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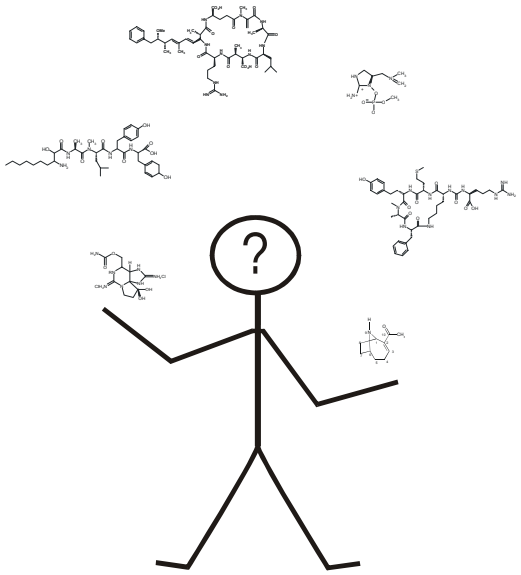
Problems during drinking water treatment of cyanobacterial-loaded surface waters: Consequences for human health



Dissertation an der Universität Konstanz

Gefördert durch die Deutsche Bundesstiftung Umwelt (DBU)

Problems during drinking water treatment of cyanobacterial-loaded surface waters: Consequences for human health

Dissertation

Zur Erlangung des akademischen Grades des Doktors der Naturwissenschaften an der Universität Konstanz
Fakultät für Biologie

Vorgelegt von
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Tag der mündlichen Prüfung: 16.07.2003
Referent: Prof. Dr. Daniel Dietrich
Referent: Dr. Eric von Elert

Quod si deficiant vires, audacia certe laus erit: in magnis et voluisse sat est.
(Sextus Propertius: *Elegiae* 2, 10, 5 f.)

PUBLICATIONS AND PRESENTATIONS

Published articles

Hitzfeld BC, Hoeger SJ, Dietrich DR**.** (2000). Cyanobacterial Toxins: Removal during drinking water treatment, and human risk assessment. Environmental Health Perspectives 108 Suppl 1:113-122.

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Hoeger SJ, Shaw GR, Hitzfeld BC, Dietrich DR (2003). Occurrence and elimination of cyanobacterial toxins in Australian drinking water treatment plants, in preparation.

Hoeger SJ, Hitzfeld BC, Dietrich DR**.** (2003). Occurrence and elimination of cyanobacterial toxins in Swiss and German drinking water treatment plants, in preparation.

Hoeger SJ, Hitzfeld BC, Dietrich DR**.** (2003) Chemical and toxicological characterisation of microcystin by-products after ozonation, in preparation.

Published abstracts, oral or poster presentations

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Hoeger SJ, DR Dietrich, BC Hitzfeld (2000). Microcystin-LR ozonation by-products: chemical and toxicological characterization, 39th Annual Meeting of the Society of Toxicology (San Francisco, USA); Toxicological Sciences **54**:330.

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Promotionsförderpreis der Universitätsgesellschaft Konstanz E.V. (20/10/2001).

Cooperations

Participant of an international intercomparison exercise:

Fastner J, Codd GA, Metcalf JS, Woitke P, Wiedner C, Utkilen H. 2002. An international intercomparison exercise for the determination of purified microcystin-LR and microcystins in cyanobacterial field material. Analytical and Bioanalytical Chemistry 374:437-44.

Participant of the DIN/ISO Microcystin working group (coordination: Prof. Dr. Bernd Luckas, University of Jena)

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ABBREVIATIONS

Adda	(2S, 3S, 8S, 9S)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid
ANA	anatoxin-a
ATP	adenosine triphosphate
BGAS	blue-green algae food supplements
COX	cyclooxygenase
CYL	cylindrospermopsin
Cys	cysteine
DAF	dissolved air filtration
DMBA	7,12-dimethylbenzanthracene
D-MeAsp	D-*erythro*-ß-methylaspartic acid
DW	dry weight
FAB-MS	fast atom bombardment-mass spectrometry
GAC	granular activated carbon
GST-P	glutathione-S-transferase placental form
GTX	gonyautoxin
HbsAG	hepatitis B antigen
HBV	hepatitis B virus
HPLC	high performance liquid chromatography
i.p.	intra-peritoneal
i.v.	intra-venous
LD	lethal dose
LPS	lipopolysaccharide
LOAEL	lowest observable adverse effect level
MAP	mitogen-activated protein
MC-AR	microcystin-AR (alanine and arginine)
MC-FA	microcystin-AR (phenylalanine and alanine)
MC-LR	microcystin-LR (leucine and arginine)
MC-YA	microcystin-YA (tyrosine and alanine)
Mdha	*N*-methyldehydroalanine
Mdhb	2-(methylamino)-2-dehydrobutyric acid
MF	microfiltration
MQ-H_2O	MilliQ-H_2O
ND	not determined
NOAEL	no-observable adverse effect level
NOD	nodularin
OATP	organic anion transporter protein
PAC	powdered activated carbon
PAF	platelet activating factor
PCC	Pasteur culture collection
PCR	polymerase chain reaction
PG	prostaglandin
PL	phospholipids
PLA_2	phospholipase A_2
PLC	primary liver cancer
PP	protein phosphatase
PPI	protein phosphatase inhibition
PSP	paralytic shellfish poison (saxitoxins)
TDI	tolerable daily intake
TNF-α	tumour necrosis factor-α
TX	thromboxane
UF	ultrafiltration
UV	ultraviolet
WHO	World Health Organisation
WW	wet weight

GENERAL INTRODUCTION

Parts of this introduction as well as additional information have been previously published in the review "CYANOBACTERIAL TOXINS: REMOVAL DURING DRINKING WATER TREATMENT, AND HUMAN RISK ASSESSMENT". The aim of this general introduction is to update the review (following in chapter III) to the state of the knowledge in 2003.

CYANOBACTERIA - ECOLOGICAL FACTS

The systematics, structure and elementary physiological findings concerning cyanobacteria are not part of this work. However, cyanobacterial blooms ("water blooms") and the reasons for this phenomenon are important for water supplies, for recreational purpose and other fields, which directly or indirectly influence human health.

Four potentially toxic genera (*Anabaena*, *Aphanizomenon, Microcystis, Planktothrix)* are known for their ability to form massive blooms near the water surface, whereby incredible cyanobacterial cell densities can occur. In 2001 over 900 tonnes of *M. aeruginosa* were removed from the Swan-Canning system, Australia. In this scum up to 1.3 x 10^8 cells/mL were counted (*5*). In South-Africa Zohary *et al.* (*6*) mentioned hyperscums of *M. aeruginosa*, with cell densities which exceeded 10^9 cells/mL. As an exception, *P. rubescens* forms blooms in deeper layers of water bodies within or near the thermocline (further information later in the text).

