tested.

Some lakes such as the alpine lakes containing P. *rubescens* further showed a rather consistent pattern peptide occurrence both within years and between the years. Consequently the same peptide-produci strains may coexist in the alpine lakes for years. Correspondingly in Lake Steinsfjorden strains produci the same peptide spectrum have been isolated over four decades (NIPH, T. Rohrlack, pers. comm.). contrast, *Microcystis* in Brno Reservoir showed seasonal shifts in chemotype composition, leading to fluctuating and unpredictable peptide net production in the reservoir.

This information on peptide occurrence is of relevance for the development of exposure scenarios, as renders the occurrence of peptides produced by *P. rubescens* more predictable from cell density, while f predicting peptides produced by other taxa, cell quotas will need to be confirmed or re-established at mo frequent intervals.

4.3 Occurrence of cyanopeptide-producing cyanobacterial genotypes

Information on the stability of genotype composition in phytoplankton in general and in cyanobacteria particular is still scarce. It has been argued that environmental factors may influence the wax and wane specific peptide genotypes and may therefore influence the peptide net production in water (Sivonen Jones 1999). The overwhelming influence of the microcystin genotype proportion on microcystin n production in water has been demonstrated (Kurmayer et al. 2003). A prerequisite for predicting pepti production from cell numbers, using the model of Orr & Jones (1998), is that the genotype composition a field population remains constant. However, if it changes seasonally and shifts occur between genotyp differing in peptide composition during certain periods of the year, levels of a given peptide per u cyanobacterial biomass would also shift. Until very recently, this mechanism could not be addressed field studies because genotypes differing in peptide production look the same and cannot be differentiat in the microscope. Thus, physiological regulation could not be distinguished from regulation through shi in genotype composition. Therefore the factors leading to a possible succession of genotype occurrence water during pre-bloom and bloom periods have not been identified.

PEPCY results particularly for Planktothrix (Fig. 8) show that although populations differ significantly in t proportion of microcystin genotypes, the average proportion remain may remain fairly constant, with lit change between pre-bloom (10-10 cells ml^{-1}) and bloom conditions (10^5 – 10^6 cells ml^{-1}). The results imp

that the increase or the decease of the populations were the strongest predictor of microcystin genotyp occurrence.

It is emphasized that these conclusions are based on average values only which do not allow f conclusions on the stability of the genotype composition in the short term, i.e. within several week However, the measurements on the proportions of microcystin genotypes between the populations of *rubescens* and *P. agardhii* in Fig. 17 are mostly separated and overlapped only occasionally, implying th the *P. agardhii* population in Wannsee (Germany) probably never consisted to one hundred percent microcystin genotypes (as observed in Mondsee, Austria) but also never contained a lowest percentage microcystin genotypes during the seasonal development (as observed in Frederiksborg Slottso, Denmarl It is concluded that the proportion of specific genotypes is stable from winter to summer and the c numbers could be used to infer the mean proportion of microcystin, aeruginosin and anabaenopept genotypes in water. These results are of relevance for our understanding of the likelihood of th occurrence of hazardous cyanopeptides under specific environmental conditions and specific waterbodie They may be used as input to the proposal of guideline values for cyanobacterial concentrations unlikely cause health problems.

The results from Finland indicating more microcystin-producers to occur in water-bodies with higher leve of total phosphorus and/or nitrogen (section 3.1.1) have interesting implications for cyanobacteri diversity. It may reflect a higher cyanobacterial species diversity in Finnland's mesotrophic water-bodies a compared to its oligotrophic ones, potentially with the number of microcystin-producing taxa and/ genotypes declining again in the very highly eutrophic water-bodies of other countries. Beside nutrie enrichment a number of other factors have been shown to influence species diversity of phytoplankton general and that of cyanobacteria in particular, i.e. predation by herbivorous zooplankton, competitio among phytoplankton for light and nutrients, physical stability of the water column and disturbance event Consequently more data on the correlation between mcy genotype occurrence and the degree eutropication are needed to generalize this implication for peptide genotype diversity.

4.4 The genetic control of cyanopeptide production

The number of new peptide structures detected in isolates and field samples indicates that apparently th peptide biosynthesis in cyanobacteria truly is combinatorial biosynthesis. The data compiled in Welker von Döhren (2006) for aeruginosin, for example, give a theoretical number of more than 500 possib congeners when the combinations of all moieties that have been found in particular positions a calculated. New congeners generally have only a few modifications compared to known peptides ar summarizing all data on particular peptide classes does not indicate obvious limits in combinatori biosynthesis. This is also supported by genetic data on the presence/absence of a modifying gene, th halogenase gene in aeruginosin and cyanopeptolin synthetases, that is not related either to the origin the strains nor to the phylogeny based on ITS-sequences. With respect to amino acid exchanges, mo previouly reported moieties in particular positions could be found in new combinations, for example cyanopeptolins, leading frequently to the identification of new congeners in various samples. Simil calculations for cyanopeptolin type peptides result in several tens of thousands of theoretical congeners: number underlining the fact that we probably are still only seeing the tip of the iceberg.

Although some permutations could be not observed in vivo, progress in structural elicudation has ofte confirmed previous theoretical considerations of possible peptide structures, and work in progres indicates that indeed the majority of theoretical congeners is likely to be produced by some strains.

Several gene clusters were found to contain very similar domains. This is an important observation wi consequences for the deduction of PCR primers to be used in the detection of specific genes. Further, suggests recombinative exchange of domains as molecular mechanism in the evolution of genes f cyanopeptides.

4.5 The physiological control of cyanopeptide production

A key result is that for none of the peptides any induction of peptide synthesis by a specific factor wa observed, and variations in the range of peptide concentrations per cell were similar to those previous reported for microcystins: For example the peptide content and the production in *Microcystis* PCC78(was higher under light limiting conditions than under light saturating conditions. Similar results we obtained by Wiedner et al (2003) who showed a maximum of total microcystin content per biovolume at 4 µmol m^{-2} s^{-1}. The findings on the effect of light intensity on the contents of the different peptide grou produced by *Anabaena* 90 are similar to the findings of Repka et al (2004) in batch cultures.

Kaebernick et al. (2000) emphasized direct effects of high to low irradiance on the transcription of th *mcyB* and *mcy*D genes encoding specific steps during microcystin biosynthesis (Tillett et al. 200(Notably, the stimulating effect of light on the transcription of microcystin synthetase genes were observe already after a few minutes of illumination arguing for a direct effect of light (Kaebernick et al. 2000). Wh the transcription level was found decreased under low light conditions the range of variation in th transcript amount in relation to the light dose did not exceed the value of three to four. In addition, n significant differences were found in microcystin content per cell between light intensities[t6].

Similarly to microcystins, changes in cellular content of the cyanopeptides studied in PEPCY were in th range of a factor of 0.4-4. Only in a few cases the magnitude of variation was higher than (anabaenopeptilide 90B and anabaenopeptin A and C in *Anabaena* 90. This suggests rather simil regulatory mechanisms for cyanopeptide production in general and that cell growth or cell biovolume ca be a reasonable parameter for the estimation of peptide net production or peptide concentration in wate Indeed in this study highly significant linear relationships have been described implying that analogous microcystin (Orr & Jones 1998), the peptide net production rates are significantly linearly correlated to th growth rate of the cyanobacterial cells (Kurmayer et al. 2003, Briand et al. 2005). It is argued that wh regulation of peptide synthesis has been observed, the influence of environmental factors on peptic concentrations found in the field is mainly indirect through the increase or decline of cells, while a dire impact of environmental effects on peptide production is of relatively minor importance.

This result contributes to cyanopeptide risk assessment by implying that the potential concentration peptides in water can be estimated cell numbers if minimum, mean and maximum cell quotas a determined at larger time intervals.

4.6 Suggestions for a potential role of cyanopeptides in aquatic systems

4.6.1 Defense against zooplankton ?

The results show that bioactivity not related to microcystin bioactivity, such as protease inhibition, can b detected in laboratory and field samples using *Daphnia* trypsin-like enzyme extracts. The data from fie samples document that peptide induced protease inhibition is a frequent phenomenon in *Planktothr* populations and in field samples dominated by *Microcystis*. This result corresponds to the freque detection of protease inhibitors in field samples. The *Daphnia* trypsin-like enzyme assay therefore prove to be a useful tool for bulk detection of potential cyanobacterial ecotoxicity in field samples.

