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Chapter 39: Toxin mixture in cyanobacterial blooms – a critical comparison of reality with current procedures employed in human health risk assessment

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Abstract

Cyanobacteria are the oldest life forms on earth known to produce a broad spectrum of secondary metabolites. The functions/advantages of most of these secondary metabolites (peptides and alkaloids) are unknown, however, some of them have adverse effects in humans and wildlife, especially when ingested, inhaled or upon dermal exposure. Surprisingly, some of these cyanobacteria are ingested voluntarily. Indeed, for centuries mankind has used cyanobacteria as a protein source, primarily *Spirulina* species. However, recently also *Aphanizomenon flos–aquae* are used for the production of so called blue green algae supplements (BGAS), supposedly efficacious for treatment of various diseases and afflictions. Unfortunately, traces of neurotoxins and protein phosphatases (inhibiting compounds) have been detected in BGAS, making these health supplements a good example for human exposure to a mixture of cyanobacterial toxins in a complex matrix. The discussion of this and other possible exposure scenarios, e.g. drinking water, contact during recreational activity, or consumption of contaminated food, can provide insight into the question of whether or not our current risk assessment schemes for cyanobacterial blooms and the toxins contained therein suffice for protection of human health.

Cyanobacterial metabolites: Health hazards for humans?

Cyanobacteria exist worldwide ubiquitously, including in extreme environments (Hitzfeld *et al.* 2000a; Wynn–Williams 2000). Some of these

cyanobacterial species produce complex compounds at great energy expense. The ecological or physiological function or advantage of these compounds for these cyanobacteria species is yet unknown. Toxic cyanobacterial blooms, whether on the surface, benthic growths or as subsurface layers of fresh, brackish or marine water bodies have been responsible for numerous acute and subacute intoxication incidents of humans and animals. Most of these toxic metabolites are very small alkaloids or peptides, with a molecular weight of <2kD, a very complex structure and, in case of the peptides, composed of uncommon amino acids. Amongst the alkaloids synthesized by cyanobacteria are e.g. saxitoxins or anatoxins, known to be toxic for humans and other organisms at very low concentrations (Hitzfeld *et al.* 2000b). The cyanobacterial peptides are not metabolized in the mammalian digestive tract due to the presence of D–amino acids and a cyclic structure. Consequently, either local toxicity, e.g. diarrhea, or systemic toxicity, e.g. hepatotoxicity, neurotoxicity, renal toxicity i.e. morbidity or even mortality are the direct consequences of acute exposure. However, the uptake of these toxins and their organ distribution appears to be mediated by specific transporters, of which most, if not all, remain unidentified in humans and other mammalian species (Fischer *et al.* 2005). An exception to the latter is the uncommon, non–protein amino acid β–N–methylamino–L–alanine (BMAA), a neurotoxin, which is probably produced by most likely all known groups of cyanobacteria (Cox *et al.* 2005).

In order to provide a reasonable hazard and risk assessment for these toxins in conjunction with acute or chronic exposures, it is prerequisite to characterize their uptake and distribution (kinetics) and consequently the toxic dose that induces *in–situ* effects (dynamics). Moreover, it is essential to understand the kinetics and dynamics and thus the hazard and risk of single toxins before the hazard and risk of toxin mixtures can be evaluated and meaningful risk management strategies can be developed.

In the environmental setting, not every cyanobacterial family or even every strain within a family synthesizes highly toxic alkaloids and peptides, meaning that the simple occurrence of cyanobacteria in a water body does not mandate the presence of cyanobacterial toxins. In order to ensure that a given water body is free of or has only a limited contamination with toxins, specific toxin analyses should be carried out. However, to do this on a routine basis can be financially problematic, thus calling for a prioritization of water bodies and definition of sampling routines that would ensure the highest degree of safety at reasonable cost. First and foremost, all water bodies in use as drinking water resources must be monitored routinely, the frequency of analyses should be determined based on the cyanobacterial history of the water body and the observation of the water body by qualified personal. However, even oligotrophic lakes can contain

blooms of toxic cyanobacteria that are not readily and routinely observable. Especially *Planktothrix rubescens*, the species having the highest microcystin concentration/cell of all known cyanobacteria, occurs specifically in the metalimnion of oligotrophic and deep lakes, sometimes even during winter. Second, water bodies used for aquaculture (fish, shrimp and shellfish) must be monitored and the products harvested must be controlled for contamination with known cyanobacterial toxins, since for some of them (e.g. BMAA) bioaccumulation and trophic transfer has been demonstrated. Especially lakes in use for harvesting and the production of cyanobacterial food supplements must be monitored and a product and consumption derived limit of acceptable contamination defined. Finally, water bodies used for recreational purposes must be monitored and the bathing areas must be closed, if the concentration of cyanobacterial toxins is considered to endanger human health, especially children and toddlers.

For risk assessment and risk management purposes one would be inclined to try to differentiate and classify species that occur specifically only in one given environment, and thus, to try to deduce the possible type(s) of toxins that could potentially evolve from a bloom of this species in the given environment. This approach, however, has so far proven to be unsatisfactory due to the following observations:

- While some cyanobacterial species appear to prefer specific environments (e.g. *Nodularia spumigena*, *Planktothrix rubescens*), others are found nearly in all environments (*Lyngbia* sp.)
- Cyanobacterial blooms may be predominated by one given cyanobacterial species, however this does not exclude the quantitatively important presence of another toxin producing species
- Blooms of different cyanobacterial species (e.g. *Anabaena circinalis* and *Microcystis aeruginosa*) have been observed to occur in the very same water body at different time–points but also sometimes overlapping when occurring in rapid succession to one another
- Many cyanobacterial species have been observed to be capable of producing several different toxin types as well as different toxin congeners (e.g. microcystin congeners)
- Bloom size, expressed as cells density (cells/ml), are not a reliable indicator for toxin production especially as large blooms have been reported that did not produce extensive toxin amounts, while conversely blooms containing low to moderate cell densities have been demonstrated to produce copious amounts of toxin.

