Chapter 21: Cyanotoxins: sampling, sample processing and toxin uptake

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Introduction

There are several cyanobacterial (blue–green algal) toxin groups which have been implicated in human and animal illnesses and mortalities. Sampling and sample processing of cyanotoxins will be discussed as well as toxin uptake in different organisms.

Cyanotoxins

The paper will concentrate on the following commonly occurring cyanotoxins: hepatotoxic microcystins/nodularins (together over 80 analogues), cytotoxic cylindrospermopsin and neurotoxic anatoxin–a (Fig. 1). In addition, there are several other cyanotoxins that deserve attention in local or national monitoring programmes: anatoxin–a(S), saxitoxin family, dermatotoxic alkaloids, lipopolysaccharides, beta–*N*–methylamino–L–alanine (BMAA) etc.

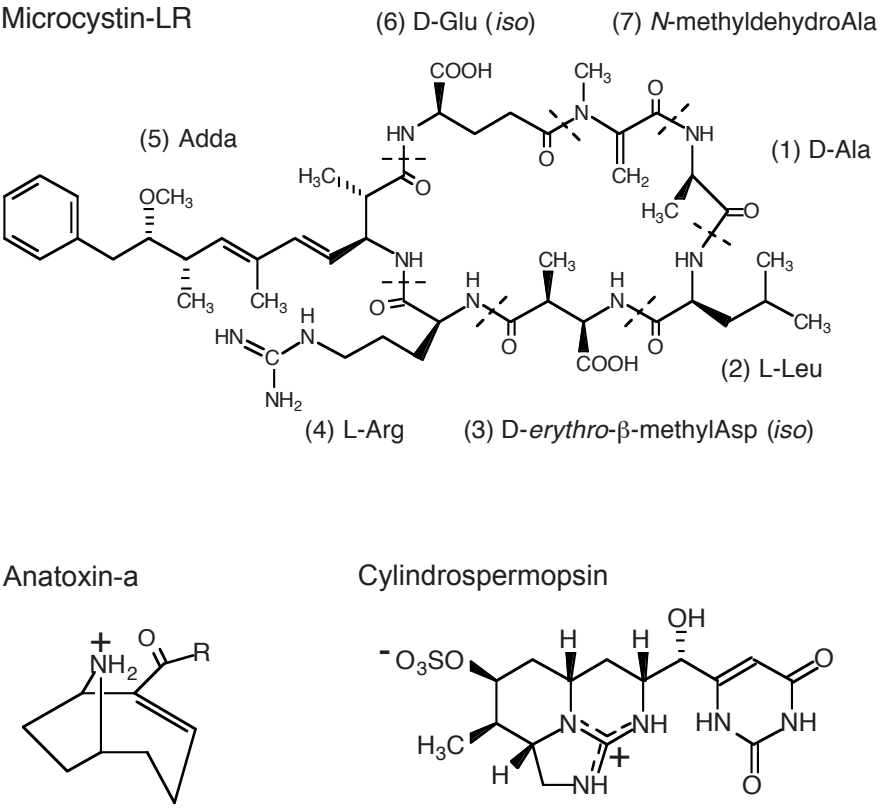

Fig. 1. Chemical structure of microcystin–LR, anatoxin–a (R=CH_3) and cylindrospermopsin.

The commercial availability of the main cyanotoxins, microcystins, anatoxin–a and cylindrospermopsin is limited. The available toxins do not always meet critical norms of purity or guaranteed quantity (Fig. 2). It was recently reported that when three commercial standards of microcystin–RR were tested only one of them actually contained microcystin–RR. The second standard was a mixture of microcystin–RR, and its variant [Dha^7]microcystin–RR, and the third one contained [Dha^7]microcystin–RR only (Kubwabo et al. 2004). When certified reference materials are unavailable, method development related to sample processing and analysis is hampered, and, from a critical viewpoint, the analytical results may not be regarded as truly quantitative.