Water blooms are defined as an accumulation of a population of buoyant planktonic cyanobacteria at the water surface previously distributed through the water column (*7*). According to Vollenweider (*8*) algal (cyanobacterial) bloom means an algal population of such density as to render it visible to the human eye. This means abundant quantities which generally consist of over 1 x 10^6 cells/L, largely of one species. Cyanobacterial blooms are also seen as the direct undesirable consequence caused by the accumulation of floating cyanobacteria (*9*). They depend on the coincidence of three preconditions: a pre-existing cyanobacterial population, a significant proportion of which have positive buoyancy and the absence of, or weak water mixing, which overcomes the tendency of the cells to float (*10*). These three preconditions can furthermore be influenced by nitrogen, phosphorus, light and micronutrients (e.g. iron, molybdenum) in the water, pH, temperature and

morphology of the water body and the prevailing hydrologic and meteorological conditions. This list shows that the often discussed reduction of the external loadings with nutrients alone does not guarantee the restoration of "non-cyanobacterial" plankton. The reasons for cyanobacterial dominance are not altogether straightforward and the comparative merits of the various explanations proffered continue to be controversially and inconclusively debated (*11*). Belov *et al.* (*12*) developed a semi-empirical mathematical model for water-column toxicity, which proposes a plausible description of the seasonal development of cyanobacteria populations and of their toxicity within the water body.

But what are the reasons for bloom development? Cyanobacteria are unique among microplanctic photoautotrophs because they possess gas vesicles, which provide buoyancy and thus the possibility for vertical migration and access to spatially separated resources (*13*). To avoid potential damage of pigments and the photosynthetic machinery at the water surface, cyanobacteria have to escape high irradiation intensity at the water surface (*14, 15*). Changes in light and/or nutrients are followed by a generation of osmotically active photosynthates and potassium cation. Thus, turgor pressure is strong enough to collapse the gas vesicles (*16-18*). In addition, carbohydrates accumulate during light exposure and act as ballast (*19, 20*). According to Stokes's law, the sinking rate is dependent on the difference in the density between the water and the cells, and on the square of the colony size. Furthermore, gas vesicle synthesis relative to growth is molecularly regulated (*21*). In deeper water layers previously synthesised carbohydrates can be used by the cyanobacteria to synthesise gas vesicles (*20*). The described mechanisms guarantee the best position in the water column with respect to light and nutrients for these cyanobacteria. But what happens in the lake during bloom formation? As a result of repeated mixing within the water column (e.g. strong wind during the day) or during low light periods (e.g. at night) (*22*), cyanobacteria synthesise gas vesicles via carbohydrate metabolism, because they are outside the euphotic zone (positive buoyancy). After stabilization of the weather or at sunrise, the cyanobacterial cells/colonies ascend and accumulate at the water surface. But, due to insufficient CO_2 the cyanobacteria cannot start the mechanisms to distribute themselves lower in the water column to avoid high irradiation (*23*). Additionally, surface scums may be blown together by wind. The high radiation at the surface and/or heat injury causes

lysis of cyanobacterial cells, blooms break down and toxins are set free into the water (*24*).

In contrast to such surface blooms is the behaviour of *P. rubescens*. This species is stratified in the metalimnion of oligotrophic and mesotrophic lakes. High densities of more than 1000 trichomes/mL can occur in seasons of low wind and stable stratification (*25*). Though this phenomenon is also called "bloom", the only parallel to the event described above is the concentration of cyanobacterial cells in distinct layers. Due to their photosynthesis apparatus including phycoerythrin and carotenoids, these cyanobacteria can use the light in deeper layers of the lake and avoid high irradiation at the surface. Their buoyancy equipment is very useful for changing depths within a deep lake without collapse of gas vesicles (*26*). However, surface blooms of *P. rubescens* have been described (see the description of the Burgunderblutalge in the following section). Sub-populations within the species *P. rubescens* seem to coexist in the same lake. The changes of populations in Lake Zurich are largely determined by interactions of light and depth distribution, whereas decreases in nutrient-loading have had little impact (*27*). What could be the reason for lysis/breakdown of these metalimnic "blooms"? After complete mixing of the lake, the layers of the lake are disturbed and no further stratification is possible. The highly concentrated *P. rubescens* population is distributed over the whole water column. However, occasionally a metalimnic bloom disappears within days, although the climatic conditions have not changed. Viruses may play an important role in this process (*28*) and may be one possible explanation for a breakdown of cyanobacterial blooms (*29*). *Bdellovibrio*-like bacteria have also often been considered as one of the causes of sudden disappearance of cyanobacterial blooms (*30*).

But how can cyanobacteria reach such high cell numbers and dominate the phytoplankton of a surface water over long periods? An explanation for the dominance of cyanobacteria in eutrophic lakes (and in case of *P. rubescens* in oligotrophic lakes) is that they are rarely used as food by zooplankton and even when ingested, they are poorly utilized. The biochemical properties of the different species and the shape and size of the cyanobacteria seem to be the most important factors concerning their suitability as food (*31-37*). Although cyanobacterial toxins are dealt with in detail later in the introduction, a short introduction seems appropriate to complete this ecological chapter.

Cyanobacterial toxins such as the microcystins may play a role as messengers or even as biological weapons (*38-40*) and may thus be a means of attaining the dominance in an aquatic system. This leads to the question which other functions the toxins produced by cyanobacteria could have.

This is another point of controversial debate and the arguments for and against the different hypotheses are discussed elsewhere in detail (*41*). In short, possible functions of MCs are for light protection and as nitrogen-storage facility. That it should be a waste compound is unlikely, as the molecule is quite "expensive" to produce and is synthesised in an extremely complex way (see also chapter III). As mentioned above, it has been suggested that these secondary metabolites may help to ward off encroachments upon their habitat by other microorganisms, thus gaining a competitive advantage. As active export is necessary for a possible function as protection against grazers, the discovery of a gene coding for an ABC transporter (mcyH) within the microcystin gene clusters (mcyA - mcyJ) makes this theory more likely (*42*). Another interesting hypothesis is the role of cyanobacterial secondary metabolites as intra-species messengers. This would allow for communication within a cyanobacterial bloom or colonies, concerning strategies for the fight against competitors for light and nutrients. Recent investigations into the influence of light on the concentration of microcystins within and outside of the cells have yielded evidence of a possible function in communication between cyanobacterial cells (*43, 44*). The ABC transporter offers another potential role. Microcystins could act as a siderophore to capture Fe^{2+} (or other essential metals) and transport this (these) into the cells (*45*). However, an intracellular binding of metal-ions may be negative for the cell due to competition with primary metabolites for the essential metals. A function as protector in case of high intracellular metal concentrations is also a possibility (*46*), but the localisation of most MCs in the thylakoid and the nucleoid regions (*47*) contradict this theory.