The following two examples may serve as an illustration of the above points:

1. In 1996 an intensive phytoplankton bloom was observed in the Montargil reservoir, Portugal. From May to June *Aphanizomenon flos–aquae* had been the predominant species and bloom extracts exhibited clear neurotoxic symptoms in a mouse bioassay. Pereira et al. (2000) analytically identified five saxitoxin variants following the isolation of an *A. flos–aquae* strain predominating the bloom. From July to August *Microcystis aeruginosa* predominated the bloom with extracts showing high hepatotoxicity in the mouse bioassay. The observed hepatotoxicity was attributed to the presence of high concentrations of microcystins. Although the two cyanobacteria species occurred in the bloom with alternating predominance, *A. flos–aquae* was still present in considerable densities during *M. aeruginosa* predominance. However, despite the presence of large numbers of *A. flos*–aquae no neurotoxicological activity (AOAC mouse bioassay) was observable suggesting that the *A. flos–aquae* strains co–occurring within the *M. aeruginosa* bloom did not produce saxitoxin at all or, at least not in relevant quantities. It seems that in the Montargil reservoir several saxitoxin producing and non–producing strains coexist within the same bloom but with varying densities. Alternatively, the *A. flos–aquae* in the Montargil reservoir was only one strain of *A. flos–aquae* with rapidly changing capabilities of producing saxitoxins.
2. In a drinking water reservoir in Australia where *M. aeruginosa* and *A. circinalis* were the predominant cyanobacteria, Hoeger et al. (2004) found that the highest concentrations of microcystins produced by *M. aeruginosa* did not coincide with the highest cell counts. This could, as suggested above, indicate the simultaneous presence of non–toxin–producing and toxin–producing strains of *M. aeruginosa*. In contrast, saxitoxin(s) concentrations found appeared to be highly correlated with the cell densities of *A. circinalis*, suggesting that only one continuously toxin–producing strain of *A. circinalis* was present in this specific water body.

The two examples above strongly illustrate the importance of detailed understanding of the limnological and toxicological characteristics of a specific water body, i.e. the spatial and temporal occurrence of toxin(s) and cyanobacterial species present in a given bloom. Consequently, generalized statements regarding the species and toxins occurring in a specific water body, or water bodies in general, is difficult if not impossible. Reliable risk characterization of possibly toxic blooms demands assessment of

the cyanobacterial species and toxins by specialists or trained personnel, and a thorough understanding of the limnological history of the respective water body concerned.

Taking the above points regarding the characterization of bloom events into consideration, it becomes stringently clear that each and every bloom event in a given water body represents a unique risk scenario for humans and animals. In order to reduce complexity and facilitate understanding of the main issues at hand, the following discussion primarily focuses on human health risks. The discussion of the possible exposure scenarios in conjunction with the toxin specific health risk extrapolations will provide insight into the question as to whether or not our current risk assessment schemes for cyanobacterial blooms suffice for protection of human health as well as pin–point areas where more profound understanding of the underlying toxicity mechanisms is needed.

General risk scenarios (cyanobacterial blooms)

Massive toxic cyanobacterial blooms of concern for humans predominantly occur in fresh water bodies used for the preparation of drinking water and recreational activities. Consequently, human cyanobacterial toxin exposure can occur either via contaminated drinking water or during recreational activities in cyanobacteria blooming water. The exposure scenarios that follow are therefore artificially divided into the two predominant human exposure routes, namely oral and inhalation exposure. Although dermal exposure and subsequent effects have been described in the literature (Pilotto *et al.* 1997; Soong *et al.* 1992; Stewart *et al.* 2001; Torokne *et al.* 2001), primarily as anecdotal reporting of incidences without a experimental follow–up and/or proof of principle analysis, the cyanobacteria and toxin(s) causally responsible for the reported dermal effects have not been established with the exception of *Lyngbya majuscule*. The contact with this species causes dermatitis, and accidental oral consumption led to an burning sensation in the mouth (Osborne *et al.* 2001).

Oral exposure

The oral exposure scenario assumes that the person is exposed voluntarily or accidentally. In both situations, it is the goal of the risk assessment to elucidate the highest toxin concentration in the water that has no health

consequences for the exposed person ingesting a given volume of water in an acute (one time ingestion) or chronic (continuous ingestion) situation.

Drinking water

For drinking water, one of the primary concerns is whether water treatment can eliminate or reduce the concentration of the cyanobacterial toxins present during a bloom event. Although this generally appears to be the case for most technically moderately modern water treatment systems (Hitzfeld *et al.* 2000b; Hoeger *et al.* 2005) and bloom situations, exceptions to the rule do occur (Hoeger 2005).