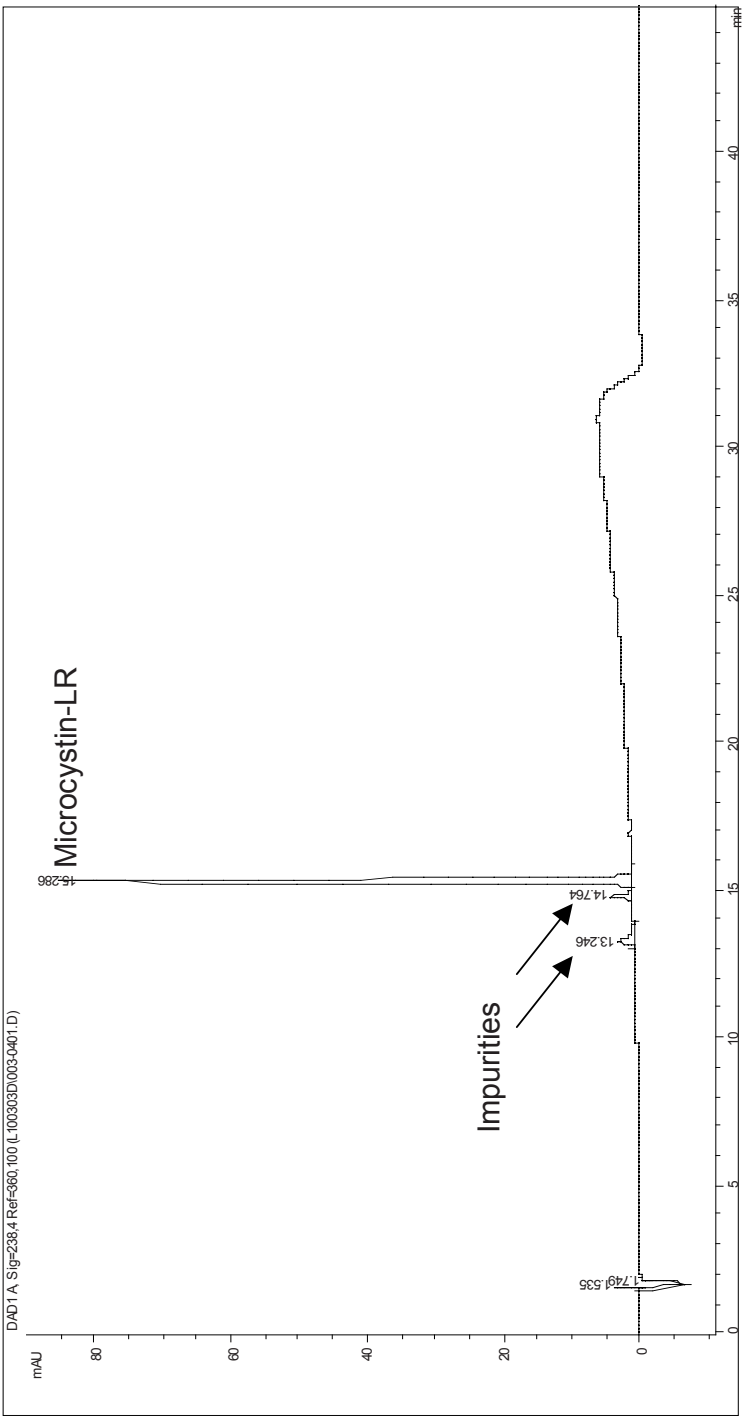

Fig. 2. Trace of commercial microcystin–LR (92%) with impurities (8%, having microcystin–like UV spectra); separation on amide–C16 phase, aqueous trifluoroacetic acid–acetonitrile gradient

Matrices; where, what, when and how to sample

Cyanotoxins are known to occur in a number of matrices and sample types: a) Water – source/recreational waters (fresh, estuarine, brackish & marine) and treated water. Sediments can contain at least stable peptide toxins, microcystins and nodularins. b) Biological materials: phytoplankton including food supplements, zooplankton, shellfish, fish, terrestrial animals including humans, sea birds, aquatic plants, agri– and horticultural products etc. The wide distribution of microcystins and nodularin in food webs is well documented. The main sources of exposure from the human health point of view are drinking water, recreational waters, shellfish and fish, and for some consumers, food supplements. The importance of crop plants as a source of exposure is unclear.

Comprehensive monitoring of lakes and reservoirs (which may be used for both water abstraction and recreational activities) requires extensive resources as the sampling should have coverage in temporal, horizontal and depth dimensions (Fig. 3). In most cases only a fraction of the waterbodies can be monitored in a satisfactory manner (i.e., Finland with its close to 200,000 lakes). In studies performed at the University of Helsinki, Finland, in the 1980s, about 50% of the cyanobacterial blooms tested contained toxins – the majority of them hepatotoxins (microcystins) (Sivonen et al. 1990). Later data from other countries corroborate these findings, and in some countries most of the studied cyanobacterial blooms have been toxic. In our studies on Åland Islands, SW Finland (Spoof et al. 2003), 113 samples taken from 93 different locations in 2001 were analysed for microcystins and nodularin–R. The purpose was to monitor the prevalence of microcystins in a set of non–selected lakes, not only in those where cyanobacterial blooms were observed. Both eutrophic and oligotrophic freshwater lakes and also some brackish waters were included. Intracellular toxins exceeding 0.2 μg L^{-1} were confirmed by three analytical techniques in samples from 14 locations (15% of the waterbodies). The highest recorded microcystin concentration in the Åland Islands lakes in 2001 was 42.0 μg L^{-1}.