A lot of work has been carried out to investigate possible factors influencing the toxin concentration in the cyanobacterial cell. These parameters have, however, not been conclusively elucidated (*48*). Although they can be considered as being closely involved in bloom formation, cell numbers and toxin levels do not usually correlate. Furthermore, few generalisations can be made from the laboratory studies that have been conducted to date (*49-53*). For example a relationship between the MC concentration and the nitrate and phosphorous concentrations in water has been

shown by many researchers (*50, 54-57*). A more detailed discussion on this topic has been attempted in chapters III and VII and also by Sivonen and Jones (*58*).
Last but not least, in addition to their talent in toxin synthesis, cyanobacteria posses the remarkable ability to synthesise ethanol by bioconversion of solar energy and CO_2 (*59*).

CYANOBACTERIAL TOXINS

History

Intoxications of animals or humans with cyanobacterial toxins have been reported for centuries. In fact, perhaps the oldest observation and report of a possible bloom of red *Planktothrix (Oscillatoria) spp*. occurred in the river Nile several millennia ago: "... all the waters that were in the river were turned to blood. And the fish that were in the river died; and the river stank, and the Egyptians could not drink of the water of the river" (Exodus 7: 20—21). Nowadays, in Egypt researchers have observed toxic *Oscillatoria (Planktothrix) agardhii* in irrigation channels (*60*) and toxic *Oscillatoria tenuis* in River Nile itself, which is still used as drinking water source and of course, for fishing (*61*). Closer to southern Germany, there are a few anecdotal reports about blooms of a red *Planktothrix* species in Swiss lakes after the Burgundian wars (*62*). The species *P. rubescens*, which still occurs in many lakes in Switzerland, was and is still called “Burgunderblutalge” (blood of Burgundians - algae). People from the region around lake Murten/Switzerland thought, that the phenomenon of this *Planktothrix*-bloom returning each spring was the blood of the Burgundians, who were killed on the shores of lake Murten or at least represented a memorial for them (*63*). To my knowledge, even the first scientific description of *P. rubescens* goes back to this lake (*64*).
In 1933, further north cattle were poisoned by blue-green algae in the Finnish Lake Vesijärvi (*65*), in which toxic *O. agardhii* can still be found (*66*). One century earlier, in 1833, J.C. Hald, secretary of the “Farm Household Society” by order of the Danish king, described a local water body in the following way: “...attaining a green colour during calm weather in summer and the water is harmful to cattle. If, for example, cattle stampede and then drink the water, they collapse immediately.....The lake contains many fish, such as perch, pike, roach eel, silver bream and a few bream, some of which drift dying or dead to the land when the lake is in the above condition. Such fish are not eaten as they are considered harmful.”(*67*). Gerald of Wales wrote in

his book The Journey Through Wales (AD 1189) about Llangorse Lake: "As I told you, it sometimes turns bright green, and in our days it has been known to become scarlet"(*12*). Early reports of animal intoxication also exist in other climatic zones. As well as the well known report of Francis from a *Nodularia* bloom in Australia (*68*), Codd (*69*) described a few observations from the 19th century about cyanobacterial blooms in Lake Alexandria and the lower river Murray (Australia) as "perceptive and prescient". To return to our journey, a description of an algal scum in the River Dnepr in AD 77 from the Roman scholar Pliny the elder is mentioned in the same article (*69*). All these reports supply evidence, that toxic cyanobacterial blooms have accompanied mankind for many centuries and are no recent development.

Toxicology

"There are no toxic materials, there are only toxic doses." Every toxicologist or even every student in a scientific subject knows this sentence from Paracelsus, one of the pioneers of toxicological science, who lived about 400 years ago. The molecules this work will discuss are considered to be secondary metabolites, which implies that they are "apparently not absolutely essential to the life and growth of the producing organism" (*70*).

In addition to these following toxic compounds, other secondary metabolites from cyanobacteria have also been associated with hormonal, antineoplastic, antimicrobial (*71, 72*), antibacterial (*73*), antifungal (*74*) and antiviral effects (*75-77*).

CYCLIC PEPTIDES

Only two "families" of cyclic peptides, synthesised by cyanobacteria are in the focus of this introduction, namely the nodularins and microcystins, the other known cyclic polypeptides (cyanopeptolines, anabaenopeptines) are less investigated but seems to be less toxic.

TOXIN SYNTHESIS

The molecular synthesis of microcystins and nodularins is similar (*78, 79*). They are synthesised non-ribosomally by a multi-enzyme complex, comprised of polyketide synthases and polypeptide synthetases. These huge enzymes (200-2000 kDa) are composed of modules. The modules (mcyA – mcyJ) are coded in one gene-cluster with ten open reading frames. Each is responsible for one synthesis step (*42*).

STRUCTURE

The molecular weight of MCs and NODs range from 800-1100 Da (*80*). The heptapeptides of the microcystin-family and the pentapeptide of the nodularin-group are cyclic molecules with the general structure cyclo-(D-alanine[1]-X[2]-D-MeAsp[3]-Z[4]-Adda[5]-D-glutamate[6]-Mdha[7]) and cyclo-(D-MeAsp[1]-L-arginine[2]-Adda[3]-D-glutamate[4]-Mdhb[5]), respectively, whereby X and Z are two variable L-amino acids, D-MeAsp is D-*erythro*-ß-methylaspartic acid, Mdha is *N*-methyldehydroalanine and Mdhb is 2-(methylamino)-2-dehydrobutyric acid. Adda is an unusual amino acid which seems to be unique to cyanobacterial toxins: (2S, 3S, 8S, 9S)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid. Sixty MC congeners were listed in 1999 by Sivonen and Jones (*58*), but since then, several new MCs have been identified and many more will probably be identified in the future. Because of the high variety of amino acids included in the scaffold of these molecules, the chemical properties are very diverse. The logarithmic $K_{o(ctanol)w(ater)}$ (estimated from molecular structure) of seven MC-congeners (*81*) range from –3.12 for MC-AR (for alanine and arginine as variable amino acids) to 1.628 for MC-FA (phenylalanine and alanine). More details concerning the structure are given in chapter III.