In the years 1985 –1988 recurring cases of severe gastro–enteritis were observed in Bahia State, Brazil, apparently closely associated with the consumption of treated and untreated water from the Itaparica Dam reservoir (Teixera *et al.* 1993). Although cases of gastro–enteritis and associated diarrhea were observed throughout the years at low incidence, increased numbers of cases with concurrent hospitalization and subsequent mortalities occurred regularly between January to May of each year. In 1988 the most severe outbreak of gastro–enteritis and associated diarrhea with fatal outcome occurred between February and May. Of approximately 2392 reported cases of gastro–enteritis, 368 were immediately hospitalized. The average monthly mortality among the hospitalized patients was 24% with a peak of 45.1% in May. No abnormalities in the frequency of diarrhea incidences arose in adjacent regions with a different drinking water source. Clinical/microbial investigations of the water from the Itaparica dam reservoir treated water did not identify the presence of fecal coliforms, salmonella, shigella, adenovirus or rotavirus. Boiling and filtration of the treated water had no impact on the occurrence of severe gastro–enteritis. Accordingly, it appeared that the agent in the treated water was heat and filtration resistant, possibly pointing to cyanobacterial toxins e.g. microcystins. Limnological analyses of the dam water in April 1988 revealed the presence of *Anabaena* and *Microcystis* species at densities exceeding the WHO maximum acceptable levels for untreated drinking water by a factor 4 – 33. Seventy percent of the hospitalized gastro-enteritis cases were children under the age of 5 in the period between March and May, 1988. The high incidence of illness among children suggests that risk calculations based on data from human adults or animal studies may not be sufficiently protective for children.

In 1999 and 2000 a survey of cyanobacterial toxins in surface waters and drinking water supplies was carried out across Florida, USA, revealing the following concentrations of cyanotoxins in raw and treated/drinking water (Burns 2004):

- concentrations of microcystins ranged from below the detection limit to 12.5 µg/l in raw and treated/drinking water samples
- concentrations of anatoxin–a ranged from below the detection limit to 8.46 µg/l in three treated drinking water samples
- concentrations of cylindrospermopsin ranged from 8.07 to 97.12 µg/l in nine treated drinking water samples. In some cases, trichomes of *Cylindrospermopsis raciborskii* in the raw water survived the treatment process and were present in the tap water.

An acute maximum dose that is not toxic to infants, small children or adults can be extrapolated from the lowest acute toxic doses of cyanotoxins observed in mouse studies using body weight assumptions (Dietrich and Hoeger 2005; Fromme *et al.* 2000). For example, a single intake of 12.5, 50 or 150 µg microcystin–LR (MC–LR) equiv. are assumed not to produce adverse effects in toddlers (< 5 kg bw), small children (>20 kg bw) and adults (60 kg bw), respectively. However, such calculations are fraught with difficulties as it is unclear:

- how much of the compounds are actually biologically available in humans
- whether or not direct extrapolations from mouse studies to humans are reliable as there are vast species differences in toxin specific transporter expression in the gastro–intestinal tract (see below).
- whether or not extrapolations from single toxin toxicity experiments are feasible, in view of the fact that multiple toxins may occur in a bloom simultaneously and because there is lack of knowledge regarding combinatorial toxicity.

For the calculation of safe levels for chronic exposure, estimates of drinking water intake and average body weights must be used. The average daily consumption of water varies between 1500 and 2000 ml of treated (drinking) water per day for adults with an average weight between 50 – 80 kg bw. For infants and small children average daily water consumption is assumed on average to be 750 ml and 1000 ml per day, respectively (Dietrich and Hoeger 2005). In all calculations, the assumptions are made that treated water is consumed, consumption will occur nearly for a life–time, and that only one of the variety of cyanobacterial toxins will occur at any one given time. However, toxic cyanobacteria are unlikely to be continuously present, different cyanotoxins toxins can be present at the same time in varying concentrations, and water treatment procedures do not always guarantee cyanotoxin removal.

Recreational Activities

All activities at, in, and on the surface of the water body that bring humans into direct contact with water are considered to be recreational activities. These include bathing, swimming incl. contests e.g. triathlons, water skiing, boating, etc. While pursuing these activities it is assumed that some toxin–contaminated water will be accidentally and involuntarily ingested. The amount of water ingested is – in a worst–case situation – approximated as ranging from 50 – 120 ml per episode. As this is usually an intermittent rather than a continuous occurrence, with the exception of people continuously swimming and bathing in a water body chronically contaminated with toxic cyanobacteria blooms, all risk calculations are driven toward an acute exposure scenario. The following episode is an example of this type of exposure:

Turner et al. (1990) reported cyanobacterial poisoning of two 16 – year old army recruits. Both became ill after a canoe exercise on a freshwater reservoir in Staffordshire, England, experiencing a massive *M. aeruginosa* bloom containing high concentrations of MC–LR. Both swallowed some of the water; both recruits developed pneumonia and other symptoms including blistering around the mouth, sore throat, dry cough, pleuritic pain, vomiting and abdominal pains requiring medical attention. One of the patients presented with temporary difficulties in walking, suggesting possibly neurotoxic effects. Subsequently another sixteen soldiers received medical attendance also after canoeing. Eight exhibited some symptoms similar to the first two cases. The disease pattern was considered to stem from microcystin intoxication, even though two weeks later a critical contamination of the reservoir with *Escherichia coli* was confirmed. The coincident presence of *M. aeruginosa* and *E. coli* could suggest a simultaneous exposure of the recruits to MC–LR and lipopolysaccharides (LPS) because the patterns of symptoms, with possibly the exception of the transient neurological disorder, are characteristic for a MC and LPS exposure. The neurological symptoms could be due either to MC (Fischer *et al.* 2005) or other neurotoxic cyanotoxins. In addition, augmentation and exacerbation (additive or synergistic) of effects and symptoms due to simultaneous exposure to different toxins (MC and LPS) cannot be excluded (Best *et al.* 2002).