For both laboratory and field samples, there was no correlation between trypsin inhibition and microcyst concentration. Consequently the inhibition of proteases by other cyanopeptides poses a mechanist explanation for the toxicity of field samples without detectable microcystins. This toxicity has long bee recognized (Jungmann & Benndorf 1994, Keil et al. 2002), however the rather efficient inhibition proteases in herbivorous zooplankton by cyanobacteria has been elucidated only recently (Agrawal et a 2001, Rohrlack et al. 2003, VonElert et al. 2005). From an ecological point of view, the inhibition zooplankton proteases may be an alternative strategy to deter zooplankton feeding similar to the tox effects induced by microcystins. So far attempts to show that zooplankton grazing may induce shifts in th proportion of microcystin-producing genotypes in water have failed. This might be due to the frequent an efficient effects of other cyanopeptides on zooplankton proteases, as observed in this study. If speci

cyanobacterial populations frequently tend to contain cyanopeptides potentially inhibiting Daphnia trypsi like enzymes, this may be a key mechanism for grazing protection and indeed potentially the ecologic function of these substances.

Trypsin inhibition may be one reason for the toxicity observed in assays with cyanobacterial crude extrac on mammalian cell lines (see section 3.9.3). If so, the Daphnia enzyme extract assay may provide a alternative to the use of mammalian bioassays.

4.6.2 Communication between cyanobacterial cells?

The presence of an ABC transporter of the FAE family of exporter proteins (Pearson et al. 2004) in ea analysed gene cluster suggest export and (a) extracellular function(s) of the cyanopeptides. On the oth hand, with a few exceptions only low amounts of microcystins and other peptides were usually fou outside the cells, and this observation does not favour a role as allelopathic compounds acting agai competitors. Rather, a possible role of these oligopeptides could be in cell-cell communications with cyanobacterial communities, alternatively or in addition to a function as toxins and deterrents.

The capacity to chlorinate a compound is rare in nature and such compounds are usually particula stable. If it is confirmed on environmental samples that chlorination of either aeruginosins or cyanoeptoli is indeed exclusive, chlorination of cyanobacterial oligopeptides may well represent a selective advanta and contribute to the fitness improvement of *Microcystis* cells for colonizing specific ecological niches for their response to specific changes or fluctuation of environmental cues.

4.7 Non-microcystin cyanopeptide toxicity – a hazard to humans ?

None of the tested peptides showed overt cytotoxic effects *in vitro.* This result is an important outcome the PEPCY project, as the data generated currently constitute the most comprehensive assessme available for cyanopeptide toxicity tested in systems designed to reflect effects on mammals. While testi the full range of cyanopeptide variants is hardly feasible, our results do cover at least one variant fro every peptide class. Thus, the negative results found in the bioassays applied suggest that major acu toxicity of cyanopeptides is unlikely. The limitation to these results is that toxicity may vary by seve orders of magnitude between variants within a peptide class. Indeed, although in the peptide class microcystins, one of the moieties determining functional toxicity is conserved (the ADDA-side-chain), oth moieties within the molecule might be very variable. This may explain the rather high variability observed toxicity amongst the different Microcystin congeners. Similarly, pronounced differences in toxic have been shown for cyanopeptolins (Czarnecky et al. 2006) and also for microviridins with microviridin causing far more pronounced effects than other microviridins (Rohrlack 2003). Therefore, as PEPCY wa able to analyse only a few of the numerous cyanopeptides, despite the lack of high toxicity of thes cyanopeptides in vivo and in vitro, results cannot exclude that some other peptides of the same pepti class could be toxic.

Due to their molecular weights, ranging between 600 and 3000 Da, and charge it must be assumed th these peptides need an active transporting system across the cell membrane in order to reach the cytos HepG2 are known to have lost some of the typical human liver transporting peptides or show an reduce expression (Kullak-Ublick et al. 1996; Lee et al. 2001), e.g. the OATPs, which have been shown to b primarily responsible for transporting large organic molecules into the cells (Hagenbuch and Meier 200 Similarly CaCo2 cells express some transporting peptides but not necessarily those required f cyanobacterial peptide transport. Therefore, the specifically OATP transfected HEK293 cell line wi apical-basal polarity appeared more suitable for demonstrating cyanobacterial peptide transport. A OATPs were shown to be responsible for the transport of MCYSTs (Fischer et al. 2005) and maybe als for nodularin, four different MCYST congeners and nodularin were used as positive control in the OATP in the transfected cell lines. MCYST-RR and nodularin, when judging from the observed cytotoxicity, see to be transported only by one of the two tested OATPs and to a distinctly lower degree than e.g. MCYS LR (Table 15 and 17). As the protein phosphatase inhibiting capacity of MCYST-RR and nodularin

comparable to that of MCYST -LR the observed differences in cytotoxicity in the OATP transfected HE 293 may be explained by differences in affinity for and capacity of these two OATPs.

No cytotoxicity could be observed for most of the cyanobacterial peptides tested with the exception of tl very mild cytotoxicity of Aeruginosamide and Oscillarin and the known cytotoxicants i.e. MCYSTs ar nodularin (Table 15). The expression of OATP1B1 or OATP1B3 did not enhance the toxicity of tl Anabaenopeptins tested in the OATP expressing HEK 293 cells. At present it cannot be deduced wheth the lacking toxicity is due to general lack of toxicity or a lack of cell accessibility of these peptides. Indee it cannot be excluded that other transporters are necessary for the passive or active transport of tl investigated cyanobacterial peptides. However, as none of the quite diverse cell systems employe demonstrated any sensitivity towards these cyanopeptides, the assumption can be made that tl cyanopetides tested are unlikely to be cytotoxic, even at very high concentrations.

Results of toxicity assays with cyanobacterial extracts confirmed earlier published observations of toxici that could not be explained by the extracts' content of known cyanotoxins, although in the extracts teste in PEPCY, effects were not very pronounced. However, as they could not be attributed to the extract cyanopeptide contents, this results implies that future research should target other groups of substances cyanobacteria. Steward et al. (2006) have shown the alkaloid cyanotoxin cylindrospermopsin to caus cutaneous effects and hypersensitivity reactions in mice, and this was confirmed by crude extracts of tl producer strain of *Cylindrospermopis raciborskii* while two other cyanobacterial strains did not produ these effects.

4.8 Characterising the human health impact of cyanopeptide toxicity

So far, most cyanopeptide hazard assessments are either based on occurrence of cyanobacteria as suc without regarding their specific toxin content, or only on MCYST-LR data, as this is the only microcystin f which adequate longer-term exposure data from an animal study exist (the Fawell et al. 1994 mous study). No microcystin with a lower acute LD_{50} value is known, and the use of the MCYST-LR guideli value for chronic and subchronic exposure scenarios (e.g. through drinking-water) has often implici assumed the ranking of microcystins according to their acute toxicity to apply to chronic toxicity as well or at least that other microcystins would not show a higher chronic toxicity. This hypothesis has bee tested only with one further study, i.e. the Falconer et al. 1994 pig study which orally administered *Microcystis aeruginosa* extract containing at least 7 different microcystis variants, the major one bei MCYST-YR. Interestingy, the results confirm those of the Fawell et al. mouse study very well, with tl difference between the NOAEL found in the Fawell study and the LOAEL found in the Falconer stu being only a factor of 2.5.

However, work in PEPCY on microcystins using the OATP1B1 and OATP1B3 system to assess transpo of microcystins into the cell has shown a different ranking for the impact of microcystin variants: f MCYST-LF and MCYST-LW the transporting capacity is 10- to 50- fold higher than for MCYST-LR. Thes two congeners are known to be synthesized by several *Microcystis aeruginosa* strains (e.g. PCC782 PCC7813) in high amounts (Metcalf and Codd 2000; Robillot et al. 2000) and are frequently found in fie samples.

Furthermore, the results with the OATP1B1 and OATP1B3 system correspond to those found for EC values with the protozoa *Tetrahymena pyriformis:* toxicity of MCYST-LF and MCYST-LW was 3 fo higher than that of MCYST-LR, and summarizing all tested endpoints, MCYST-LF could be shown to b the most toxic congener (Ward and Codd 1999). The results obtained in PEPCY suggest that the high toxicity of MCYST-LW and MCYST-LF to eukaryotes of lower taxonomic orders may well be the result of higher transporting rate into the cell.

Though it cannot be excluded that in vivo the expression of respective transporting peptides in vario animals or humans is different to the situation *in vitro* with transfected cell lines, the results presented he give strong evidence that MCYST-LR may not be the most toxic microcystin for animals/human particularly as the protein phosphatase inhibition capacity of the microcystins tested was very similar ar

cannot explain the differences in toxicity. If these differences in microcystin transport are confirmed *in viv* this potentially would have major implications for assessing the human health hazard from exposure water containing mixtures of microcystin variants, as well as for the choice of MCYST-LR as key variant f which a guideline value for drinking water exists.