EXPOSURE ROUTE, TRANSPORT AND DISTRIBUTION OF MICROCYSTINS IN HUMANS AND ANIMALS

In humans microcystins and every other toxin produced by cyanobacteria are adsorbed mostly via the oral route. Drinking water, contaminated food, algal supplements or accidental ingestion during recreation can be routes of uptake for cyanobacterial toxins. Dermal contact causes skin irritations such as allergic reactions (*82*), but, due to the hydrophilic character of most microcystins, anatoxins, cylindrospermopsins, and saxitoxins (PSPs), an uptake via the skin is unlikely. Another possible route of uptake is the nasal mucosa. For microcystins the LD_{50} values of intranasal/intratracheal administration into mice are similar to those of the i.p. route (*83, 84*). During recreational activities in cyanobacterial contaminated water bodies toxins might flow into the respiratory tract. MC-LR is 30-100 times less toxic, when administrated orally than intraperitoneally (*85*). The main exposure route is, however, via the stomach. Only a portion of the ingested cyanobacterial cells die during the passage. The percentage differs from species to species of ingested cyanobacteria as well as amongst the exposed organism (cows, sheep, humans etc.).

Microcystis is more stable against digestion due to the mucous layer around the cell colonies. *Aphanizomenon*, in contrast lacks such a protection. No reliable information is available concerning differences between the exposed organisms. As the dissolved toxins are stable against low pH and enzymatic degradation, they remain intact within the digestive tract. The enzymes in the digestive tract can crack peptide bonds, in which D-amino acids participate only very slowly if at all. The toxins are absorbed via the stomach (*86*) but mostly via bile acid transporters of intestinal cells (*86, 87*) into the portal vein. The condition of the capillaries in the small intestine was shown to affect the percentage of MC-uptake (*88*). Isolated intestinal enterocytes from chickens were deformed and even killed by exposure to MCs (*89*).

In summary, only a part of the free toxin passes this barrier. The majority remains in the intestinal tract and is excreted in faeces (*90*). The MCs reach the liver via the portal vein. After i.p., i.v. or intra-tracheal administration, roughly 50-70% has been shown to accumulate in the liver. Significant amounts can also be found in the kidney and intestine, but not in other organs (*91-93*). The first-pass effect and the active uptake into parenchymal liver cells via multi-specific bile acid transporters is responsible for this specific distribution (*91, 94, 95*). The specific transporters have not yet been described in detail. As a consequence of active transport, the toxicity of microcystins and nodularins is restricted to organs expressing a suitable transporter, such as the organic anion transporter in cell membranes. Using a *Xenopus* oocyte expression system, the widely expressed human organic anion transporting polypeptide (OATP) has been shown to be able to transport MCs (*96*). OATP is expressed in human brain, lung, liver, kidney and testis (*97*). Within the cytosol the MCs can interact with suitable molecules such as protein phosphatases.

TARGETS OF MICROCYSTIN

One significant mode by which toxicants can perturb cellular homeostasis is by modification of the phosphorylation-based signalling (*98*). Microcystins are very potent inhibitors of the ubiquitous serine/threonine protein phosphatases PP1, PP2a and PP3 to PP6 but not PP2B, PP2C and PP7 (*98, 99*). There are many potential targets (*100, 101*), which are controlled by reversible phosphorylation within the cell including ion channels, metabolic enzymes, controllers of the cell cycle (*102, 103*), cytoskeletal proteins and even processes such as memory formation (*104*). Reversible

phosphorylation acts analogous to a switch, that turns these biological processes on and off. It has been shown *in vitro* that MCs interact with these protein phosphatases at the molecular level (*105-107*). Specifically, the toxins bind covalently to cysteine-273 of PP1c and Cys-266 of PP2Ac (c means the catalytic subunits) via the N-methyl dehydroalanine (*108-111*). It has been shown that inhibition of the enzyme activity results from an initial non-covalent interaction, which is mediated by the hydrophobic Adda side chain of MCs and two potential hydrogen bonding sites (*112-114*). Nodularin and some of the MC congeners (*115*) lack the methyl dehydroalanine and thus they are most probably unable to form covalent complexes with the protein phosphatases (*111*). Nevertheless, these molecules are highly toxic and nodularins are furthermore known for tumour initiating activity (*116*). How can toxicity without the postulated prerequisite covalent binding to protein phosphatases take place? It has been demonstrated that nodularin and MC-YR (tyrosine, arginine and of course methyl dehydroalanine) differ in their actions when injected i.p. in mice. While MC-YR damages the membranes of lysosomes, nodularin induces the synthesis of hydrolytic enzymes resulting in a disturbance of cellular homeostasis through protein degradation. The effect of MC-YR could be a consequence of the inhibition of PP2A and PP2A is responsible for the activation of a transporter for lysosomal enzymes into the lysosomes. Therefore, the two toxins have the same consequence for the liver cell but through different pathways. Thus it is unlikely, that the covalent bond between the toxins and the PP is the only and most important factor for the observed toxicity, although a more than 10-fold reduction of MCs affinity occurs if covalent binding is abolished by mutation of Cys-273 to leucine, alanine or serine (*110, 117*). At this point a connection to the ecological role of toxins can be made: the protein phosphatases of some MC-producing cyanobacteria are resistant to MC-LR (*118*). It seems wise to prevent an inhibition of "self" protein phosphatases. However, the protein phosphatases from MC-producing genera *Anabaena* (*119*) and *Nostoc* (*120*) have been shown to be very similar to the serine/threonine phosphatases of eukaryotes and thus perhaps a target for MCs, but binding of MCs to these PPs has not been investigated. Interestingly, *Anabaena* and *Nostoc* are not known for producing MCs in remarkable amounts in contrast to *Microcystis* and *Planktothrix*.

The observed toxicity of MCs is certainly not only based on inhibition of protein phosphatases of the serine/threonine class. A wide spectrum of other effects have been reported in the literature. However, no clear connection can be drawn between

the many molecular changes after MC administration although a few of them can be coupled to the inhibition of PPs, because these enzymes are involved in almost every cascade in the cell. Nevertheless, the findings are outlined below to update the knowledge of today. A direct action of MCs on cell membranes has been reported (*121*) as well as the inhibition of protein synthesis followed by a mobilisation of glucose-6-phosphate (*122, 123*). Also, glutathione pools are depleted after MC administration, whereas serum glucose (*124*) and cytoplasmic calcium levels in hepatocytes are increased (*94*). After administration of MC serum sorbitol dehydrogenase (*124*) and serum lactate dehydrogenase increase (*125, 126*). It has been observed that oxidative stress is closely associated with the hepatotoxicity of cyanobacteria (*126*). In addition, rat hepatocytes treated with microcystin show mitochondrial damage, which seems to be related to cell injuries (*127*). With techniques such as proteomics, other targets of MCs can be detected. The ATP-synthase beta subunit was found to be another binding partner for MCs, which could be associated with the suggested apoptosis-inducing potential of MCs (*128*).
An overview of the known targets of MC and the consequences thereof is shown in Figure 1 adapted from Kaya *et al.* (*2*).

