Toxic and Bioactive Peptides in Cyanobacteria

PEPCY

SUMMARY of the Final Report

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Objectives

Cyanobacterial toxins ("cyanotoxins") are amongst the most ubiquitously found hazardous substances in surface waters used by humans. Though these substances are natural toxins, eutrophication (*i. e.* excessive loading with fertilising nutrients) has caused massive cyanobacterial proliferation throughout Europe. Thus, cyanotoxins now occur at unnatural frequencies and concentrations. While some cyanotoxins have been known for 2-3 decades and are rather well-studied, a much wider range of structurally-related substances is found in cyanobacteria, but little is known about their toxicity and occurrence. Many of these are peptides.

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

Results

PEPCY contributed to cyanotoxin research in two ways: most importantly, the consortium's concerted research on cyanobacterial peptides produced a comprehensive understanding of cyanopeptides, *i. e.* mechanisms governing production, occurrence in nature, and impact on other biota. PEPCY further systematically developed methods and produced materials necessary for cyanopetide research. In addition to this scientific outcome, PEPCY developed and promoted a risk-based and setting-specific approach to protecting public health from cyanotoxins which is in line with the current approach of the 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 competitive advantage that cyanobacteria gain from producing cyanopeptides, remains a target for future research. Hypotheses proposed include signalling between cells within colonies as well as deterring zooplankton grazing. Gaining a better understanding of the biological function is likely to be of practical use, as this 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, and more than 3000 masses of cyanobacterial metabolites were entered into the data base of MALDI-TOF mass spectra. The internal data base of PEPCY now contains data of more than 250 samples from 37 water-bodies. The large number different peptide classes and the huge diversity of congeners within each 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. A prominent result is that anabaenopeptins B and F occurred in all samples. Specific aeruginosins occurred only with *Planktothrix* (*P. agardhii* or *P. rubescens*) and *vice versa*, of more than 1000 single *Planktothrix* filaments analysed, 90% contained these two peptides as well as [Asp3]-microcystin-RR, so that these may be considered characteristic for these two cyanobacterial species. In contrast, *Microcystis* colonies showed much more diverse peptide patterns. Detailed results from one reservoir indicate that they may be used as markers for the fate of the *Microcystis* populations during seasonal succession. Field samples dominated by *P. rubescens* and *Microcystis* have the highest probability to contain microcystins and anabaenopeptins, and frequently contain aeruginosins and cyanopeptolins. *Anabaena* and *P. agardhii* have not been sampled sufficiently to draw general conclusions on their peptide production.

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

How is cyanopeptide production and occurrence regulated?

PEPCY characterised biosynthetic gene clusters of the major peptides identified in *Microcystis* PCC 7806, *Anabaena* strain 90 and *Nostoc* strain 152. Cyanobacterial genes encoding nonribosomal peptides are now well known – a precondition for the elaboration of DNA- and RNA-based methods to detect and quantify potentially toxic genoytpes in the environment. Moreover, these clusters provide insight into the biosynthetic pathways and, together with the mutants, should be useful for elucidating the function of the cyanopeptides. Essential evidence for the proposed function of identified gene clusters was obtained from the generation of mutants. These also support the identification of toxic peptides in cells or extracts containing several candidate substances.

A number of discrepancies between PCR data and peptide composition as determined by MALDI–TOF-MS and/or HPLC of some stains were found. For many peptides putative gene clusters were documented to be involved in the synthesis of specific peptides by gene knock out experiments and *in silico* biochemical analyses. Investigating a larger number of strains revealed that (i) genetic diversity within the analysed peptide synthetase gene is often high (when compared to 16S rDNA genes and variable interspacer sequences) and (ii) peptide synthetase genes may be inactive, since the corresponding peptide could not be found. These discrepancies imply that peptide synthetase genes may undergo rapid alterations in structure and sequence and may, at least in the investigated populations of *P. rubescens*, frequently become inactivated. Recently, insertion elements of the IS4 family have been shown to inactivate the pathway of microcystin synthesis, i.e. the investigated *P. rubescens* strains contain active transposases (Christiansen et al., 2006). A number of recombination processes including either whole 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: (i) cyanobacteria invest heavily in the re-organisation processes of peptide synthetases, *i. e.* transposases and gene exchange via homologous recombination, and (ii) PCR probes used to detect peptide synthetase genotypes in nature need to be carefully designed and validated using a larger number of strains in the laboratory.