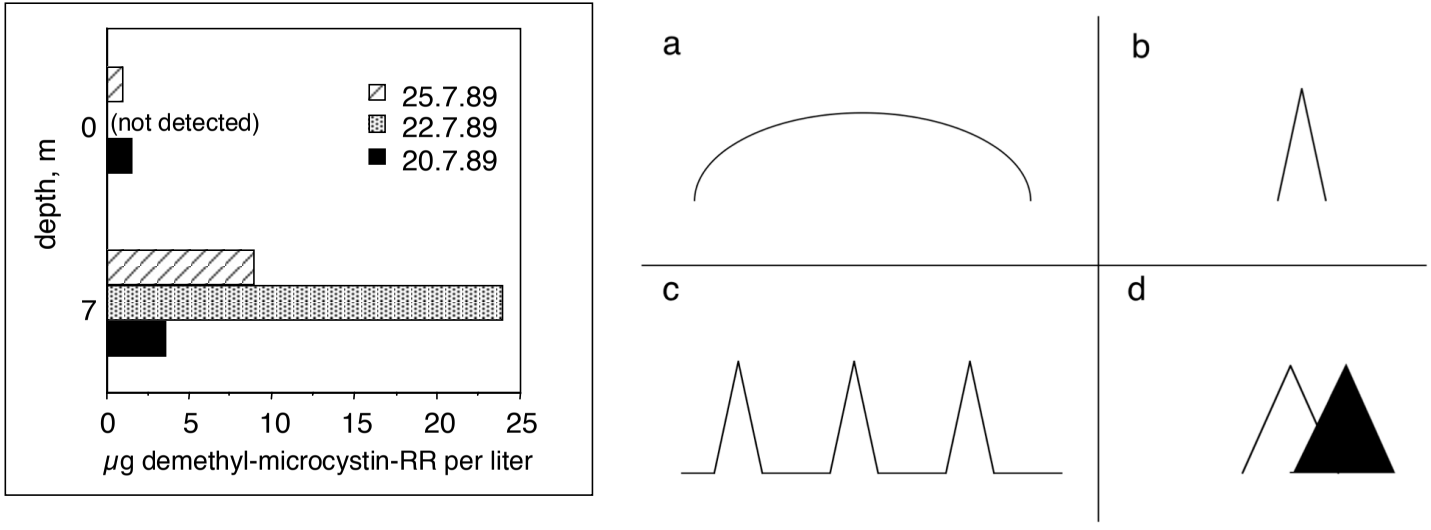

Fig. 3. Left panel: Vertical movement of a *Planktothrix* population in a lake, measured as microcystin at different depths. Right panel: Cyanobacterial blooms can be (a) persistent, (b) intermittent and/or (c) recurrent. Situation (d) represents two toxin profiles at two locations of a waterbody or two populations producing different toxins.

Chlorophyll–a, a measure of phytoplankton in routine water monitoring, can be useful as a first estimation of maximum intracellular microcystin concentration (Fastner et al. 1999, Lindholm and Meriluoto 1991). When microcystin concentrations in German fresh water bodies were studied (Fastner et al. 1999), intracellular microcystin concentrations were below 10 μg l^{-1} in over 70% of the pelagic water samples. The microcystin to chlorophyll–a ratio in the German study varied usually between 0.1–0.5 with maxima of 1–2. The extracellular toxin (for microcystins, <10% of the total toxin in log phase populations, but much higher for cylindrospermopsin) is usually relatively quickly degraded by biotic and abiotic factors, diluted or sedimented in a non–specific manner. Nevertheless, release of cyanotoxins during water treatment, induced by e.g. pre–oxidation, can lead to problems in the production of safe drinking water as extracellular toxins are difficult to remove with conventional treatment technology.

Foodstuffs in contact with cyanobacteria such as shellfish, fish, and agri– and horticultural products irrigated with cyanotoxin–containing water should be monitored for toxins at least occasionally during the bloom periods of cyanobacteria and immediately after blooms. Intensive blooms of known toxin producers require higher levels of vigilance.