CONSEQUENCES

By inhibiting the ubiquitous and high basal activities of PP1 and PP2A the normally reversible phosphorylation of cytosolic, cytoskeletal and cytoskeletal-associated proteins increases, followed by a redistribution of these proteins. Toivola (*129, 130*) demonstrated the hyperphosphorylation of the intermediate filament proteins keratin 8 and 18 induced by MC-LR. In addition, increased phosphorylation of dynein heavy and intermediate-chains results in inhibition of dynein ATPase activity, and reduces motor-dependent avidity of endosomal/lysosomal membranes for microtubules (*131*). In general, the microtubule-based membrane vesicle transport is significantly inhibited (*132*). Gosh (*133*) showed that the collapse of cytoskeletal actin filaments occurs in rat hepatocytes prior to the dislocation of the associated proteins α-actinin and talin, rather than being caused by their dislocation. The cytoskeletal disruption might also cause alternation of the coupling of G-proteins and phospholipase C (*134*). This reduces the receptor-mediated and G-protein-mediated phosphoinositide turnover in rat hepatocytes. Nassem (*135*) reported increased prostaglandin formation in rat hepatocytes and thus proposed a release of

arachidonic acid by phosphatidylinositol metabolism. By inhibiting protein phosphatases, MCs activate phosphorylase A (*136*). This is probably one of the reasons for the observed depletion of glycogen in the liver (*124, 137*).

ACUTE AND SUBACUTE SYMPTOMS

The consequent protein phosphorylation imbalance causes disruption of the cytoskeleton, which leads to massive hepatic haemorrhage which is the cause of death (*138-140*). Death of the organism through haemorrhage and shock is rapid, occurring within about 3 hours in the case of mice. Pathological and ultrastructural features commonly observed in the liver are centrilobular hepatic necrosis, destruction of sinusoidal endothelium, disruption of bile canalicular function, loss of microvilli, bleb formation in hepatocytes, and hepatocyte necrosis (*88, 141-145*). Symptoms of sub-acute intoxication with MCs are diarrhoea, vomiting, piloerection, weakness and pallor (*146*).

One milligram of microcystin was estimated (*147*) to be necessary to completely saturate the protein phosphatases 1 and 2A of an adult female human liver (if the complex toxin-PP is stable). One has to drink two litres of water contaminated with 32 µg toxin/L per day for two weeks or has to eat 760 g mussels (WW) with 16 µg/g DW to reach this level (*148*). The concentrations to which the majority of people are exposed are a lot lower. Concentrations of 0.1 – 1.0 µg/L in drinking water (see chapters III, VI, VII) and up to 20 µg/d through dietary (*149*) supplements are regularly achieved. However, even then complete uptake of the toxin is unlikely. Thus a much higher concentration would have to be ingested. What percentage of the liver protein phosphatases must be inhibited to cause death of the organism? Is the inhibition of the protein phosphatases responsible for the acute effects after intoxication or do other interactions with fatal consequences exist? The answers to these questions have not been given yet.

TUMOUR PROMOTING ACTIVITY

The major health problems with microcystins are probably those associated with chronic intoxications with low MC concentrations.

A possible reason for the tumour promoting activity of MCs is the inhibition of PP2A, since this phosphatase is the negative regulator of several steps of the mitogen-activated protein kinases (MAP kinases) signalling pathway (*150*). As this pathway

regulates the transcriptional onset of several genes activated during cell proliferation (e.g. c-jun), chronic exposure to MCs may significantly affect cell proliferation. MAP kinase activation cannot only result in increased cell proliferation, but also in inhibition of apoptosis (*151*). Both are able to promote tumour formation. Furthermore, weakening of cell-cell contacts by disturbance of the cytoskeleton may result in the loss of contact-inhibition and thus increase proliferation.

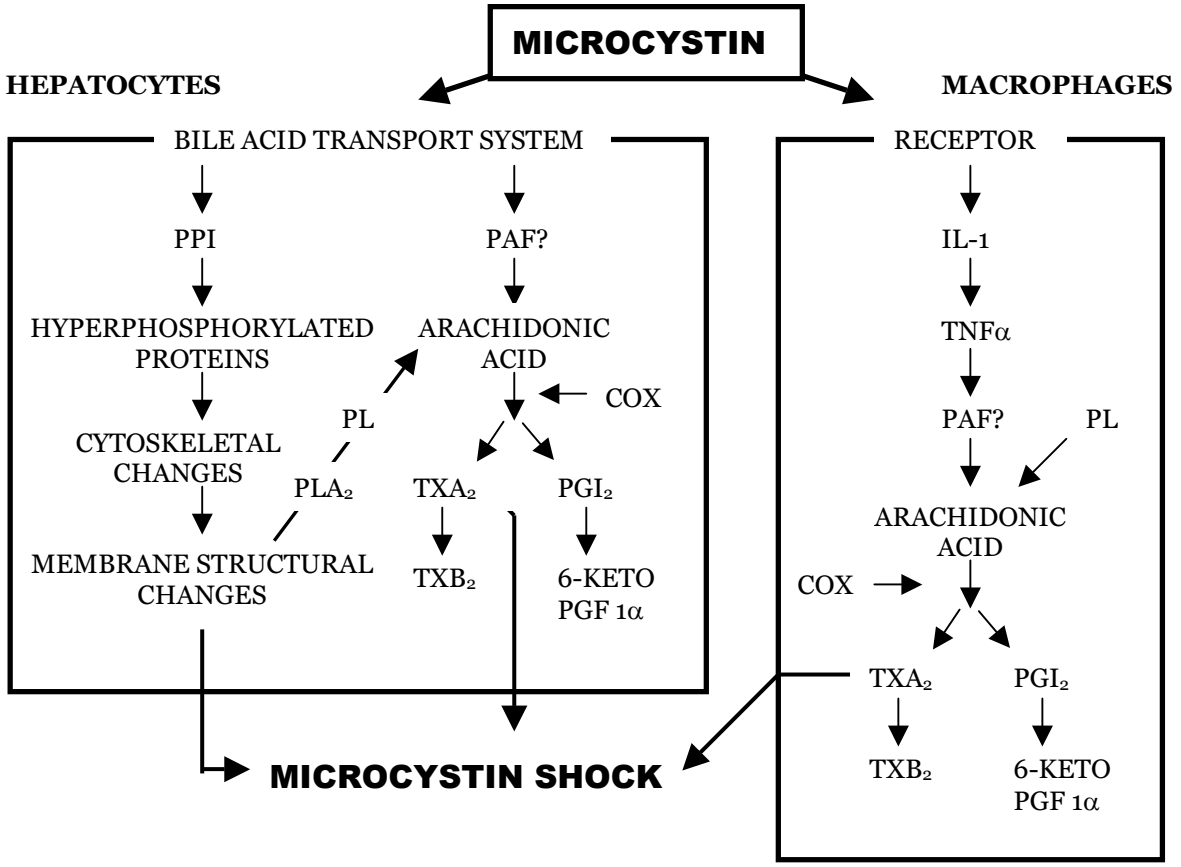

Figure 1. Toxic mechanism of microcystin by Kaya (2); PPI Protein phosphatase inhibition, PAF Platelet activating factor, COX Cyclooxygenase, PL Phospholipids, PLA_2 Phospholipase A_2, TX Thromboxane, PG Prostaglandin.