Culture experiments revealed that the following peptides are physiologically regulated but are constitutively produced: ananabaenopeptin A, B, C, F (*Planktothrix*), aeruginosin 102A, aphapeptin?, cyanopeptolins A/D, C and 970 (*Microcystis*, *Anabaena*), microviridin J (*Microcystis*), nostophycin (*Nostoc*), microcystin LR, RR (all three genera), oscillamide Y, microginin. While those peptide classes are clearly secondary metabolites and synthesized by different synthesis pathways, all these pathways are closely linked to the primary 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*, *Microcystis* and *Anabaena* has been shown to occur frequently in water samples and from an ecological perspective 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 of cyanobacterial peptides and therefore, if toxic, the availability of the single peptides within the single organ systems. Similar to drugs, cyanobacterial peptides can only be specifically cytotoxic if they are capable to dynamically interact with their respective partners *e. g.* phosphatases, tyrosinases, elongases, proteases etc. within the cells. Most of the cyanobacterial peptides appear not to be able to reach the cytosol without being actively transported *via* (un)specific transporting systems. Therefore, knowledge concerning the ability of transporting peptides, such as the organic anion transporting polypeptide family to transport cyanobacterial peptides into the cytosol of various organs, is very helpful for risk assessment. The results obtained with microcystins as model peptides demonstrated the importance of transfected cell line studies for microcystins human risk assessment.

The cytotoxicity of the cyanopeptides and extracts tested were all observed for concentrations in the high µM range with the exception of those of the microcystins and nodularin, which were in the nM range. Thus, the peptides and extracts tested appear either not to be transported with high efficiency, or not to have a high cytotoxicity and therefore do not *a priori* appear to have high toxicological relevance for human risk assessment. A caveat remains that although at least 13 peptides from 4 defined peptide classes (as classified by Welker & von Döhren, 2006) were tested, in face of the huge number of variants within each class and the variability of their specific bioactivities these results cannot exclude more pronounced toxicity 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: microcystins, cyanopeptolins and aeruginosins. A collection of mutants with defects in the biosynthesis of common cyanopeptides has been established, which proved valuable particularly for experiments aimed at the determination of toxicity of strains and peptides.

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

Methods for the monitoring of cyanopeptides in laboratory experiments and environmental samples are now in place and available to practitioners, including for the revision of the WHO Guidebook "Toxic Cyanobacteria in Water". These provide a basis for further research on the natural function of this vast 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 by demonstrating the adequacy of approaches addressing cyanobacterial cells as a whole, with quantitative data on the occurrence of known cyanotoxins contributing to the assessment by increasing the certainty with which the likelihood of a public health impact may be classified as high. PEPCY contributed to risk management by demonstrating how the Water Safety Plan approach can be applied to cyanobacteria and by providing guidance materials with which practitioners may develop setting-specific risk assessments and the cyanobacterial component of their own Water Safety Plan.

Implications of the PEPCY outcomes for future research

The low indication of toxicity found for the cyanopetides tested in PEPCY with the test systems available would imply a "de-warning" for this group of compounds for human risk assessment. However, the latter suggestion certainly requires substantiation by further and more detailed research, especially as the systems employed only provide for a first rough estimation of overt toxicity. Two hypotheses remain to be 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 effects because cell systems were employed that did not express the "right", *i. e.* effective transporter systems that would facilitate entry of bioactive peptides into the cells. The toxicological results of PEPCY imply that future research on cyanopeptide toxicity to mammals should focus on mechanisms of peptide transport into mammalian cells (toxicokinetic aspects) as well as on the physiological activity (toxicodynamic aspects) of these peptides, primarily using structural activity relationship (SAR) analyses, prolonged 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 on other groups of cyanotoxins, *e. g*. alkaloids. Recent results on the occurrence of cylindrospermopsin show that this is widely produced by the common species *Aphanizomenon flos-aquae* (Preussel et al., 2006) and is likely to occur in European settings with a frequency similar to that of microcystins (Fastner et al., accepted; Rücker et al., submitted). Possibly, cylindrospermopsin accounts for part of the bioactiviy observed in earlier work which at the time could not be attributed to known cyanotoxins in the extracts tested, as no quantitative method for cylindrospermopsin analysis was available before LC-MS. Possibly also, further toxic and bioactive cyanobacterial metabolites remain to be discovered.

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

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

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

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