None of the peptides or extracts tested appeared to demonstrate increased cytotoxicity in OAT transfected HEK293 cells, suggesting that OATPs may not be responsible for their transport. Converse in some cases a higher cytotoxicity for peptides and extracts was observed in the CaCo2 cells, indicati that transporters other than the OATPs may be responsible for cross-membrane uptake.

4.9 Cyanopeptide risk assessment and risk management

The implication of the PEPCY results for hazard assessment is twofold:

- For microcystins, the result that the efficacy of transport of variants across cell membranes may b independent of their acute toxicity indicates that variants other than MCYST-LR (the only one f which a toxicologically derived Guideline value exists) may be just as relevant for human healt even if their acute toxicity is lower. This strongly supports the approach of including all microcysti in hazard assessment and highlights the need for improving the toxicological knowledge base f variants other than MCYST-LR. This is important for the regulatory debate over setting standar for microcystin – i.e. whether they should be set only for MCYST-LR because the toxicity data bas is lacking for other variants, or whether they should nonetheless be valid for the sum of all variants
- Toxicity testing in PEPCY did not show pronounced effects of any of the cyanopeptides tested. Th toxicity assessment results for the cyanopeptides tested in PEPCY therefore do not indicate a rationale for addressing them specifically in cyanotoxin hazard analysis. This result does n generally exclude a toxic potential from variants not tested in this project, but results from testi crude extracts indicate other cyanobacterial compounds to be the more likely cause of th unexplained toxicity seen with crude extracts.

For cyanotoxin hazard assessment, these results imply that looking at exposure to cyanobacterial cells a a whole is more likely to ensure safety than focusing exclusively on the known toxins. Nonetheless, hig concentrations of microcystins (or any of the alkaloid toxins) would imply a higher ranking on the "severi of publich health impact" axis of Figure 17, as this renders the probability of exposure being hazardous a "certain" rather than as "possible" or "likely". The presence of high cell density itself, however, indicates a elevated likelihood of a hazardous situation.

PEPCY provided guidance for including potentially toxic cyanobacteria when developing a Water Safe Plan. This approach to risk management shifts attention both of system operators and of the authoriti responsible for surveillance from the current culture of compliance to standards towards an improving th understanding of water supply systems and setting priorities. In this approach, guideline values serve help assess the potential hazard, but hazard assessment is not limited to compliance to a number. Rathe it requires taking into account new knowledge arising from research such as that from PEPCY.

For recreational exposure to toxic cyanobacteria, the newly revised EU Bathing Water Directive is in li with the new risk-based WHO approach. Interestingly, the discussion at the EU workshop that develope the text for the Directive's specific article on toxic cyanobacteria very clearly revealed the inadequacy of a approach driven by compliance to standards for cyanotoxins: (i) the scientific basis for setting standards as of yet lacking for most cyanotoxins (and unlikely to be developed in the near future, as this wou require long-term toxicity trials with large numbers of animals); (ii) standardized analytical methods as w as certified analytical standards and reference materials are largely lacking, and (iii) cyanobacterial bloo situations shift within hours, i.e. too quickly for any prescriptive monitoring scheme to follow. It was on th background of these deficits that the expert group decided that setting cyanotoxin standards in bathi water is neither possible nor adequate, and the risk-based approach is the way forward. Site-speci knowledge and understanding of the ecology of the waterbody as well as its cyanobacterial blooms oft

allows narrowing down risk situations, so that periods of interventions (e.g. warning against use temporary site closure) can be minimized on the basis of flexible risk assessments specific to th respective setting. It is therefore the more appropriate approach.

5 Summary and conclusions

PEPCY contributed to cyanotoxin research in two ways: most importantly, the consortium's concerte research on cyanobacterial peptides produced a comprehensive understanding of cyanopeptides, i. mechanisms governing production, occurrence in nature, and impact on other biota. PEPCY furth systematically developed methods and produced materials necessary for cyanopetide research. addition to this scientific outcome, PEPCY developed and promoted a risk-based and setting-speci approach to protecting public health from cyanotoxins which is in line with the current approach of th WHO for drinking-water quality and the EU Bathing Water Directive.

The question of the biological function of cyanopeptides for the producer cells, i.e. the competiti advantage that cyanobacteria gain from producing cyanopeptides, remains a target for future researc Hypotheses proposed include signalling between cells within colonies as well as deterring zooplankt grazing. Gaining a better understanding of the biological function is likely to be of practical use, as th understanding why they are produced is likely to provide a point of access to suppressing their formation.

More specifically, PEPCY's provided answers to the following questions:

Which peptides do cyanobacteria contain?

An enormous range of cyanopeptides was found from several hundred cyanobacterial strains studied, ar more than 3000 masses of cyanobacterial metabolites were entered into the data base of MALDI-TO mass spectra. The internal data base of PEPCY now contains data of more than 250 samples from 3 water-bodies. The large number different peptide classes and the huge diversity of congeners within eac class (e.g. more than 500 congeners may exist alone of aeruginosins) became evident.

What have we learned about cyanopeptide occurrence?

Results show anabaenopeptins, microcystins and aeruginosins to be the most frequent peptides. prominent result is that anabaenopeptins B and F occurred in all samples. Specific aeruginosins occurre only with *Planktothrix* (*P. agardhii* or *P. rubescens*) and *vice versa*, of more than 1000 single *Planktothr* filaments analysed, 90% contained these two peptides as well as [Asp3]-microcystin-RR, so that thes may be considered characteristic for these two cyanobacterial species. In contrast, *Microcystis* coloni showed much more diverse peptide patterns. Detailed results from one reservoir indicate that they may b used as markers for the fate of the *Microcystis* populations during seasonal succession. Field sampl dominated by *Planktothrix rubescens* and *Microcystis* have the highest probability to contain microcystin and anabaenopeptins, and frequently contain aeruginosins and cyanopeptolins. *Anabaena* and *P. agardh* have not been sampled sufficiently to draw general conclusions on their peptide production.

The average proportion of microcystin, aeruginosin and anabaenopeptin genotypes in specific populatio of *Planktothrix* spp. has been found to be stable and the population increase/decrease has been identifie as a significant variable to predict the number of peptide synthetase genotypes in water both through pr bloom and during bloom conditions. Although populations have been shown to consist of numero peptide chemotypes (Fastner et al. 2001), some populations appear to be more variable in chemoty composition than others.

How is cyanopeptide production and occurrence regulated?

PEPCY characterised biosynthetic gene clusters of the major peptides identified in *Microcystis* PCC 780 *Anabaena* strain 90 and *Nostoc* strain 152. Cyanobacterial genes encoding nonribosomal peptides a now well known – a precondition for the elaboration of DNA- and RNA-based methods to detect ar quantify potentially toxic genoytpes in the environment. Moreover, these clusters provide insight into th

biosynthetic pathways and, together with the mutants, should be useful for elucidating the function of th cyanopeptides. Essential evidence for the proposed function of identified gene clusters was obtained fro the generation of mutants. These also support the identification of toxic peptides in cells or extrac containing several candidate substances.

A number of discrepancies between PCR data and peptide composition as determined by MALDI–TO MS and/or HPLC of some stains were found. For many peptides putative gene clusters were documente to be involved in the synthesis of specific peptides by gene knock out experiments and *in silic* biochemical analyses. Investigating a larger number of strains revealed that (i) genetic diversity within th analysed peptide synthetase gene is often high (when compared to 16S rDNA genes and variab interspacer sequences) and (ii) peptide synthetase genes may be inactive, since the correspondi peptide could not be found. These discrepancies imply that peptide synthetase genes may undergo rap alterations in structure and sequence and may, at least in the investigated populations of *P. rubescenc* ,frequently become inactivated. Recently, insertion elements of the IS4 family have been shown inactivate the pathway of microcystin synthesis, i.e. the investigated *P. rubescence* strains contain activ transposases (Christiansen et al. 2006). A number of recombination processes including either whc enzyme domains (Kurmayer et al. 2005) or shorter fragments of approximately 1000 bp (Kurmayer Gumpenberger 2006) have been reported. These results have two very different implications: cyanobacteria invest heavily in the re-organisation processes of peptide synthetases, i.e. transposase and gene exchange via homologous recombination, and (ii) PCR probes used to detect peptide synthetas genotypes in nature need to be carefully designed and validated using a larger number of strains in th laboratory.