OTHER EFFECTS

The negative influences of cyanobacterial ingestion to the intestinal tract have already been described in the section "Exposure route, transport and distribution of microcystins".

Several authors have also mentioned MC-mediated renal toxicity in different cell systems (*152*) and *in vivo* in fish (*153-155*) and mammals (*142, 156-158*). A chronic study using low concentrations of MC-LR (10 μg/kg i.p.) in rats induced damage to

the kidney cortex and medulla (*158*). The authors concluded a considerable risk of kidney damage during chronic exposure to MCs. As in the liver (Figure 1), phospholipase A_2 and cyclooxygenase have been shown to be involved in the toxic effects of MCs in kidney (*157*).

No increase in foetal death or abnormalities could be observed after administration of MC-LR to pregnant mice (*85, 159*). However, in a preimplantation mouse embryo model Sepulveda *et al.* (*160*) demonstrated an inhibition of early embryo development in a dose–response relationship by a toxin purified from a *Microcystis*-bloom. An epidemiological study in Australia yielded no clear evidence for an association between cyanobacterial contamination of drinking water sources and adverse pregnancy outcomes in humans (*161*).

DETOXIFICATION

The phase II enzyme glutathione-S-transferase, uses the same binding site on MCs as the protein phosphatases to add MCs to glutathione (*162, 163*): The electrophilic α/β–unsaturated carbonyl of the methyl-alanine binds nucleophilically to the cysteine of the tripeptide glutathione. As already discussed, not every MC and no NOD (*105, 111*) contains this binding site. Thus, these Mdha-free molecules are not expected to bind protein phosphatases and glutathione in the same way as “normal” MCs. Strong evidence for the importance of glutathione in detoxification has been shown through a complete protection of mice from MC-lethality after pretreatment with glutathione (*125*). Administration of glutathione conjugate and a MC-cysteine was 12 times less toxic than MC-LR alone. An explanation for this could be that the transport system into the liver does not function well with the conjugates of the toxins or they are fast and effectively detoxicated by an appropriate system (*164*). The latter could be interpreted as further evidence for the detoxification of MC by glutathione.

NEUROTOXINS

There is a wide variety of paralytic shellfish poisons (PSPs), which block the sodium voltage-gated channels of the axonal membrane by reversible binding to the receptor. PSPs have been detected in *Aph. flos-aquae*, *A. circinalis* and *Lyngbya wollei* (*165*). The toxins are known for their occurrence in dinoflagellates (*166*). PSPs as well as anatoxins are discussed in chapter III and VII.

CYLINDROSPERMOPSINS

These tricyclic alkaloids with protein synthesis inhibiting activity mainly occur in tropical and subtropical areas and are therefore mentioned and further described in occurrence and elimination of cyanobacterial toxins in two Australian drinking water treatment plants (chapter VII).

LIPOPOLYSACCHARIDES (LPS)

LPS consists of an innermost lipid moiety, a core oligosaccharide and a polysaccharide in the outermost moiety. The cyanobacterial LPS differs from those of Enterobacteriaceae. The lipid and sugar components are different and more variable (*2*). High endotoxin concentrations detected in bloom samples are probably due to the presence of gram negative bacteria which coexist with cyanobacteria. Using the Limulus amoebocyte lysate assay, 26 axenic strains from different cyanobacterial genera show very low endotoxin activity (*167*). Cyanobacteria can stimulate the growth of aquatic bacteria. Especially the heterocysts of N_2–fixing cyanobacteria seem to attract heterotrophic bacteria (*48, 167, 168*), which can be the reason for high concentrations of LPS.

Cyanobacterial LPS has been demonstrated to affect the phase-II microcystin detoxication mechanism in zebra fish (*Danio rerio*). LPS preparations from cyanobacteria significantly reduce activity of both the soluble and the microsomal glutathione S-transferases (*169*). The latter findings are important for estimating the health risks for humans, because a cyanobacterial bloom always contains a cocktail of different cyanotoxins like LPS and MCs. In addition, the dermal route of uptake and allergic/irritant reactions probably caused by LPS, may pose a hazard during the recreational use of water bodies contaminated with cyanobacterial blooms (*170, 171*).

OTHER TOXIC COMPOUNDS SYNTHESISED BY CYANOBACTERIA

Every year dozens of new secondary metabolites with “toxic” or allelopathic effects to other organisms are isolated from cyanobacteria. To give a short overview, a few of them are listed here without guarantee of completeness:

Anabaenopeptins (oscillamides, nodulapeptides) are hexapeptides with variable amino acids (*172-174*). Some are inhibitors of protein phosphatase 1 and 2A, but at 1.0 mg/L the IC_{50} value is 1000 fold higher than that of MC-LR (*175, 176*). Known

producers of this group of metabolites are *P. rubescens/agardhii*, *M. aeruginosa*, *N. spumigena* and several species of *Anabaena*. Microginins (oscillaginin) are linear peptides produced by *Microcystis* (*177, 178*), with an IC_{50} for the angiotensin-converting enzyme of 1.6×10^{-5} M (*179*). No inhibitory effect could be observed during embryologic development of zebra fish up to concentrations of 10 mg/L. The C_{18} lipid mueggelone has been described as toxic to embryologic development of zebra fish at concentrations of 10 mg/L (*180*). Aeruginosins (*181*), cyanopeptolins (anabaenopeptilides, micropeptins, aeruginopeptins, nostopeptins) (*182, 183*) and microviridins (*184*) are also mentioned, but without detailed toxicological or pharmacological descriptions (*165, 185*). This huge variety of compounds with unknown toxicological and pharmacological effects must be considered, when cyanobacterial extracts or natural blooms are investigated regarding their effects in different test systems.