Specific health risks from contaminated food and cyanobacterial food supplements

Contrary to the direct exposure of humans to cyanobacterial toxins via contaminated water, the risk situation involving exposure via food and food supplements is much more complex. Worst–case exposures can be in-

terpolated from assumed daily or weekly consumption of specific food sources e.g. fish, crayfish, shellfish, vegetables, salads, etc. for the general populace as well as for populations at high risk, e.g. indigenous tribes predominantly existing on a specific food source (Dietrich and Hoeger 2005; Ernst *et al.* 2005). However, the potential human toxin exposure via food that provides the basis for risk calculations is also largely determined by the degree of toxin contamination of a given food source as well as by the bioavailability of the toxin from the food type. Furthermore, although most likely an exception to the rule, bioaccumulation of cyanotoxins in the food chain, as is the case with β–N–methylamino–L–alanine (BMAA), may provide for an additional element of risk (Cox *et al.* 2005).

Exposure considerations similar to those for drinking water also apply to the voluntary consumption of cyanobacteria (blue–green alga) food supplements on the basis of cyanobacteria, also know as blue–green alga supplements (BGAS). The main difference is that the daily consumption of pills, powders and drinks is extremely difficult to estimate. Furthermore, these products are often handled as if they were pharmaceuticals. Therefore, the consumption per person does not correspond to body weight as is the case for real food sources, e.g. fish, crayfish etc. A detailed discussion of this issue can be found in Dietrich & Hoeger (2005), a publication that also estimates the effects of varying levels of microcystin contamination of food and food supplements and the corresponding limit amount that can be safely consumed by infants, children and adults (Table 1).

BGAS are usually produced from *Spirulina maxima* or *platensis* and *Aphanizomenon flos–aquae*. Although *Spirulina maxima* is considered as being non–toxic (Salazar *et al.* 1996; Salazar *et al.* 1998), Draisci et al. (2001) identified Epoxyanatoxin–a and Dihydrohomoanatoxin–a at concentrations ranging from non detectable to 19 μg g^{-1} dw in *Spirulina*–based BGAS.

In alkaline crater lakes in Kenia, *Arthrospira fusiformis* (syn. *Spirulina fusiformis = platensis*) was found to produce small amounts of both, microcystins and anatoxin–a (Ballot *et al.* 2004; Ballot *et al.* 2005). Positive ELISA– results were evidence for ~~a~~ possible contamination of Spirulina supplements with microcystins (Gilroy *et al.* 2000). In addition, Spirulina based BGAS is suspected to be responsible for the liver injury of a 52–year old Japanese (Iwasa *et al.* 2002). Further corroboration of these data is necessary before proper evaluation of the potential hazard to human health in the context of a risk assessment can be carried out.

Contrary to *Spirulina*, where direct production of toxin is yet uncorroborated, *A. flos–aquae* is known to be capable of producing the neurotoxic alkaloids anatoxin–a (Rapala *et al.* 1993) and saxitoxins (Adelman *et al.*

1982; Ferreira *et al.* 2001; Mahmood and Carmichael 1986; Pereira *et al.* 2000), as well as the neurotoxic, nonprotein amino acid BMAA (Cox *et al.* 2005). Maatouk et al. (2002) assumed that a mono–specific *Aph. flos–aquae* bloom was responsible for the microcystin content of a *Aph. flos–aquae* sample from Saint–Caprais reservoir in France. The cytotoxic and most likely carcinogenic alkaloid cylindrospermopsin has yet not been detected in *Aph. flos–aquae*, however its presence in *Aph. ovalisporum* is confirmed (Banker *et al.* 1997; Shaw *et al.* 1999).

In contrast to Spirulina, *Aph. flos–aquae* is generally harvested from natural lakes. One of its biggest sources is Lake Klamath, Oregon, where *Aph. flos–aquae* co–exists and coincides with *Microcystis sp*. blooms (Carmichael *et al.* 2000), this coexistence can also be observed in other lakes (Ekman–Ekebom *et al.* 1992; Teubner *et al.* 1999). Consequently, consumers of Spirulina– and *Aph. flos–aquae* –BGAS are potentially exposed to toxins produced by all three of the cyanobacteria species.

Although neurotoxic alkaloids (anatoxins and saxitoxins) have not been identified to date in Klammath Lake, Sawyer et al. (1968) reported on an aqueous extract of an Klamath Lake *Aph. flos–aquae* bloom that was nearly instantaneously lethal to mice after i.p. injection. In addition, *Aph. flos–aquae* was reported to be responsible for massive fish kills (Barica 1978) and being generally highly toxic to members of the freshwater fauna (Gentile and Maloney 1969). Out of 88 bloom samples from Finland in which *Aph. flos–aquae* was one of the predominating species, 11 were determined to be neurotoxic and 25 to be hepatotoxic (Sivonen *et al.* 1990). Acute toxicity testing of 23 populations of *Aph. flos–aquae* from 12 localities of inland waters in South Norway, resulted in protracted toxic response in the test animals with 60% of the samples tested (Underdal *et al.* 1999).