Culture experiments revealed that the following peptides are physiologically regulated but are constitutive produced: ananabaenopeptin A, B, C, F (*Planktothrix*), aeruginosin 102A, aphapeptin?, cyanopeptolin A/ C, 970 (*Microcystis*, *Anabaena*), microviridin J (*Microcystis*, nostophycin (Nostoc), microcystin LR, RR (three genera), oscillamide Y, microginin. While those peptide classes are clearly secondary metabolite and synthesized by different synthesis pathways all these pathways are closely linked to the prima metabolism of the producing organism.

How do cyanopeptides impact on other biota, including humans?

The inhibition of proteases such as trypsin via a number of peptides produced by *Planktothrix*, *Microcysti* and *Anabaena* has been shown to occur frequently in water samples and from an ecological perspectiv may be of high relevance to understand interactions between cyanobacteria and herbivorous zooplankton

Transfected cell lines proved to be an excellent tool to learn more about the possible kinetics cyanobacterial peptides and therefore, if toxic, the availability of the single peptides within the single orga systems. Similar to drugs, cyanobacterial peptides can only be specifically cytotoxic if they are capable dynamically interact with their respective interaction partners e.g. phosphatases, tyrosinases, elongase proteases etc. within the cells. Most of the cyanobacterial peptides appear not to be able to reach th cytosol without being actively transported via (un)specific transporting systems. Therefore, knowledg concerning the ability of transporting peptides such as the organic anion transporting polypeptide family transport cyanobacterial peptides into the cytosol of various organs is very helpful for risk assessment. Th results obtained with microcystins as model peptides demonstrated the importance of transfected cell lin studies for microcystins human risk assessment.

The cytotoxicity of the cyanopeptides and extracts tested were all observed for concentrations in the hig µM range with the exception of those of the microcystins and Nodularin, which were in the nM rang Thus, the peptides and extracts tested appear either not to be transported with high efficiency, or not have a high cytotoxicity and therefore do not *a priori* appear to have high toxicological relevance for huma risk assessment. A caveat remains that although at least 13 peptides from 4 defined peptide classes (classified by Welker & von Döhren 2006) were tested, in face of the huge number of variants within eac class and the variability of their specific bioactivities these results cannot exclude more pronounced toxici to occur from cyanopeptides not isolated and tested within the PEPCY project.

Materials and methods produced in PEPCY

Genes for the most common cyanopeptides in fresh water bodies are now available: microcystin cyanopeptolins, aeruginosins. A collection of mutants with defects in the biosynthesis of comm cyanopeptides has been established, which proved valuable particularly for experiments aimed at t determination of toxicity of strains and peptides.

PEPCY supported the improvement of methods for the detection of peptides and of potentially to genotypes. MALDI-TOF-MS has been established as a rapid screening tool for cyanopeptides., particula useful in combination with the database generated in the frame of PEPCY. PCR assays are now availab to screen for producers of several common cyanopeptides in environmental samples. Real-time PC could be demonstrated to reliably quantify microcystin producing cells in field samples. LC-MS and L MS-MS methods are now available for cyanopeptide detection, and purified gravimetric standards a available as reference materials for LC-MS quantification of important cyanopeptides. The range and hi quality of reference materials produced are a unique resource, previously unavailable in the field cyanobacterial toxin research.

Methods for the monitoring of cyanopeptides in laboratory experiments and environmental samples a now in place and available to practitioners, including for the revision of the WHO Guidebook "To Cyanobacteria in Water". These provide a basis for further research on the natural function of this va array of novel bioactive cyanopeptides.

Protecting public health from exposure to toxic cyanobacteria

PEPCY contributed to consolidating the scientific evidence base for cyanotoxin risk assessment demonstrating the adequacy of approaches addressing cyanobacterial cells as a whole, with quantitati data on the occurrence of known cyanotoxins contributing to the assessment by increasing the certain with which the likelihood of a public health impact may be classified as high. PEPCY contributed to ri management by demonstrating how the Water Safety Plan approach can be applied to cyanobacteria a by providing guidance materials with which practitioners may develop setting-specific risk assessmer and the cyanobacterial component of their own Water Safety Plan.

6 Implications of the PEPCY outcomes for future research

The low indication of toxicity found for the cyanopetides tested in PEPCY with the test systems availab would imply a "de-warning" for this group of compounds for human risk assessment. However, the latt suggestion certainly requires substantiation by further and more detailed research, especially as t systems employed only provide for a first rough estimation of overt toxicity. Two hypotheses remain to substantiated or falsified: (i) PEPCY did not find overt effects because it happened to pick up the "wrong i.e. non-toxic structural variants within each peptide class, and (ii) PEPCY did not find pronounced effec because cell systems were employed that did not express the "right", i.e. effective transporter systems th would facilitate entry of bioactive peptides into the cells. The toxicological results of PEPCY imply th future research on cyanopeptide toxicity to mammals should focus on mechanisms of peptide transp into mammalian cells (toxicokinetic aspects) as well as on the physiological activity (toxicodynam aspects) of these peptides, primarily using structural activity relationship (SAR) analyses, prolonge exposure in vitro systems and biochemical determination of overt changes in cellular physiology.

The PEPCY results indicate that future research for cyanobacterial hazard assessment should focus other groups of cyanotoxins, e.g. alkaloids. Recent results on the occurrence of cylindrospermopsin sh that this is widely produced by the common species *Aphanizomenon flos-aquae* (Preussel et al. 2006) a is likely to occur in European settings with a frequency similar to that of microcystins (Fastner et a accepted; Rücker et al., submitted). Possibly, cylindrospermopsin accounts for part of the bioacti observed in earlier work which at the time could not be attributed to known cyanotoxins in the extrac tested, as no quantitative method for cylindrospermopsin analysis was available before LC-MS. Possib also, further toxic and bioactive cyanobacterial metabolites remain to be discovered.

Cyanopeptide research will remain important irrespective of cyanopeptide toxicity to humans. Results o the impact of cyanopeptides on *Daphnia,* further developed in PEPCY, now strongly indicate a ecotoxicological relevance of cyanopeptides which should be pursued further. Elucidating the biologic function of cyanopeptides remains an important challenge, as this knowledge is likely to provide a usef basis for controlling the occurrence of the toxic ones, particularly microcystins, and the materials an methods developed in PEPCY will be useful for such research. Progress in the molecular understanding cyanopeptide production useful for such future research includes the gene clusters identified in PEPC which provide insight into the biosynthetic pathways and mutants developed. More specifically, future wo should address (i) the physiological responses to peptides of invertebrates such as *Daphnia,* (ii) their us as early warning tools and/or toxicity tests, (iii) field studies on the implication of toxic cyanobacteria on th wax and wane of zooplankton populations and other aquatic biota; (iii) scaling up of lab findings to re situations by modelling.

A further scientifically exciting field for future research is the quantification of the occurrence of peptic genotypes within cyanobacterial populations towards elucidating why a population of a give morphospecies in a given reservoir consists of a mixture of peptide genotypes and what regulates the respective shares. The primers now used in conventional PCR could be optimized for use in revers transcriptase and quantitative real-time PCR applications with environmental RNA as a template. Thes methods would enable the determination and quantification of the taxa actively producing microcystins other cyanopeptides.

The production of halogenated compounds by cyanobacteria is of particular interest for agrochemical an pharmaceutical applications and the understanding of the biosynthesis of halometabolites may provid useful information for establishing biotechnological methods for the halogenation of organic compounds.

Beyond its use in PEPCY, the set of sequence data established in this project can be used in futu studies to establish DNA-based methods for the detection of potentially toxic genotypes and identificatio of cyanobacterial taxa in environmental samples.

7 Exploitation and dissemination of results

The following media were used to disseminate the results and knowledge gains of PEPCY to oth scientist, administrators and to the general public.

7.1 The Website www.pepcy.de

The PEPCY website provided current information about the aims, background and the results of tl project, the list of publications, links to other websites and the project partners (with postal and e-ma addresses). It further gives information about cyanobacteria, peptides, peptides groups, toxicity ar different methods for analyzing peptides. It also links to the PEPCY dissemination products presented this chapter, i.e. PEPCY practical guidelines and manuals, the PEPCY Decisions Support Tool, the flyer and the publications in scientific journals listed with a link to the email address of the authors. Images cyanobacterial blooms, cyanobacterial filaments and cells visualize the objects of PEPCY's work for tl general public. Fig. 25 gives a screen shot of the starting page of the website.