Sample extraction and clean–up for microcystins, anatoxin–a and cylindrospermopsin

Effective toxin recovery from cyanobacterial cells is impossible without disruption of the strong cell wall structure. Most procedures use freeze–drying or freeze–thawing for this, and the extraction of toxins is enhanced by ultrasonication in a bath or probe–type sonicator. Toxins can be extracted from filter discs containing harvested cyanobacterial cells or from weighed lyophilized bloom material. Most cyanotoxins can be extracted with (acidified) aqueous methanol. Extraction of tissue samples usually resembles that of phytoplankton samples except for longer extraction times. Adequate toxin recovery has been seldom validated. The complete recovery of microcystins from tissues is practically impossible as microcystins form (non–covalent and) covalent bonds with their target proteins (Ernst et al. 2005).

The sample concentration and clean–up methodology developed for main microcystins/nodularins, anatoxin–a and cylindrospermopsin in phytoplankton and water samples is reasonably robust but still requires great carefullness when naturally occurring concentrations of cyanotoxins (in most cases below 10 μg/L) are analysed. Microcystins and nodularins

are usually concentrated on reversed–phase solid–phase extraction cartridges (SPE; C18 or polymeric materials) which have limited clean–up capacity. For microcystins in raw and treated waters, there is an ISO standard (ISO 20179:2005). The ISO method specifies the reversed–phase SPE and HPLC conditions and it was validated for microcystin–RR, –YR, and –LR through an international intercalibration exercise. Immunoaffinity cartridges, now commercially available, can offer superior clean–up for the peptide toxins in water (Rivasseau and Hennion 1999, Lawrence and Menard 2001, Aguete et al. 2003, Aranda-Rodriguez et al. 2003) and more difficult tissue matrices (Harada et al. 2001). Molecular imprinted polymers, still experimental, allow selective molecular recognition of certain known compounds and can be useful for pre–treatment and concentration of microcystins (Chianella et al. 2002, Kubo et al. 2004). Anatoxin–a can be concentrated on either C18 (after pH adjustment to 9.6) or on weak cation exchange sorbents (James and Sherlock 1996). Cylindrospermopsin is very hydrophilic and can be concentrated on graphite carbon SPE columns (Norris et al. 2001, Metcalf et al. 2002).

Many of these sample preparation and analytical methods have been discussed and presented in a detailed manner in the book "TOXIC: Cyanobacterial Monitoring and Cyanotoxin Analysis" published by Åbo Akademi University Press in 2005 (eds. J Meriluoto and GA Codd 149 pp ISBN 951–765–259–3). The book was one of the deliverables of the research project "TOXIC – Barriers against Cyanotoxins in Drinking Water". The TOXIC project was funded by the European Commission under the Fifth Framework Programme (contract number EVK1–CT–2002–00107) in 2002–2005. The TOXIC project involved ten European research groups in nine countries and comprised four programmes focussing on Raw Water Quality, Analysis, Treatment and Exploitation. The manual presents the core methods which have been developed, standardised and used within the Raw Water Quality and Analysis programmes. Contributions to the manual have been made by researchers at Åbo Akademi University (FIN), University of Dundee (UK), University of Lodz (PL) and DVGW Technologiezentrum Wasser (D). The manual contains comprehensive chapters on the identification and sampling of cyanobacteria, and analytical methods for microcystins, anatoxin–a and cylindrospermopsin in phytoplankton and water. Moreover, 22 standard operating procedures (SOPs) for the actual monitoring and analysis work are included. HPLC–diode–array detection (HPLC–DAD) was chosen as the main analytical tool because a) HPLC–DAD (but not HPLC–FL, cf. anatoxin–a, or LC–MS) was available at all partner laboratories b) HPLC can be used as a general method applicable to several toxin classes and c) HPLC can separate individual toxin variants and degradation products which was important because TOXIC

aimed at the identification of toxin derivatives formed during water treatment.

Simultaneous SPE of several cyanotoxin classes has been a challenge due to the large differences in analyte structure (Fig. 1) and polarity (Fig. 4). Cylindrospermopsin is very hydrophilic and carries both a positive and a negative charge at neutral pH. Anatoxin–a is hydrophilic and has a positive charge at neutral pH. Microcystins have the Adda residue with a lipophilic side chain, two negatively charged carboxylic acid functions at neutral pH, and arginine–containing microcystins have a positive charge in the guanidino group (pKa > 12). Taken together, cation–exchange could be an alternative for SPE for anatoxin–a and cylindrospermopsin and also for many, but not all, microcystins. The elution of the cation–exchange sorbent should avoid high pH due to the instability of anatoxin–a at basic pH. The trapping of all microcystin variants can be done by reversed–phase materials.