Several investigations report high concentrations of microcystins in BGAS products (Hoeger and Dietrich 2004; Lawrence *et al.* 2001; Saker *et al.* 2005; Yu *et al.* 2002), often dramatically exceeding the provisional guidance value of 1 µg/g dw set by the Oregon Health Division and the Oregon Department of Agriculture (Gilroy *et al.* 2000). Based on some major weaknesses in the assumptions underlying the risk assessment leading to this provisional guidance value, Dietrich and Hoeger (2005) concluded that BGAS could pose a serious health risk to consumers, especially children. One of the main weaknesses in the risk calculation process is the assumption that all congeners of a toxin, in this case microcystins have the same toxicokinetic and –dynamic properties, i.e. that all congener concentrations in a given example can be added up to give one single MC–$LR_{equiv.}$ concentration. Another weakness is that all of the risk calculations are focused on risk of a single compound exposure situation but not, as

was shown above, on the potential simultaneous exposure to multiple toxins of vastly different structure, kinetics and dynamics, ignoring possible additive or even synergistic effects, as will be discussed later in the text. The toxicity of BGAS supplements is particularly worrisome because the benefits of the consumption of BGAS are still unclear and could not be confirmed scientifically (Vitale *et al.* 2004).

Inhalation exposure through drinking/hygienic water

Inhalation exposure to cyanobacterial toxins stems primarily from three exposure situations, namely habitation near chronically contaminated surface waters, use of contaminated water for hygienic purposes (showering and sauna), and finally during recreational activities as already indicated above. The problem is that good exposure models are lacking. Only rough assumptions can be made for the amount of toxin and toxic cyanobacterial cells available in an aerosolic form and for the average inhalation rate (liters minute^{-1}) of the persons potentially exposed to the cyanobacteria and their toxins. Needless to say that the average inhalation rate of a person involved in a high–energy recreational activity e.g. swimming/triathlon competition, water skiing or those involved in a moderate activity e.g. swimming leisurely, boating, etc or those living at the coast–line of a chronically bloom contaminated surface water will be entirely different. The following examples illustrate situations in which adverse effects from inhalation exposure was very likely although proper documentation is missing:

Hoppu et al. (2002) reported cases where cyanobacterial contaminated water was used for washing and for producing hot steam in a sauna in Finland. By pouring cyanobacteria containing water on heated stones, cyanobacterial cells may be lysed, toxins released and aerosolized. As a consequence, people could experience both dermal and inhalation exposure to cyanotoxins via contaminated water and aerosols. Subsequent to sauna and bathing, 48 persons developed gastrointestinal, dermal and neurological symptoms most likely attributable to cyanobacterial toxin exposure. Unfortunately no information was available on the cyanobacteria species or the cyanotoxins present in the water used for bathing and the sauna.

A similar situation may have occurred in Florida, where copious amounts of microcystins, anatoxin–a and cylindrospermopsin as well as trichomes of *Cylindrospermopsis raciborskii* were reported in raw and treated water (Burns 2004). Inhalation exposure could have taken place especially in showers, as shower water usually exceeds 70°C during the heat-

ing process, cyanotoxins are released from cells due to heat lysis, and aerosolized with the steam in the shower cabin. Up to 10^4 cells ml^{-1} could be counted in the tap water in a town in Queensland/Australia (Hoeger *et al.* 2004). Although no exposure related effects were reported for residents in either case, the above examples demonstrate that high cell numbers of potentially toxic cyanobacteria can pass water treatment and provide a basis for inhalation, dermal and oral toxin exposure.

Furthermore, the above examples demonstrate that exposure to multiple toxins via inhalation can most likely occur. Heating of cyanobacteria contaminated water and aerosolization of cyanobacterial components may provide a form of exposure unlikely to occur under other circumstances (bathing, etc.). Consequently it is at present unclear whether the risk assessment calculations carried out with known cyanobacterial toxins offer any safety for the inhalation exposure scenario.

Human health risk assessment single vs. multiple compounds: toxicokinetic and –dynamic considerations

Investigations of cyanobacteria intoxication events have so far focused on MCs due to their frequent occurrence, high toxic potential, and clear association, if not causal relationship, with severe poisonings of humans (Kuiper–Goodman *et al.* 1999) and animals (Landsberg 2002). As most of these incidences were related primarily to ingestion of contaminated water, the WHO (Chorus and Bartram, 1999) developed a provisional guidance value of 1 µg MC–LR_{equiv} L^{-1} for drinking water, based on data from a mouse study using purified MC–LR. A similar study with pigs and MC containing extracts resulted a nearly the same no/lowest observed adverse effect value (Dietrich and Hoeger 2005).

Toxicological studies using MC containing extracts inherently appear to have a greater relevance for assessment of potential toxicological hazards to human health than those using one purified congener. To date numerous studies on the toxicity of purified MCs, MC–LR in particular, and of MC containing extracts have been carried out using different test organisms and different routes of administration. Despite that microcystins have generally been characterized as hepatotoxins, there is also evidence for neurotoxicity, as suggested in the Caruaru incident, where more than 60 dialysis patients died following i.v. treatment with MC contaminated dialysis water (Azevedo *et al.* 2002; Fischer *et al.* 2005; Jochimsen *et al.* 1998; Pouria *et al.* 1998), and for nephrotoxicity, as indicated by several animal experiments (Bhattacharya *et al.* 1997; Fischer and Dietrich 2000; Khan *et al.*

1996; Milutinovic *et al.* 2002; Milutinovic *et al.* 2003; Moreno *et al.* 2005; Stotts *et al.* 1997). Studies on organ distribution support these findings and suggest that, beside liver and kidney, the brain and the lungs (Picanco *et al.* 2004) can also be affected to a varying extent (Dietrich and Hoeger 2005; Meriluoto *et al.* 1990; Pace *et al.* 1990; Robinson *et al.* 1989; Robinson *et al.* 1991; Stotts *et al.* 1997).