Toxin release

Most of the cyanotoxins remain in the cell until the cell is lysed. As mentioned earlier in this text, evidence exists, that the toxins may be actively transported into the surrounding media. But even if this transport can be confirmed, whether for capturing iron or communicating with other life forms, the concentration of toxins remains low. Problems for drinking water treatment plants occur, if high extracellular toxin concentrations are to be expected in the raw water. Under natural circumstances high concentrations appear during the breakdown of a cyanobacterial bloom. Cyanobacterial cells are also lysed in the presence of chemicals (*186, 187*) which inhibit new cell wall synthesis, enzymatic reactions, or photosynthesis (e.g. reglone A, potassium permanganate, chlorine, ozone, simazine). Copper sulphate also leads to a lysis of cyanobacterial cells, which is followed by the release of the intracellular toxins into the water (*186, 188-190*). The use of e.g. copper sulphate or reglone A to stop algal growth and “clean” the water body has led to several intoxications of livestock with cyanobacterial toxins (*53*). A case of severe intoxication of humans has also been reported after treatment of the drinking water dam with copper sulphate (*191*). In addition, lysis of cyanobacterial cells has been observed as a result of passage through a cooling system in a nuclear power plant in Illinois, USA (*192*). A detailed introduction and discussion of problems with cell lysis during the drinking water treatment is presented in the following chapters.

Degradation of cyanobacterial toxins

One point of interest of this thesis is the degradation of cyanobacterial toxins, particularly MCs. If the cyanobacterial cells are not removed by flocculation, by back-washing of the fast-filter or by a change of the filter material, the cells and therefore the toxins remain in the drinking water and must be degraded to non-toxic compounds. The toxins themselves can not be removed by flocculation nor by sand filtration. Activated carbon has the ability to adsorb the toxins, but the adsorption capacity is limited and the hydrophilicity/adsorbability of the MCs varies but can be estimated using the K_{ow} (*81*). The PSP variants also differ in their hydrophilicity and no generalisation can be made. Anatoxins and cylindrospermopsin are highly water soluble and therefore insufficiently adsorbed by activated carbon or removed by other filtration steps and flocculation. Oxidation steps or bacterial degradation are necessary during drinking water treatment to remove these cyclic peptides. Such a "biofilm" exists in activated carbon and slow sand filters. The mineralisation of MCs and other cyanotoxins and the degradation process during oxidation is mentioned in chapters III, IV and VIII.

The following paragraph gives an overview of the natural degradation of MCs in the environment and by biofilm during drinking water treatment. Once MCs have been released into the water body, the toxins can persist for weeks (*188*) before they are degraded by e.g. bacteria of the genus *Sphingomonas* (*193*). Different MC-congeners have been demonstrated to be degraded after incubation with water from a lake in Japan which is frequently contaminated with cyanobacteria (*194*). A more effective degradation was observed after adding bed sediment or mud from the lake, whereas no degradation could be observed after incubation with boiled water. Christoffersen *et al.* (*195*) also mentioned that bacteria can efficiently degrade MCs in natural waters with previous cyanobacterial histories and that the degradation process can run quickly and without lag phase. In another study with MC-LR incubated with water samples from 14 different water bodies, degradation proceeded rapidly after the lag phase and was completed within a few days. A lag phase was apparent in all cases, this, however, ranged in length from a few days to several weeks, depending on the water body in question (*196*). Further investigations have led the authors to the hypothesis that organic compounds induce the growth of bacteria, which are able to degrade MCs independent of the presences of MCs. Thus, MC-degrading bacteria are

common in surface waters, independent of former contaminations of this water body with cyanobacteria or MCs.

Seventeen strains of gram negative bacteria with the ability to degrade MCs were isolated by Lahti *et al.* (*197*). While *Sphingomonas sp*. is not the only bacteria genus which is responsible for degradation of MCs, the following details are worth mentioning. At least three intracellular hydrolytic enzymes have been shown to be involved in the degradation of MC-LR by *Sphingomonas sp*. (*3, 198*): the metalloprotease microcystinase (MlrA) catalyses the ring-opening at the Adda-arginine peptide bond, the putative serine peptidase MlrB cuts the linear peptide, such that a tetra-peptide is generated, and a putative metallopeptidase MlrA splits the tetra-peptide into smaller peptides or amino acids (Figure 2).

Saitou *et al.* (*199*) found microcystin degrading bacteria with 98.5% homology to *Sphingomona stygia* on a 16s rRNA basis. These bacteria were able to degrade MC-LR, MC-YR and MC-RR and indeed to use MCs as their sole carbon source.

The alkaline protease of *Pseudomonas aeruginosa* is another example of how bacteria can cut the peptide bonds of MCs (*200*).

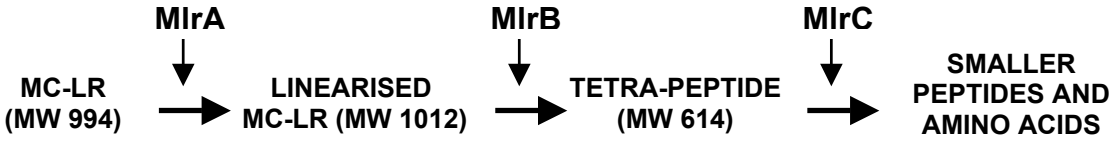

Figure 2. MC-LR degradation pathway by *Sphingomonas sp.* Strain ACM-3962 (adapted from Bourne *et al.*(*3*)). MlrA-C microcystinases A-C.

Another possible pathway of degrading MCs within a water body is via sunlight. Because the absorption maxima of MCs are in the UV-C-range and UV-C radiation is absorbed by the ozone molecules of the stratosphere and does not reach the (water) surface, natural sunlight cannot be responsible for direct degradation of MCs. Thus no overlap exists between the solar spectrum and the absorbable spectrum of MCs and only the absorbance of photons can cause transformations in a molecule (*201*). Humic substances can however act as photosensitisers by absorption of sunlight in the range above UV-B to the wavelengths of photosynthetically active radiation. The energy can be transferred to other molecules while the humic substances return to the ground state. Different radicals are generated during these processes. These can react with other photochemically inert molecules (e.g. MCs). The rate of

photochemical degradation of MCs depends on the surface to volume ratio and the radiation energy reaching the water body. In addition, the percentage of reflected radiation differs according to the angle water surface-sunlight between 2 and 40% and in an oligo- to mesotrophic lake the light intensity is halved at one meter depth. The degradation by sunlight is thus clearly lower than that which can be achieved by bacteria.