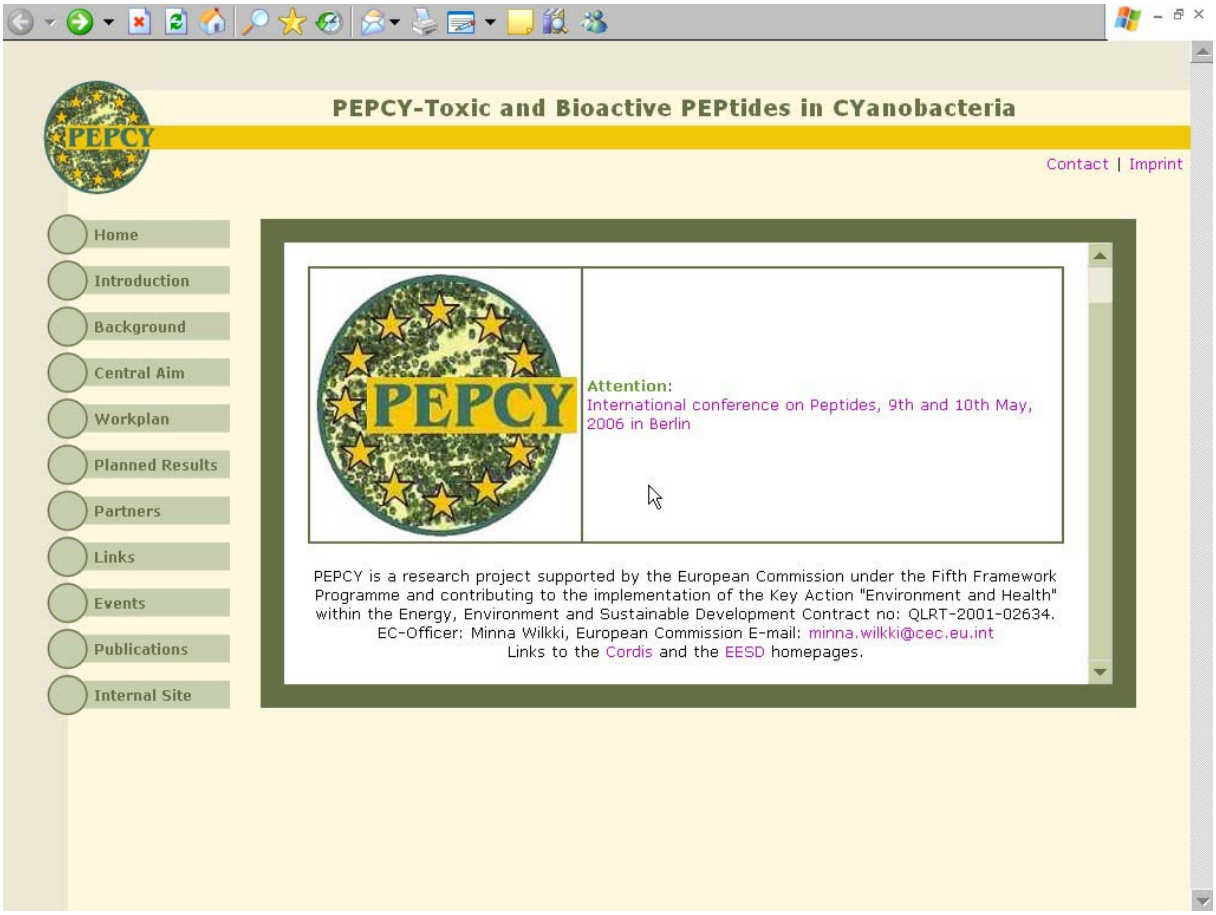


Fig. 25: Starting page of the PEPCY website

7.2 The PEPCY conference

PEPCY organized an international conference in Berlin at Umweltbundesamt in May 2006 title "Conference on Peptides in Cyanobacteria: Occurrence, regulation, hazard analysis" (Fig. 26). Tl PEPCY conference had 65 participants, 20 from the PEPCY project and 45 other experts interested in tl PEPCY results and presenting posters of their own research. Participants came from several Europea countries (Austria, Belgium, Brazil, Bulgaria, Czech Republic, Denmark, Finland, France, Germany, Ital

Netherlands, Norway, Scotland, Sweden) and also from Japan and Brasil. Most of the participants worke in the area of cyanbacterial peptides and toxins.

The conference presented 14 lectures, presented by PEPCY scientists, to disseminate PEPCY results c the topics: a) occurrence and physiological regulation, b) genetic regulation and molecular tools, c) toxicit hazard, risk assessment and implications for management and d) cyanopeptide detection and analys (see the annex to this report for the conference programme). These were followed by a poster session.

Fig: 26: Impressions from the PEPCY conference – the auditorium and discussions at the poster session

The second part of the conference was a workshop splitting participants into four groups to discuss ar develop working topics more specifically, i.e.:

- What are the key perspectives for genetic approaches and methods in cyanobacterial metaboli research for the next 5 years ?
- Which knowledge gaps on occurrence of cyanobacterial metabolites are critical?
- Which are the most pressing unresolved issues in understanding cyanobacterial toxicity?
- In the light of the current uncertainties, how can cyanotoxin risks best be assessed and manage locally? These suggestions for further research and the enumeration of research gaps are shown the “Key perspectives for research on cyanobacterial metabolites and cyanotoxin risk assessme for the next 5 years “(Annex).

The PEPCY conference was assessed as major success due to the high international interest particular from younger scientists, demonstrated in the large number of posters presented.

7.3 Scientific publications

The list of PEPCY publications in the annex shows that …[C7] scientific papers were published international journals.

7.4 Practical guidelines

One of the deliverables of PEPCY was the preparation of practical guidelines (“PPGs”) for optimizi methods and techniques for cyanopeptide analyses, in the first instance for harmonization within PEPC but also for further use by other researchers. Eight PPGs about methods for analysing cyanopeptides ar cyanopeptide-producing genotypes, written by different authors from the PEPCY consortium, are availab as pdf at the website and as paper versions in the annex (Table 6 on page 39).

7.5 Booklet on “Current approaches to cyanotoxin risk assessment, risk management and regulation in different countries”

This booklet, edited by the PEPCY coordinator, gives an overview over the variety of the approaches different countries. The booklet comprises the approaches from Australia, Belgium and Luxembour Brazil, Canada, Czech Republic, Denmark, Finland, France, Germany, Greece, Hungary, Italy, Ne Zealand; Poland, South Africa, Spain, and United Staes of America. It is the outcome of PEPCY outreach to public authorities in the partner countries of the consortium through a questionnaire on the current approaches as well as of a special session on this topic organized at the VIth Internatior Conference on Toxic Cyanobacteria in Bergen in 2004.

7.6 Public, non scientific lectures

Cyanopeptides and cyanobacteria blooms are often objects of public interest, particularly during summe because of the water quality of bathing waters. In this context scientists of the PEPCY project we frequently interviewed for their assessment of the situation in specific and in general. Some of th presentations by PEPCY scientists on the radio as well as on the television are still available in the intern (Table 22).

Table 22: Public communication via radio and television by PEPCY partners in their own countri available through the internet[n8]

7.7 PEPCY Decision Support Tool

PEPCY developed a Decision Support System (DSS) for the development of a setting-specific strategy manage the occurrence of cyanotoxins in drinking-water, from catchment to consumer. This was tested b PEPCY partners and their local or national networks and is now being advanced for international use, als in the context of the revision of WHO Guidance (Chorus and Bartram 1999). The target of the DSS is analyze, assess and manage the risk of cyanotoxin occurrence. Figure 23 (section 3.12) shows a scree shot of the starting page, and the full version is available on CD and on the pepcy website.

7.8 PEPCY Flyers

Several flyers were produced with the aim to make the knowledge about cyanopeptides and the PEPC project available for use in public authorities, the interested public and in the scientific communit Information is presented in easy-to-understand language. Some of the flyers are intended for loc adaptation as needed for use in specific settings and therefore offer images and text modules for use e. by public authorities. Flyers are available through the PEPCY website.

7.8.1 PEPCY flyer

A general flyer about the aims and purposes of PEPCY was provided at the beginning of the project. gives the PEPCY partners with the names and addresses of the project leaders. Images of cyanobacteri blooms, chemical structure of the peptides and experimental designs are shown to give an impressio about the research topic “Cyanopeptides”. The flyer was distributied by the PEPCY partners in their hon countries and at several international conferences.