We suggest the further exploration of (polymeric) mixed–mode materials with both reversed–phase and cation–exchange functionalities for the simultaneous SPE of microcystins, nodularins, anatoxin–a and cylindrospermopsin. Our very preliminary experiments consisted of the following steps: 1) activation of a 60 mg Waters Oasis MCX cartridge with 2 ml methanol and 2 ml water, 2) loading of known amounts (a few µg) of microcystin–RR, –YR and –LR, anatoxin–a and cylindrospermopsin in cyanobacterial extracts, either separately or in mixtures (loading solvent was ultrapure water or, for microcystins, up to 20% aqueous methanol), 3) washing step with 0–5 ml water, 4) vacuum suction drying for 1 min, 5) elution with 2 ml methanol – aqueous $CaCl_2$ (0.3 g ml^{-1}) 8:2 (vol:vol), 6) evaporation of solvent at 50 °C with nitrogen or argon gas, 7) reconstitution in water or 30 % aqueous methanol 8) sample clarification by centrifugation and 9) HPLC (according to Fig. 4 or by using HPLC conditions optimised for individual analytes). We received satisfactory recoveries (>75%) for all three analyte types when the SPE loading was performed in a small volume (<2 ml), with minimal breakthrough. However, cylindrospermopsin was lost completely when the loading volume was high (100 ml), indicating weak binding of cylindrospermopsin onto the SPE material. The elution of the cartridge must be carefully optimised as the most cationic of the microcystins studied, microcystin–RR, had the lowest recoveries of the microcystins (the use of a weaker cation exchanger with reversed-phase properties might also be an option).