Since microcystins are charged and spatially large molecules they cannot cross cell membranes via simple diffusion, thus cellular uptake is most likely mediated by energy dependent transporters. Indeed, Runnegar (1995) assumed sinusoidal, bile–salt, organic–anion transporters, including members of the superfamily of organic anion transporting polypeptides (human: OATP; rodent: oatp), are the unlikely transporting route for mediating the uptake of microcystins into hepatocytes, because rifamycin inhibited the MC uptake in hepatocytes (Runnegar *et al.* 1995), whereas rifampicin, a member of the same antibiotic family as rifamycin, did not inhibit the transport capacity of the investigated oatp (Jacquemin *et al.* 1994; Jacquemin *et al.* 1991). However, more detailed investigation by Fischer et al. (2005) demonstrated the uptake of [^{3}H]–dihydro–microcystin–LR in *Xenopus laevis* oocytes via human OATP1B1 and OATP1B3, both located at the basolateral (sinusoidal) membrane of hepatocytes, as well as OATP1A2, located in liver and brain, possibly responsible for the observed hepatotoxicity and neurotoxicity, respectively. Transport could be inhibited by co–incubation with the known OATP/oatp substrates taurocholate and bromosulfophthalein.

OATPs/oatps could be detected in nearly all tissues of humans and rodents (Hagenbuch and Meier 2003; Meier and Stieger 2002; van Montfoort *et al.* 2003) and therefore might be responsible for the observed microcystin mediated effects in the various organs of the respective species. Whether only OATPs/oatps mediate the transport of microcystins is presently unknown. However, this appears rather unlikely as not every organ is observed to express OATPs, at least according to current knowledge. Generally OATPs are responsible for importing substrates into cells, although bidirectional transport was also observed in some cases. Multidrug Resistance Proteins (MDRs) and Multidrug Resistance associated Proteins (MRPs), both members of the ATP Binding Cassette superfamily (ABC transporters), are other potential mediators for the export of microcystins for the following reasons:

- MCs fit into the molecular weight spectrum of MDRs and MRPs substrates (table 1)

- The uptake of MCs was shown to be inhibited after protein phosphatase inhibition, suggesting that at least some of the responsible carriers are controlled via phosphorylation (Runnegar *et al.* 1995)
- Most of the MRPs are known to transport glutathione conjugates and MCs were demonstrated to be conjugated with glutathione (Pflugmacher *et al.* 1998)
- Glutathione conjugated microcystins were observed in the bile of microcystin exposed animals (Sahin et al. 1994)
- MRD and MRP are expressed at the apical side (hepatocyte/bile membrane) of hepatocytes as well as the basolateral side of bile cells (Hagenbuch *et al.* 2002; Hagenbuch and Meier 2003, 2004; van Montfoort *et al.* 2003).

The assumption that these ABC transporters and OATPs (Ballatori *et al.* 2005; Faber *et al.* 2003; Hagenbuch and Meier 2003; Ho and Kim 2005; Kunta and Sinko 2004; Meier and Stieger 2002; Miyazaki *et al.* 2004; Nies *et al.* 2004; Shitara *et al.* 2005; van Montfoort *et al.* 2003) are the responsible carriers for MCs would potentially explain some of the organ distribution characteristics of MC–LR after oral intoxication. However, none of these investigations was carried out using other MC congeners. Indeed, *in vivo* evidence from Milutinovic et al. (2002; 2003) strongly suggests that transport of microcystins via ABC transporters and OATPs is largely congener dependent, and thus, results in a congener dependent organ distribution and distribution–mediated intensities of adverse effects. Further characterization of the transport of microcystins via OATPs, as well as other potential transport systems, is of prime importance for improving better understanding of the species– and congener–specific kinetics and thus for improved risk extrapolation and human risk assessment.

Based on the current state of the science, the risk assessment of microcystins is hampered by the following inadequacies as summarized below:

- >80 different microcystins congeners are known to date and most likely more will be discovered in the near future. However, the risk assessment calculations are based on the toxicokinetic and -dynamic property of one single MC congener, namely MC–LR
- Multiple MC congeners can occur simultaneously in a cyanobacterial bloom, emphasizing the uncertainty in estimating true toxic potency in a bloom situation
- MC–congeners display distinct differences in organ distribution and location of toxic effects (Gupta *et al.* 2003; Milutinovic *et al.* 2002;

Milutinovic *et al.* 2003) strongly suggesting that these differences are governed primarily by the affinities of the respective microcystin congeners for the transporting proteins present in the different organs, i.e. also on their expression levels ((Fischer *et al.* 2005), see above)

- The comparison of different species (rodents versus humans) strongly suggests that microcystin transporting proteins are neither identical nor comparable amongst species; neither in their level of expression and distribution amongst organs of a given species nor in their respective microcystin transporting capabilities. This strongly indicates that risk extrapolation from rodents to the humans, despite the inclusion of a safety factor of 10 for inter–species extrapolation (Dietrich and Hoeger 2005), may underestimate the potential risk posed by the different toxins to humans.
- Additive, synergistic or antagonistic effects of the various microcystin congeners have so far not been investigated.
- Current risk assessment schemes, although incorporating allocation factors for contribution to total toxin exposure from sources other than the main source (drinking water), fail to consider route and matrix differences in bioavailability that may exist.

In order to understand the toxicities, bioavailabilities and toxicokinetics and –dynamics of single toxins and extracts, additional studies are needed and are essential for an improved risk assessment.