7.8.2 Flyer for medical professionals

Most PEPCY partners confirmed a stark lack of information of the medical sector on cyanobacteri toxicity. Therefore, PEPCY developed a flyer to inform the medical sector about the potential impact cyanobacteria on human health.

7.8.3 Safe recreational water use flyer

This flyer informs the general public on the potential health hazards caused by toxic cyanbacteria a shows how to recognize when and where swimming may involve exposure risks. A discussion in PEPC showed a need for local adaptation of the information presented (particularly providing addresses of loc information centres for suspected intoxication, i.e. where to go if hazardous exposure is assumed to hav occurred), and for this purpose, this flyer is provided not only as pdf- but also as powerpoint file.

7.9 Key perspectives for research on cyanobacterial metabolites and cyanotoxin risk assessment

This document presents the outcome of the workshop of the PEPCY conference, summarizing th knowledge gaps and research needs perceived by the participants. It is also available on the PEPC website.

8 Policy related benefits

PEPCY contributed to the background information for the upcoming revision of the EU Drinking Wat Directive, for which EU discussions since 2003 have identified the need to address cyanobacterial toxin by demonstrating that the risk-based Water Safety Plan approach is the best way forward. It allows f sufficient flexibility to set local priorities for achieving the best progress in improving drinking-water qualit and as a continuous process involving periodic review and validation, it allows for rapid integration of ne results on cyanobacterial toxicity assessment.

During the run-time of PEPCY, the EU Bathing Water Directive was revised. The PEPCY coordinat participated in the EU workshop drawing up the first text for the article on Cyanobacteria and continued review changes in the draft through the national representative in this EU process. All suggestions f corrections and improvements of the draft could be implemented through this pathway. This input dre heavily on the risk management developments in PEPCY, and the Directive thus benefited from PEPCY.

On a global scale, the guidance developed in PEPCY for integrating cyanotoxin risk assessment and ri management into Water Safety Plans will be useful for the further development of this approach in WH and for its implementation. Specifically, this PEPCY output is currently feeding into the revision of th WHO Guidebook “Toxic CYanobacteria in Water” (Chorus and Bartram 1999) towards producing a secon edition. This approach to contextualising the cyanotoxin hazard in relation to other public heath hazards particularly relevant for developing countries, where the occurrence of pathogenic microorganisms drinking-water or exposure of children to open sewage channels often still present the major heal hazards. It is also important for industrialised countries, though in a different way: in such settings pub perception of hazards often heavily fears “chemicals”, whereas “natural substances” are perceived a beneficial or at worst harmless. For many water supplies using surface water as resource, setting-specif risk assessment ranking hazards from substances in the water is likely to result in a high priority f cyanotoxins in relation to other chemicals, merely because of their frequency of occurrence concentrations in a range with potential health implications.

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10 Annexes

1 Overview Table of Cyanopeptide Bioactivity
2 List of scientific publications in PEPCY
3 Booklet on Current approaches to cyanotoxin risk assessment, risk management and regulation different countries
4 PEPCY Practical Guidelines
5 PEPCY Flyer
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7 PEPCY Flyer for Medical Professionals
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10 PEPCY Summary

Annex 1: Known effects of cyanobacterial peptides beside microcystins and nodularins

TPS: trypsin; TRB: thrombin; PSM: plasmin; CTPS: chymotrypsin; ELA: elastase; PAPN: papain; ACE: angiotensin converting enz
LAPM: leucine aminopeptidase; CPEP: carboxypeptidase

Peptide	**Genera/Species**	**Effects**	**No effects**	**Mass (m/z) ε/log ε (λmax;r**	**References**
Microviridins					
Microviridin (not specified)		Disruption of the molting proces spp.		m/z 1684	(Kaebernick et al. 2001)
Microviridin A	M. viridis (NIES-102)	Tyrosinase inhibitor (IC_{50}: 3.3 x	TPS, ELA, TRB		(Ishitsuka et al. 1990)
Microviridin B	Microcystis aeruginosa (NIES-298)	Inhibit TPS (IC_{50}: 58µg (0.044µg/ml), CTPS (2.5µg/ml)			{(Okino et al. 1995; Na Rinehart 1996)
Microviridin C		Inhibit TPS (IC_{50}: 32µg (0.084µg/ml), CTPS (4.9µg/ml)			(Okino et al. 1995; Na Rinehart 1996)
Microviridin D	Oscillatoria agardhii (NIES 204)	Inhibit ELA (0.7µg/ml) CTPS (1.			(Murakami et al. 1997; Rada
Microviridin E	Oscillatoria agardhii (NIES 204)	Inhibit ELA (0.6µg/ml) CTPS (1.			(Murakami et al. 1997; Rada
Microviridin F	Oscillatoria agardhii (NIES 204)	Inhibit ELA (5.8µg/ml)			(Murakami et al. 1997)
Microviridin G	Nostoc minutum (NIES-26)	Inhibit ELA (0.019µg/ml), CTPS	TPS, PAPN	m/z 1806 4.67 (221) 3.73 (280)	(Murakami et al. 1997)
Microviridin H	Nostoc minutum (NIES-26)	Inhibit ELA (0.031µg/ml), CTPS	TPS, PAPN	m/z 1838 4.67 (221) 3.85 (280)	(Murakami et al. 1997)

Microviridin I	Oscillatoria agardhii	Inhibit ELA (0.34µg/ml), CTPS TPS (26.2µg/ml)		m/z 1765	(Fujii et al. 2000)
Microviridin J	Microcystis aeruginosa	Daphnia TPS like proteases (3.9 CTPS (2.8µg/ml).	Porcine ELA	m/z 1684	(Rohrlack et al. 2003)
Peptide	Genera/Species	Effects	No effects	Mass (m/z) ε/log ε (λmax;nr	References
Aeruginoguanidines					
Aeruginoguanidines 98-A	Microcystis aeruginosa (NIES-98)	Cytotox against P388 leuk 26µg/ml		m/z 978 1010 (268)	(Ishida et al. 2002)
Aeruginoguanidines 98-B	Microcystis aeruginosa (NIES-98)	Cytotox against P388 leukemia		m/z 914 1420 (269)	(Ishida et al. 2002)
Aeruginoguanidines 98-C	Microcystis aeruginosa (NIES-98)	Cytotox against P388 leukemia		m/z 997 1030 (265)	(Ishida et al. 2002)
Aeruginosins (microcin, spumigin)					
Aeruginosin 98-A	M. aeruginosa NIES-98	Inhibit TPS (IC_{50}: 0.6µg/ml), P and TRB (7µg/ml)	ELA, CTPS		(Murakami et al. 1995; Rada
Aeruginosin 98-B	M. aeruginosa NIES-98	Inhibit TPS (IC_{50} 0.6µg/ml), P TRB (10µg/ml)	ELA, CTPS		(Murakami et al. 1995; Rada
Aeruginosin 98-C		Inhibit TPS (IC_{50}: 3.9µg/ml) , PS TRB (3.3µg/ml)	ELA, CTPS		Cited in (Namikoshi and Rine
Aeruginosin 102-A	Microcystis viridis (NIES-102)	Inhibit TPS (IC_{50}: 0.2µg/ml) , PS TRB (0.04µg/ml)	ELA, CTPS	m/z 733 275 (1500)	(Matsuda et al. 1996; Na Rinehart 1996)
Aeruginosin 102-B	Microcystis viridis (NIES-102)	Inhibit TPS (IC_{50}: 1.1µg/ml) µg/ml), TRB (0.1µg/ml)	ELA, CTPS	m/z 733 275 (2300)	(Matsuda et al. 1996; Na Rinehart 1996)
Aeruginosin 103-A	*10.1.1.1.1.1 Microcystis viridis (NIE*	Inhibit TRB (IC_{50}: 9.0µg/ml), TPS (51µg/ml), PSM (68µg/ml)		m/z 681 224 (11600)	(Kodani et al. 1998)