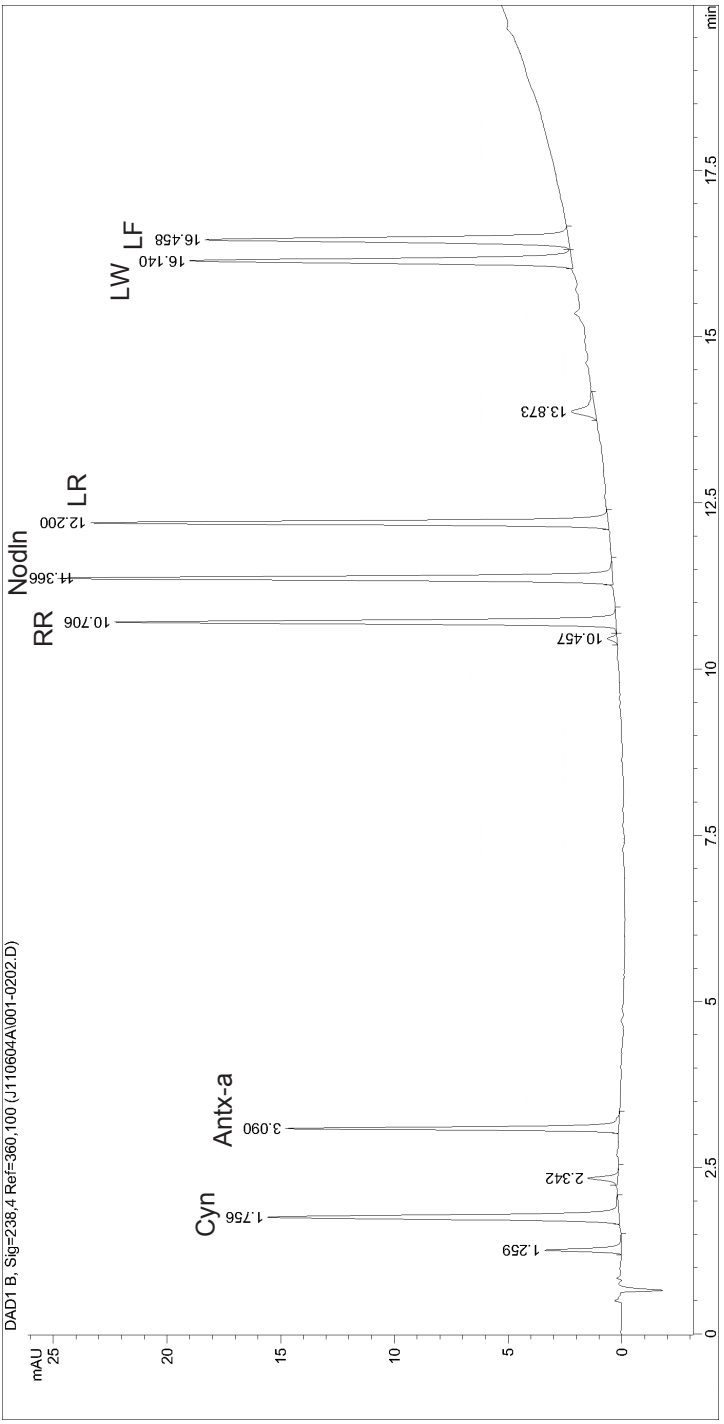

Fig. 4 There are considerable polarity differences between cylindrospermopsin (Cyn), anatoxin–a (Antx–a), microcystins (–RR, –LR, –LW, –LF) and nodularin (Nodln). Chromatographic conditions: Merck Purospher STAR C18e column 55 mm x 4 mm I.D., 3 µm particles, eluent A: 0.05% TFA (trifluoroacetic acid), B: 0.05% TFA in acetonitrile, gradient 1–70% B over 20 min, flow rate 1 ml min^{-1}, temp. 40 °C, detection at 238 nm (optimal for microcystins and nodularin: 238 nm, anatoxin–a: 227 nm, cylindrospermopsin: 262 nm).

Matrix effects

Various matrix components and co–extracted substances make the analyte clean–up, identification and quantitation difficult. These interfering substances can be of either organic (e.g. humic substances, proteins and other biological macromolecules) or inorganic (for microcystins e.g. chlorine, Fe^{3+}, Al^{3+}) nature. It is difficult to get rid of humic substances which co–chromatograph with e.g. microcystins in water samples. Acidic extraction conditions suppress unwanted extraction of many proteins and liquid–liquid extraction of aqueous extracts with non–polar solvents can be used to reduce the concentration of lipophilic substances in relation to microcystins. The processing of tissue samples is facilitated by the use of methods common in forensic science such as tissue digestion with trypsin or pronase. Cyanotoxins are not affected by these enzymes. The reactivity of chlorineous substances in tap and process waters can be quenched by the addition of sodium thiosulphate. Fe^{3+} and Al^{3+} ions have been suggested to interfere with microcystin detection, e.g. by complex formation or by microcystin destruction (Oliveira et al. 2005).

In LC–MS both severe suppression (more common) and enhancement (less common) of the analyte signal are possible, especially with tissue samples (Dionisio et al. 2004, Karlsson et al. 2005). These effects should be assessed by spiking of the reference matrix with analyte at different stages of sample processing (Karlsson et al. 2005), and corrected for. In order to achieve reasonably low LOD values the use of solid–phase extraction is always recommended for tissue samples.

Cyanotoxins in shellfish and fish

Microcystins/nodularins have been observed in laboratory and field experiments in many aquatic animals including mussels, clams, fish species, crab larvae, prawns, crayfish and zooplankton. There are severe methodological difficulties in the bioaccumulation studies and, because of unclear extraction efficiency and matrix effects in the analytical methods, the reported amounts should be considered as minimum amounts in many cases, especially in the older literature. There are no enforced methods for the tissue analysis of cyanotoxins with the exception of the saxitoxin family (saxitoxin derived from e.g. marine dinoflagellates is regulated in seafood).

Immunoaffinity purification is a very promising form of clean–up for tissue samples (Harada et al. 2001).

Nodularin does not bind covalently to protein phosphatases (Craig et al. 1996, Bagu et al. 1997) like most microcystins do and it may therefore be easier to extract from tissue material than microcystins. Typical levels of extractable microcystins and nodularins detected in tissue materials of aquatic organisms have ranged from undetectable (below 10 ng g^{-1} tissue dry weight) to several tens of µg/g tissue dry weight. The highest levels have been recorded in the hepatic (or hepatopancreatic) tissues of fish and mussels while concentrations in fish muscle have usually been substantially lower or undetectable. It was proposed that a large proportion of the microcystin is bound covalently and irreversibly to the tissue matrix, and cannot be fully extracted from the tissue with methanol (Williams et al. 1997a, Williams et al. 1997b).

Shellfish are readily contaminated by many cyanotoxins and other phycotoxins as they can filter large volume of toxin–containing water without being affected by many substances harmful for higher animals. Mussels exposed to cyanobacterial blooms consisting of over 20000 cells ml^{-1} have been shown to accumulate toxins in sufficient amounts to be unsafe for human consumption (Van Buynder et al. 2001). There are considerable differences in the toxin accumulation characteristics of different bivalve species (Yokoyama and Park 2002) and also in the experimental settings in the reported research making comparisons and predictions difficult. Freshwater mussels *Anodonta cygnea* were demonstrated to accumulate microcystin (70–280 µg per mussel) when they were kept in a culture of *Planktothrix agardhii* (40–60 µg intracellular demethyl–microcystin–RR per litre) (Eriksson et al. 1989). The mussels did not suffer from any obvious toxic symptoms. *Mytilus edulis* mussels were recommended not to be be collected for human consumption during a water bloom of *Nodularia* (Falconer et al. 1992). The gut and associated tissues showed hepatotoxicity to mice. Fragments of *Nodularia* were also detected in the gut of the mussels confirming ingestion of the cyanobacteria. *Anodonta grandis simpsoniana* accumulated microcystins by grazing on toxic phytoplankton and only minimally via uptake of the dissolved toxin (Prepas et al. 1997). In the Baltic Sea food web blue mussels accumulate nodularin present in the cyanobacterium *Nodularia spumigena*. The toxin accumulated in the mussels (recorded maximum concentration in mussels 2150 µg kg^{-1}, (Sipiä et al. 2001)) can be transferred to other organisms consuming mussels, e.g. flounders (recorded maximum concentration in the liver 399 µg kg^{-1} dry weight in the same paper, (Sipiä et al. 2001)) and eiders (sea birds, recorded maximum concentration in the liver 180 µg kg^{-1} dry weight, (Sipiä et al. 2004)).