Obviously, the above uncertainties in the risk assessment of microcystins also emphasize the problems of establishing highly reliable guidance values for drinking water, inhalation exposure, or voluntary exposure via consumption of BGAS. Moreover, similar risk calculations for other toxic compounds e.g. cylindrospermopsin (CYN), anatoxins (ANA) saxitoxins (STX) and β–methylamino–L–alanine (BMAA), as well as the ubiquitously present lipopolysaccharides (LPS) are still under development but also fraught with the problems described for microcystins. Indeed, with the exception of BMAA (see below), a good understanding of the uptake and distribution kinetics of the other cyanobacterial toxins is nearly completely lacking to date. Thus, all calculations in the risk assessment process would have to be based on physico–chemical characteristics of the toxins.

Anatoxin–a, homoanatoxin–a, anatoxin–a(S), saxitoxins and cylindrospermopsin are very small molecules, with molecular weights of 165, 179, 252, 299 and 415, respectively, and thus are assumed to be able to cross the cell membranes via diffusion or facilitated diffusion. However, to date

there is no proof for these assumptions, emphasizing again the dearth of information available for risk assessment purposes.

BMAA (β–N–methylamino–L–alanine), a cyanobacterial, neurotoxic, nonprotein amino acid, acts as an agonist of animal glutamate receptors (Brenner *et al.* 2003) and is chemically related to excitant amino acids. Its uptake into brain is most likely mediated by the large neutral amino acid carrier of the blood–brain barrier (Smith and Shine 1992). BMAA has been associated with the significantly increased incidences of amyotrophic lateral sclerosis/parkinsonism–dementia complex (ALS/PDC) among the Chamorro people in Guam and other islands of the Pacific. BMAA, originating from cyanobacterial cycad root symbionts of the genus Nostoc, was demonstrated to bioaccumulate at various trophic levels including in some of the traditional foods of these inhabitants of these islands (Cox *et al.* 2003). However, recently Cox et al. (2005) revealed that BMAA production by cyanobacteria appeared most likely to be a general phenomenon, as BMAA was detected in 95% of 21 cyanobacterial genera. Indeed, most recent GC–MS/MS analyses of *Aph. flos–aquae* and *Spirulina spec.* containing BGAS demonstrated large quantities of BMAA (Dietrich unpublished data). If BMAA is indeed involved in the aetiology of ALS/PDC, the findings of BMAA in BGAS may potentially explain the detection of BMAA in brain tissues of Canadians with Alzheimer's disease (Cox 2005). Although it would have to be established whether or not these patients had consumed BGAS at any given time. The presence of BMAA, although not highly toxic in an acute scenario, profusely demonstrates that indeed, cyanobacterial bloom toxicity is NOT a single toxin scenario but most likely a combinatorial toxicity of many toxins present. Consequently, risk assessments must take these issues into consideration, even though to do so may prove extremely difficult.

Extract toxicity and synergisms

As a consequence of the limited knowledge on cyanotoxins other than MCs and BMAA, e.g. CYN, ANA, etc., the additional factors influencing potential risk i.e. antagonistic, additive or synergistic toxicity of simultaneously occurring cyanobacterial toxins has largely been ignored. Although antagonistic effects have so far not been reported they have also not been subject of specific toxicological studies. Additive or synergistic toxicity has been suggested by a number of observations. Indeed, cyanobacterial extracts have shown much greater toxicity then what would have been expected from the amounts of toxins (e.g. MCs) contained in the respective samples.

The following examples may serve as an illustration the potential presence of additive or synergistic toxicity of extracts: Majsterek et al. (2004) reported on the increased toxicity of a MC–LR containing extract compared to a microcystin–LR standard employing a cytochrome c oxidase assay using mammalian mitochondria from *Bos taurus*. The extract inhibited the activity of mitochondrial oxidase to a much higher extent than the same concentration of purified MC–LR standard. These authors assumed that the extract contained additional congeners of MC not detected with their means of chemical analysis.

Another potential explanation for the increased extract toxicity was provided by Best et al. (2002), who investigated the effect of lipopolysaccharides (LPS) from axenic cyanobacteria and from natural blooms on the activity of microsomal (m) and soluble (s) glutathione *S*–transferase (GST) of zebra fish embryos (*Danio rerio*) *in vivo*. Both activities were significantly reduced by cyanobacterial LPS, as well as by co–exposure to cyanobacterial LPS and MC–LR. Hence, they concluded that LPS may potentially cause inhibition of the GST catalyzed conjugation of MCs to glutathione, which represents the first step in detoxification of MCs and therefore may prolong residence time of MCs in the zebrafish resulting in a much higher toxicity and what was expected from pure MC–LR.

Fitzgeorge et al. (1994) compared the toxicities of anatoxin–a and MC–LR with the toxicity of both toxins administered simultaneously. An intranasal LD_{50} of 2000 µg kg^{-1} bw for anatoxin–a and a non–lethal dose of 31.3 µg kg^{-1} bw for MC–LR were determined in mice. Upon administration of 31.3 µg kg^{-1} bw MC–LR 30 minutes prior to anatoxin–a, the LD_{50} for anatoxin–a was lowered to 500 µg kg^{-1} bw. This potential synergistic effect of anatoxin–a and MC–LR was further investigated by Rogers et al. (2005) using oral toxin administration. Mice were gavaged with either 0, 500 or 1000 µg microcystin–LR kg^{-1} bw, followed by 0, 500, 1000 or 2500 µg anatoxin–a kg^{-1} bw 50 minutes later. Despite the high concentrations used, no deaths, no clinical signs of intoxication and no differences in weight between pre– and post–treatment were reported. It was concluded that the failure in demonstrating the synergistic effect was most likely due to the different routes toxin administration. Indeed, intranasal application of toxins resulted in similar LD_{50}s as did i.p. application, while the oral LD_{50} of MC–LR in mice was approximately 12–fold higher than the i.p. or intranasal administration (Fitzgeorge *et al.* 1994). These findings, although yet uncorroborated in a more wide assessment of toxin interactions, suggest that the route of exposure as well as the toxin composition may be critical for the onset of adverse effects. In view of the vast differences of toxin transporter presence and level of expression in the different organs,

as is the case for MCs and BMAA, it is not surprising to see route of administration dependent differences in LD_{50}s as well as differences in the potential for additive or synergistic effects. Consequently, extrapolation from various routes of exposure as a means for an overall risk assessment may be quite problematic.