				274 (2100)	
Aeruginosin 298-A	M. aeruginosa NIES-298-A	Inhibit TPS (IC_{50} : 1µg/ml), TRB	ELA, CT PAPN		(Murakami et al. 1994; Rios Steiner et al. 1998)
Spumigin A	*10.1.1.1.1.2 Nodularia spumigena*	Inhibit TRB (IC_{50}: 16.1µ (4.6µg/ml), PSM (4.9µg/ml)		m/z 613	(Fujii et al. 1997; Radau 200
Peptide	Genera/Species	Effects	No effects	Mass (m/z) ε/log ε (λmax;nr	References
Spumigin B1/2	Nodularia spumigena AV1	Inhibit TRB (IC_{50} :20.7µg/ml)		m/z 627	(Fujii et al. 1997; Radau 200
Spumigin C	Nodularia spumigena AV1	unknown		m/z 613	(Fujii et al. 1997)
Cryptophycins					
Cryptophycin A-G	*Nostoc* sp. GSV 224 Nostoc sp. ATCC 53789	Cytotoxic activity (0.0002-2.0 ng (IC_{50} 5-2000 pg/ml), LoVo ce 2000 pg/ml)			(Smith et al. 1994; Trimurtul Falch 1996; Moore 1996)
Family of Cyanopeptolins (e.g. Oscillapeptin, Oscillapeptilide, Planktopeptin, Micropeptin, Aeruginopeptin, Nostocyclin, Nostopeptin)					
Oscillapeptin A	Oscillatoria agardhii	CTPS (2.2µg/ml), ELA (0.3µg/m			(Itou et al. 1999)
Oscillapeptin B	Oscillatoria agardhii (NIES-204)	CTPS (2.1µg/ml), ELA (0.05µg/r		2600 (278)	(Itou et al. 1999)
Oscillapeptin C	Oscillatoria agardhii (NIES-205)	CTPS (3.0µg/ml)	ELA	278 (1100)	(Itou et al. 1999)
Oscillapeptin D	Oscillatoria agardhii (NIES-205)	TPS (IC_{50} :1.3 x 10^{-8} M); CTP ELA (30µg/ml)		m/z 1089 log ε 3.2 (279) 1800 (278)	(Sano et al. 1998; Itou et al.
Oscillapeptin E	Oscillatoria agardhii (NIES-205)	CTPS (3.0µg/ml), ELA (3.0µg/m		2400 (278)	(Itou et al. 1999)

Oscillapeptin F	Oscillatoria agardhii (NIES-596)	TPS (0.2µg/ml), PSM (0.03µg/m	CTPS, ELA	1300 (278)	(Itou et al. 1999)
Peptide	Genera/Species	Effects	No effects	Mass (m/z) ε/log ε (λmax;nı	References
Cyanopeptolins (e.g. Oscillapeptin, Oscillapeptilide, Planktopeptin, Micropeptin, Aeruginopeptin, Nostocyclin, Nostopeptin)					
Oscillapeptin G	*Oscillatoria agardhii* (NIE 1459/22=NIVA CYA 18 + NIVA CYA	Inhibit tyrosinase (tyrosine to m 1 x 10^{-4} M), ELA (1.12µg/ml), CTPS (11.4µg		m/z 1112 log ε 3.5 (279)	(Sano and Kaya 1996; Fujii e
Oscillapeptin J	Planktothrix rubescens	LC_{50} (*T. platyurus*): 15.6µM		m/z 1093 λmax 200, 278	(Blom et al. 2003)
Oscillapeptin	Oscillatoria agardhii (NIES-204)	Inhibit ELA (IC_{50}: 0.3µg/ml) (2.2µg/ml)	PAPN, TPS	m/z 1166.5 13900 (223) 2700 (278)	(Shin et al. 1995)
Micropeptin A	M. aeruginosa	Inhibit TPS (IC_{50}: 0.071µg/m (0.026µg/ml)	TRB, ELA,		(Okino et al. 1993; Nai Rinehart 1996)
Micropeptin B	M. aeruginosa	Inhibit TPS (IC_{50}: 0.25µg/ml (0.035µg/ml)	TRB, ELA,		(Okino et al. 1993; Nai Rinehart 1996)
Micropeptin 90	M. aeruginosa (NIES-90)	Inhibit TPS (IC_{50}: 2µg/ml) (0.1µg/ml)	ELA, CTP 10µg/ml	m/z 960 2673 (279)	(Ishida et al. 1995; Radau 20
Micropeptin 103	M. aeruginosa (NIES-103)	Inhibit CTPS (IC_{50}: 1.0µg/m (9.0µg/ml)	TPS, ELA, PAPN		(Murakami et al. 1997)
Micropeptin 88-A	M. aeruginosa (NIES-88)	Inhibit CTPS (IC_{50}: 0.4µg/m (3.5µg/ml)	TPS, TRB, PSM, PAPN at 100µg/ml	m/z 884 1170 (278)	(Ishida et al. 1998)
Micropeptin 88-B	M. aeruginosa (NIES-88)	unknown	TPS, TRB, PSM, CTPS, ELA, PSM,	m/z 1079 1100 (278)	(Ishida et al. 1998)

			PAPN at 100µg/ml		
Micropeptin 88-C	M. aeruginosa (NIES-88)	Inhibit CTPS (IC_{50}: 5µg/ml)	TPS, TRB, PSM, PAPN, ELA at 100µg/ml	m/z 1095 3000 (278)	(Ishida et al. 1998)
Micropeptin 88-D	M. aeruginosa (NIES-88)	Inhibit CTPS (IC_{50}: 10µg/ml)	TPS, TRB, PSM, PAPN, ELA at 100µg/ml	m/z 1117 1600 (278)	(Ishida et al. 1998)
Peptide	Genera/Species	Effects	No effects	Mass (m/z) ε/log ε (λmax;nı	References
Cyanopeptolins (e.g. Oscillapeptin, Oscillapeptilide, Planktopeptin, Micropeptin, Aeruginopeptin, Nostocyclin, Nostopeptin)					
Micropeptin 88-E	M. aeruginosa (NIES-88)	Inhibit CTPS (IC_{50}: 5.2µg/ml)	TPS, TRB, PSM, PAPN, ELA at 100µg/ml	m/z 1063 1830 (278)	(Ishida et al. 1998)
Micropeptin 88-F	M. aeruginosa (NIES-88)	Inhibit CTPS (IC_{50}: 3.4µg/ml)	TPS, TRB, PSM, PAPN, ELA at 100µg/ml	m/z 1109 2700 (278)	(Ishida et al. 1998)
Micropeptin T-20	M. aeruginosa	Inhibit CTPS (IC_{50}: 2 x 10^{-9} M, T 10^{-3} M)		m/z 1011 2800 (278)	(Okano et al. 1999)
Micropeptin T1	cyanobloom	Inhibit CTPS (IC_{50}: 2.0µg/ml)	TPS, PSM, ACE, CPEP at 100µg/ml	m/z 1011 2800 (278)	(Kodani et al. 1999)
Micropeptin T2	cyanobloom	Inhibit TPS (IC_{50}: 0.1µg/ml), PSI	ACE, CPEP,	m/z 1011 2800 (278)	(Kodani et al. 1999)

			CTPS, LLAPM at 100µg/ml		
Micropeptin 478-A	M. aeruginosa (NIES-478)	Inhibit PSM (0.1µg/ml)	TPS, ELA, C TRB	m/z 976 950 (281)	(Ishida et al. 1997)
Micropeptin 478-B	M. aeruginosa (NIES-478)	Inhibit PSM (0.4µg/ml)	TPS, ELA, C TRB	m/z 1055 1140 (282)	(Ishida et al. 1997)
Nostopeptin A	Nostoc minimum (NIES-26)	Inhibit ELA (1.3µg/ml), CTPS (1	PAPN, TPS,	$[M - OH]^+$ 937 1200 (278)	(Namikoshi and Rinehart 19 al. 1997)
Nostopeptin B	Nostoc minimum (NIES-26)	Inhibit ELA (11µg/ml), CTPS (1.(	PAPN, TPS,	m/z 927 1300 (278)	(Okino et al. 1997)
Nostocyclin	*Nostoc* sp. (DUN901)	unknown	No effect to brine shrimp	m/z 1099 2500 (278)	(Kaya et al. 1996)
Oscillapeptilide 97-A	O. agardhii	Inhibit ELA (0.73µg/ml), CTPS (		m/z 1046	(Fujii et al. 2000)
Oscillapeptilide 97-B	O. agardhii	Inhibit ELA (0.41µg/ml), CTPS (		m/z 1032	(Fujii et al. 2000)
Peptide	Genera/Species	Effects	No effects	Mass (m/z) ε/log ε (λmax;nı	References
Cyanopeptolin S	Microcystis sp.	Inhibit TPS (IC_{50} <0.2µg/ml) , P and TRB (<5µg/ml)		m/z 925 λmax 218	(Jakobi et al. 1995)
Cyanopeptolin SS		Inhibit TPS (IC_{50} <0.2µg/ml), P and TRB (<5µg/ml)			(Jakobi et al. 1996)
Aeruginopeptin 917S-A	Microcystis sp.	Weak inhibition of CTPS	No toxicity 1 mg/kg	m/z 1072 4586 (278) 22869 (225)	(Harada et al. 2001)