The derived health alert levels for microcystin and nodularin for adults were 250 μg kg^{-1} for fish, 1100 μg kg^{-1} for prawns and 1500 μg kg^{-1} for mussels (Van Buynder et al. 2001). Boiling of the seafood can redistribute toxins between viscera and flesh as reported for nodularin in prawns (Van Buynder et al. 2001). The depuration mechanism of microcystins when bivalves are placed in pure water have been shown to be biphasic (Prepas et al. 1997), a initial fast decline in toxin concentration is followed by a very slow elimination (duration weeks–months). The depuration of toxins by bivalves may be temperature dependent, being more effective in higher temperatures (Yokoyama and Park 2003).

Bivalves are also resistant to toxic effects of high concentrations of cylindrospermopsin. Cylindrospermopsin from *C. raciborskii* accumulated in exposed *Anodonta cygnea* with the highest concentration in the haemolymph (Saker et al. 2004). The derived health alert levels for cylindrospermopsin were 158 μg kg^{-1} for fish, 720 μg kg^{-1} for prawns and 933 μg kg^{-1} for mussels (Saker et al. 2004).

A coarse generalisation, with many exceptions and reservations, of microcystin, nodularin and cylindrospermopsin levels found in shellfish within 2–3 weeks of exposure: Biomass–bound toxin level in water, *N* μg L^{-1}, equals roughly to the typical maximum toxin level in shellfish soft tissue, *N* μg g^{-1} dry weight. Much inter–species variation is expected. The highest toxin levels have been found in the hepatopancreas/viscera (microcystin/nodularin) and haemolymph (cylindrospermopsin), less in the muscle.

Fish flesh is usually safe for consumption (there has been some exceptions) but stomach/intestinal content and internal organs, especially the liver, can contain considerable amounts of cyanotoxins. Phytoplankton–eating fish are exposed to toxins in cyanobacterial biomass. *Tilapia rendalli* was collected from a Brazilian coastal lagoon (phytoplankton dominated by the genus *Microcystis*, average microcystins 46 μg L^{-1}, highest 257 μg L^{-1}) (Freitas de Magalhaes et al. 2001). Microcystins were detected in 57% of the fish liver (concentration of microcystins 0–15 μg g^{-1}) and viscera samples (0–72 μg g^{-1}). In muscle tissue the microcystin concentration peaked at 337 ng g^{-1} (the values concern an intensive bloom period of cyanobacteria in 1999).

In conclusion, the overall message found in the literature is that the consumption of fish muscle tissue cannot be considered a major hazard to human health but there are fish species and/or fish organs which may contain considerable amounts of cyanotoxins. Consumption of mussels and clams collected during cyanobacterial blooms or immediately after blooms should definitely be avoided.

Cyanotoxins in plants

Toxin accumulation in plants could either reduce crops or cause health problems for plant consumers. The best documented cyanotoxin group in this context is microcystins which are known to be taken up and affect a number of terrestrial and aquatic plants (MacKintosh et al. 1990, Kurki-Helasmo and Meriluoto 1998, Pflugmacher et al. 1999, McElhiney et al. 2001, Chen et al. 2004, Mitrovic et al. 2005). Seedling growth is usually inhibited at micromolar ($\mu g\ mL^{-1}$) microcystin concentrations which are seldom found in nature. At micromolar toxin exposure the recorded toxin concentrations in the plant seedlings have been up to a few $\mu g\ kg^{-1}$. Plant exposure to toxins is more probable in hydroponic cultures than in soil–grown cultures. It is currently unclear whether the main crop plants grown in natural conditions can contain high enough concentrations of cyanotoxins to cause a health hazard for consumers. It is possible that the only scenario which can lead to serious toxin contamination of terrestrial plants is spray irrigation with a heavy bloom of toxic cyanobacteria. Especially the contamination of leafy plants where the aerial part is eaten, e.g. lettuce, is possible (Codd et al. 1999).