Conclusions

The present analysis of the data on cyanobacterial toxins and their use within the context of risk assessment demonstrates that the dearth of information precludes development of simple safety assumptions. Although the WHO drinking water guidance value for MCs may provide a little more certainty due to the physiologically limited quantity of drinking water per person per day, the simultaneous presence of other toxins in the drinking water questions the reliability of this guidance value as it is expressively specified for only one (MC–LR) of the > 80 presently known toxin congeners and does not take other congeners or other toxins types into consideration. The provisional guidance value for BGAS as proposed by the Oregon Department of Health of 1µg MC–$LR_{equiv.}$ g^{-1} dw BGAS, when considering the potential for additive or synergistic effects stemming from the presence of other cyanobacterial toxins in bloom situations in Klamath Lake, as well as the nearly unlimited voluntary daily uptake of these compounds by the public and especially children, is even more problematic. The latter situation appears even more of a concern because recent analyses confirmed the presence of BMAA and microcystins in these BGAS products, thus clearly emphasizing the high potential for the onset of hepatic, renal and neurological disorders in these children, whether or not this may be of the subacute/subchronic (MCs), or the delayed (MCs and BMAA) type. While sale of BGAS should be severely controlled or even restricted, authorities and academia should be supported in developing additional data pertaining to the inhalation and the food contamination exposure scenarios.

Table 1. Calculated possible daily ingestion to avoid acute health problems according to the calculations of Fromme *et al.* (2000). For details, see Dietrich et al (2005)

Ingestion route	MC concentrations	Infants 5 kg = 12.5µg	Children 20 kg = 50 µg	Adults 60 kg = 150 µg
Food	100 µg kg^{-1}	125 g	500 g	1,500 g
	10000 µg kg^{-1}	1.25 g	5 g	15 g
Cyanobacterial bloom in lake/river	100 µg l^{-1}	125 ml	500 ml	1,500 ml
	1000 µg l^{-1}	12.5 ml	50 ml	150 ml
Drinking water	1.0 µg l^{-1}	12,500 ml	50,000 ml	150,000 ml
	100 µg l^{-1}	125 ml	500 ml	1,500 ml
BGAS	1.0 µg g^{-1}	12.5 g	50 g	150 g
	10 µg g^{-1}	1.25 g	5 g	15 g

Table 2. Known and possible transporters of microcystins and their known distribution in human and rodent organ system. oatp: organic anion transporting polypeptide; mdr: multidrug resistance proteins; mrp: multidrug resistance associated Proteins, human transporters are generally written in upper case, rodent transporters in lower case.

Organs	Transporter	Human	Rat/ Mouse	References
Gastro-Intestinal Tract	OATPs/Oatps	2A1, (2B1), 3A1, 4A1	1a5	(Cheng et al. 2005; Hagenbuch and Dawson 2004; Ho and Kim 2005)
	MDR/Mdr	1	1a, 1b	(Faber et al. 2003; Ho and Kim 2005)
	MRP/Mrp	1–3, 5, 7–9	1–3	(Faber et al. 2003; Glavinas et al. 2004; Ho and Kim 2005)
Liver	OATPs/Oatps	1A2*, 1B1*, 1B3*, 2A1, (2B1), 3A1, 4A1	(1a1), (1a4), 1b2*, 2b1	(Cheng et al. 2005; Hagenbuch and Dawson 2004)
	MDR/Mdr	1, 2/3	1b, 1a, 2	(Faber et al. 2003; Glavinas et al. 2004; Ho and Kim 2005; van Montfoort et al. 2003)
	MRP/Mrp	1–3, 5–9	1–3, 6	(Faber et al. 2003; Glavinas et al. 2004; Ho and Kim 2005; van Montfoort et al. 2003)

Organs	Transporter	Human	Rat/ Mouse	References
Kidney	OATPs/Oatps	1A2*, 2A1, 3A1, 4A1, 4C1	(1a1), 1a3, 1a6, 3a1, 4c1	(Cheng et al. 2005; Hagenbuch and Dawson 2004)
	MDR/Mdr	1	1a, 1b	(Glavinas et al. 2004; Ho and Kim 2005; van Montfoort et al. 2003)
	MRP/Mrp	1–9	2	(Glavinas et al. 2004; Ho and Kim 2005; Robertson and Rankin 2006; van Montfoort et al. 2003)
Blood–Brain	OATPs/Oatps	1A2*, 1C1, 2A1, 3A1, 4A1	(1a4), 1c1	(Cheng et al. 2005; Hagenbuch and Dawson 2004)
	MDR/Mdr	1	1a	(Glavinas et al. 2004; Hagenbuch et al. 2002; Ho and Kim 2005)
	MRP/Mrp	1, 5, 7–9	2	(Glavinas et al. 2004; Hagenbuch and Dawson 2004; Hagenbuch et al. 2002; Ho and Kim 2005)

* dihydro – MC–LR transport demonstrated by Fischer et al (Fischer *et al.* 2005)

() no dihydro – MC–LR transport

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