Aeruginopeptin 917S-B	Microcystis sp.	Weak inhibition of CTPS	No toxicity 1 mg/kg	m/z 1076 3997 (277) 21904 (224)	(Harada et al. 2001)
Aeruginopeptin 917S-C	Microcystis sp.	Weak inhibition of CTPS	No toxicity 1 mg/kg	m/z 1022 2516 (276) 16431 (224)	(Harada et al. 2001)
Anabaenopeptins (Oscillamide, Nodulapeptin, Ferintoic acid)					
Anabaenopeptin	Oscillatoria agardhii (NIES-595)	CPEP A inhibitor			(Itou et al. 1999)
Anabaenopeptin A	Anabaena flos-aquae (NRC 535-17	produce relaxation concentration-dependent	Serine prote	m/z 844 15000 (225) 2417 (276)	(Harada et al. 1995)
Anabaenopeptin B	Anabaena flos-aquae (NRC 535-17 agardhii (NIES 204)	produce relaxation (NO re endothelial cells) concentration-dependent	Serine prote	m/z 837 8833 (225)) 1583 (277) log 3.22 (279)	(Harada et al. 1995; Mur 1997; Fujii et al. 2000)
Anabaenopeptin F	Planktothrix rubescens (NIES-610)	Inhibit PP1 and 2A IC_{50}: 100µg/ml	Tyrosine P specific PP	m/z 851	(Fujii et al. 2000; Sano et al.
Peptide	Genera/Species	Effects	No effects	Mass (m/z) ε/log ε (λmax;nı	References
Anabaenopeptin T	cyanobloom	Inhibit CPEP 2.0µg/ml	PSM, TP LAPM, ACE	m/z 866.5 3800 (278)	(Kodani et al. 1999)
Oscillamide B	Planktothrix agardhii (CCAP 1459/1	Inhibit PP1 and 2A IC_{50}: 100µg/ml	Tyrosine P specific PP	m/z 869.4	(Sano et al. 2001)
Oscillamide C	Planktothrix rubescens (CCAP 1459/14)	Inhibit PP1 and 2A IC_{50}: 1µg/ml	Tyrosine P specific PP	m/z 957.5	(Sano et al. 2001)

Oscillamide Y	Oscillatoria agardhii (NIES 610)	Inhibit CTPS (1.0 x 10^{-5} M)	Tyrosine P specific PP	m/z 858	(Sano and Kaya 1995; Fuji Radau 2000; Sano et al. 200
Microcystilide A	Microcystis aeruginosa NO-15-1840	i.p. injection to mice → spasms or convulsions (0.5-100µg), weakly cytotoxic, active in cell differentiation assay (0.5mg/mL)		m/z 1055 log ε 4.28 (22 (278) log ε 3.44	(Tsukamoto et al. 1993)
Microginins (Cyanostatin, Oscillaginin, Nostoginin)					
Microginin	M. aeruginosa (NIES-100)	inhibit ACE IC_{50}: 7.0µg/ml, LAPM	PAPN, TPS at 100µg/m	m/z 714.4 15400 (224) 2200 (276)	(Matsuda et al. 1996; Ma 1996)
Microginin FR1	Microcystis	inhibit ACE (IC_{50}: 6 x 10^{-5} M)		m/z 728 12238 (224) 2035 (276)	(Neumann et al. 1997)
Microginin T1	cyanobloom	Inhibit LAPM (2.0µg/ml), ACE (5	TPS, PSM at 100µg/m	m/z 732 2500 (278)	(Matsuda et al. 1996; Kodani
Microginin T2	cyanobloom	Inhibit LAPM (2.0µg/ml), ACE (7	TPS, PS CPEP at 10	m/z 698 3500 (278)	(Matsuda et al. 1996; Kodani
Peptide	Genera/Species	Effects	No effects	Mass (m/z) ε/log ε (λmax;nı	References
Microginin 51A	M. aeruginosa (NIES-299)	Inhibit LAPM (6.5µg/ml)	ACE	m/z 917 4100 (278)	(Matsuda et al. 1996)
Microginin 51B	M. aeruginosa (NIES-299)	Inhibit LAPM (2µg/ml)	ACE	m/z 931 4000 (278)	(Matsuda et al. 1996)
Microginin 91A	M. aeruginosa (NIES-299)	Inhibit LAPM (6.5µg/ml)	ACE	m/z 575	(Matsuda et al. 1996)
Microginin 91 B	M. aeruginosa (NIES-299)	Inhibit LAPM (2µg/ml)	ACE	m/z 609	(Matsuda et al. 1996)}

Microginin 91C	M. aeruginosa (NIES-299)	Inhibit LAPM (6.5µg/ml)	ACE	m/z 704 1000 (276)	(Matsuda et al. 1996)
Microginin 91D	M. aeruginosa (NIES-299)	Inhibit LAPM (2µg/ml)	ACE	m/z 738 1400 (276)	(Matsuda et al. 1996)
Microginin 91E	M. aeruginosa (NIES-299)	Inhibit LAPM (2µg/ml)	ACE	m/z 772 3700 (276)	(Matsuda et al. 1996)
Microginin 99-A	M. aeruginosa (NIES-99)	unknown	LAPM, ACE	773 2200 (278)	(Matsuda et al. 1996; Ishida
Microginin 99-B	M. aeruginosa (NIES-99)	unknown		m/z 806 2200 (278)	(Matsuda et al. 1996; Ishida
Microginin 299-A	M. aeruginosa (NIES-299)	Inhibit LAPM (4.6µg/ml)	ACE, PAPN PSM, CTF 100µg/ml	m/z 887 2950 (279)	(Ishida et al. 1997)
Microginin 299-B	M. aeruginosa (NIES-299)	Inhibit LAPM (6.5µg/ml)		m/z 921 2090 (278)	(Ishida et al. 1997)
Microginin 299-C	M. aeruginosa (NIES-299)	Inhibit LAPM (2µg/ml)	ACE	852 3100 (279)	(Ishida et al. 1998)
Microginin 299-D	M. aeruginosa (NIES-299)	Inhibit LAPM (6.4µg/ml)	ACE	m/z 757 3400 (271)	(Ishida et al. 1998)
Microginin 478	M. aeruginosa (NIES-478)	Inhibit LAPM (100µg/ml), ACE		m/z 769 3100 (279)	(Matsuda et al. 1996)
Peptide	Genera/Species	Effects	No effects	Mass (m/z) ε/log ε (λmax;nr	References
Others					
Kasumigamide	M. aeruginosa (NIES-78)	Antialgal activity against gre *neglecta* (minimum effective dos		m/z 787 3840 (282) 3340 (291)	(Ishida and Murakami 2000)

Kawaguchipeptin B	Microcystis aeruginosa (NIES-88)	Inhibit Staphylococcus aureus (1		m/z 1286 7390 (282)	(Ishida et al. 1997)
Nostophycin	*Nostoc* sp. 152	Weakly cytotoxic, growth inhib lymphocytic mouse leukaemia (10µg/mL)	no activi various b fungi (20µg/	m/z 889 λ max 210 nm	(Fujii et al. 1999)
Oscillatorin	Oscillatoria agardhii	CTPS inhibitor (IC_{50}: 1.3 x 10^{-5} M		m/z 1240 log ε 3.4 (283)	(Sano and Kaya 1996; Rada
Oscillacyclin	Oscillatoria agardhii			m/z 995	(Fujii et al. 2000)
Prenylagaramides A	Oscillatoria agardhii (NIES 205)	No inhibitory activity against proteases, n against P-388 leukemia cells, no antimicr against several gram-positive and gram nega		m/z 1081 2800 (278)	(Murakami et al. 1999)
Prenylagaramides B	Oscillatoria agardhii (NIES 205)			m/z 929 2400 (279)	(Murakami et al. 1999)
Laxaphycin	Anabaena laxa FK 1-2	Antifungal			(Moore 1996)
Circinamide	Anabaena circinalis (NIES-41)	PAPN 0.4µg/ml,	TPS, TRB, ELA at 100µ	m/z 387 530 (250)	(Shin et al. 1997)
Radiosumin	Plectonema radiosum (NIES-515)	TPS (0.14µg/ml), PSM (6.2µg/ml), TRB (88µg/ml)	PAPN, CT 200µg/ml	m/z 433 15000 (239)	(Matsuda et al. 1996)
Radiosumin B	Microcystis aeruginosa		No antifungal	m/z 445 15000 (239)	(Coleman and Wright 2001)