Plant–based biotests have been suggested for the detection of microcystins (Kós et al. 1995) and cylindrospermopsin (Vasas et al. 2002). Microcystins can either enter the plants through roots (Kurki-Helasmo and Meriluoto 1998) or remain on (in) the leaves after spray irrigation with toxin–containing water (Codd et al. 1999, Siegl et al. 1990, Abe et al. 1996). Translocation of microcystin within the plant is possible (Kurki-Helasmo and Meriluoto 1998). Cylindrospermopsin, a protein–synthesis inhibitor, has been suggested to affect the pollen germination if high enough concentrations of the toxin are present in spray irrigation water (Metcalf et al. 2004).

Biomagnification of cyanobacterial toxins in the cyanobacteria–plant–animal food web was demonstrated in the case of beta–*N*–methylamino–L–alanine (BMAA), a neurotoxic amino acid (Cox et al. 2003, Cox et al. 2005). BMAA was originally discovered in cycads and later in their cyanobacterial root symbiont, the cyanobacterium *Nostoc*. BMAA is suggested to be the cause of neurological disease in Chamorro people in Guam, the diet of whom include cycad seeds and flying fox bats. Nearly a 100–fold increase in BMAA for each trophic level, cyanobacteria – cycad/cyanobacteria symbiosis – flying foxes was observed. BMAA production in cyanobacteria was demonstrated to be ubiqitous, comprising both symbiotic and free–living cyanobacteria (Cox et al. 2005). This finding warrants a global risk assessment for human health. Indeed BMAA has

been found in the frontal cortex of Canadian Alzheimer's patients (Cox et al. 2003).

Microcystin/nodularin conjugates

Conjugation of xenobiotics is a common detoxification strategy for different organisms. There is some direct and indirect (elevation of glutathione *S*–transferase activity) evidence of glutathione and cystein conjugates of microcystins and nodularin in animals and plants (Karlsson et al. 2005, Kondo et al. 1992, Pflugmacher et al. 2001, Pflugmacher 2004, Sipiä et al. 2002).

Cyanotoxins in food supplements

Most blue–green algal food supplements collected from the North American market contained microcystins (Gilroy et al. 2000, Lawrence et al. 2001) with concentrations up to 35 μg g^{-1}. Tolerable daily intake of microcystin–LR (0.04 μg kg^{-1} body weight) is easily exceeded by the consumption of contaminated food supplements (Dietrich and Hoeger 2005).

Cyanotoxins in sediments

Some aquatic animals, including different invertebrates and fish, live in or feed on sediment materials and become in contact with sedimented cyanotoxins and cyanobacterial cells. It was shown that 1 ml of autoclaved (sterilised) sediment could adsorb 13–24 μg of microcystins and 50–82 μg of anatoxin–a (Rapala et al. 1994). Several sediment samples fom Japanese lakes were successfully studied using the ozonolytic MMPB method combined with GC–MS detection (Harada et al. 2001, Tsuji et al. 2001). This strategy was chosen instead of conventional HPLC approach with intact microcystins. Especially the hydrophilic microcystins such as microcystin–RR were strongly adsorbed on the sediment and were found difficult to extract. Several extraction methods were tested. The best recovery of intact microcystins (60% for MC-LR) was obtained by extraction with 5% acetic acid containing 0.1%TFA under ultrasonication. The extract was filtered, evaporated, redissolved in water, applied on C18 SPE and eluted with 90% methanol. The extract still contained several interfering impurities in HPLC–UV. Microcystins were found to interact with humic and fulvic substances, with suspended particulate matter and sediments, however

preferably remaining in the aqueous phase (Rivasseau et al. 1998). In river water spiked with microcystin–LR, –YR and –RR, 5 µg L^{-1}, only 9–13% of microcystin was adsorbed on particles and 7–8% on sandy sediment within three days. Nodularin is present in the Baltic Sea sediments (Kankaanpää et al., Finnish Institute of Marine Research).

Conclusion

The frequent presence of cyanotoxins in phytoplankton and aquatic animal specimens is well documented. There is also increasing evidence of toxin contamination of aquatic and terrestrial plants. Sample processing of most toxins in phytoplankton is reasonable robust and at least partially validated in a few intercalibration exercises whereas sample processing of tissue samples, animal and plant, still requires much standardization. The lack of certified reference materials (CRMs) of most cyanotoxins calls for immediate attention as the toxin analyses cannot be considered truly quantitative without access to CRMs.

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