Effects of toxins on different organisms

Why do cyanobacteria produce expensive secondary metabolites? This question has already been discussed earlier in the introduction. While it is not likely, that cyanobacteria produce their toxins to harm mammals, herbivore fish are potential grazers of cyanobacterial colonies. Cyanobacteria which can avoid being eaten by fish have survival advantages. Therefore the answer to the question asked at the beginning of this paragraph could be easy:

Cyanobacteria do not want to be eaten by fish or maybe livestock.....

Fish: The silver carp *Hypophthalmichthys molitrix* and the tilapia *Oreochromis niloticus* have the ability to differentiate between toxic and non-toxic strains of *M. aeruginosa*. Beveridge (*202*) supposed that this may be determined by very low levels of extracellular microcystins or/and other features which distinguish toxic from non-toxic strains, such as cell surface compounds.

Oreochromis mossambicus uses *M. aeruginosa* as a major component of its diet at certain times of the year. Investigations show a breakdown of 75% of the cells during digestion, whereas 25% appear intact after passing through the fish. The fish initially lose weight, but after 21 days on the *M. aeruginosa* diet a slight gain in weight occurs (*203*). *Rutilus rutilus* prefers *Aph. flos-aquae* as a food source if the choice between the MC-containing *M. aeruginosa* and the MC-free *Aph. flos-aquae* is given (*204*).

Fish kills have been reported in conjunction with cyanobacterial blooms and have often resulted in significant economic losses (*205-209*). A massive fish kill of more than 6 tons occurred after treatment of a *Aph. flos-aquae* bloom (3.8 x 10^5 cells/mL) with copper sulphate in Kezar Lake, New Hampshire, USA (*210*). In the early 20th century Seydal (*211*) described a fish-kill caused by *Anabaena* and *Polycystis* (now known as *Microcystis)*, however at the time an oxygen deficiency was the supposed reason. In Lake Sempach, Switzerland, tons of fish were victims of cyanobacterial blooms, probably through cyanobacterial toxins from *Aph. flos-aquae* (*212*). *Aph.*

flos-aquae was also supposed to be responsible for a fish-kill in the Yahara River, Wisconsin (USA). After the *Aph. flos-aquae* -bloom has collapsed, carp (*Cyprinus carpio*) were the predominant fish affected (*213*).

Livestock: Almost every kind of livestock has been killed by different cyanobacterial species during the last centuries. To give examples, cattle (*214-221*), sheep (*218, 219, 222, 223*) and pigs (*224, 225*) died after ingesting cyanobacterial scums.

But wildlife is also affected: birds (*225-229*), bats (*230*) rodents, zebras and rhinoceros (*225*). Why do these animals ingest the cyanobacterial scums? In some regions the animals have no other drinking water source than the cyanobacterial contaminated water. However, it has been observed that dogs are attracted by scums (*231*). The smell of the blooms and the rotting scums seem to be attractive for animals. Although dogs are known for their ability to differentiate between "healthy" and "unhealthy" water (e.g. most of them refuse to drink alcohol), several cases of deaths after ingesting cyanobacteria have been described (*232-238*). Drink selection experiments have shown a preference of mice to drink *M. aeruginosa* contaminated water instead of limpid water (*239*). Perhaps this preference is common among other species, which could explain the huge number of intoxications of livestock with cyanotoxins. *Homo sapiens sapiens* can also come into contact with cyanobacteria and meet their weapons. Therefore warning signs (picture 1 and (*240*)) in case of the recreational waters and an adequate drinking water treatment and monitoring is necessary.

Cyanobacteria and their secondary metabolites in contact with humans

CYANOBACTERIA IN WATER

It is not always obvious if an illness is a result of an intoxication with cyanobacteria and/or their toxins. Symptoms such as headache, diarrhoea (*241-244*), vomiting, skin rashes or fever are possible consequences of a sunstroke, spoiled food or other organisms in or around the water. Only if toxins and/or cyanobacteria are determined in blood, gastrointestinal tract, urine or faeces, can the involvement of the "blue-greens" be proven. To my knowledge, only one tragic example for this exists. In Brazil, 76 patients died after receiving insufficiently filtered water via hemodialysis from a reservoir contaminated with cyanobacteria (*245*), whereby MCs in amounts of several µg/g liver were deemed to be responsible for the so called

"Caruaru syndrome". Reports from Portugal and USA (*246*) about pyrogenic reactions after dialysis also mentioned cyanobacteria in the water source before treatment.

Examples for supposed or confirmed toxic effects of cyanobacteria to humans are listed in Table 1. Humans generally avoid contact with thick cyanobacterial surface blooms and an intoxication is not to be expected. Nevertheless, some ignore warnings and swim in water bodies contaminated with cyanobacterial blooms, as reported by Dillenberg and Dehnel (*247*). Pilotte *et al.* (*248*) found a correlation between the occurrence and the severity of symptoms such as diarrhoea, vomiting, skin rashes, fevers or ear irritations and the duration of water contact and cyanobacterial cell density. This epidemiological study was carried out with 852 participants in Australia in 1995. Allergic and toxic reactions have been observed after swimming in several rivers and lakes around Berlin, Germany (*249, 250*). The persons concerned were mainly children younger than 10 years. Several studies have described allergic reactions (*216, 251, 252*) and hay-fever (*247, 252*) after contact with cyanobacteria. In several regions a lack of knowledge exists about the possible toxic effects after contact with cyanobacterial blooms. Pictures and reports about children swimming and playing in *Microcystis*-green water are frightening. The consequences of chronic contamination with low concentrations of MCs or other cyanotoxins are still hard to estimate. Studies from China give evidence of a tumour promoting activity of MC even in concentrations of 0.1 µg/L (*253-255*). Using the Geographic Information System for risk evaluation, it was found that in Florida a significantly increased risk of primary hepatocellular carcinoma for residents within the service area of a surface treatment plant exists, compared to persons living in areas contiguous to the surface treatment plant (*256*). More interesting facts about cyanobacteria and their toxins in drinking water are reported in chapters III-VIII.

Picture 1. Cartoon from an Australian pamphlet.

	Water treatment/ exposure route	Cyanobacteria	Health outcomes	References
Australia, Solomon Dam Palm Island, 1979	copper sulphate	*Cylindrospermopsis*	GI,LD,KD,ID	(*191, 257, 258*)
Australia, Malpas Dam, Armidale, 1981	copper sulphate	*Microcystis*	LD	(*259*)
Australia, Lake Alexandria	NR	*Microcystis, Nodularia*	HF	(*260*)
Australia, northern and central Australia, 1992	NR	SND	"Barcoo fever", GI	(*261*)
Australia, towns at River Murray, 1992	river and drinking water contact	*Anabaena circinalis*	Multiple symptoms	(*262*)
Brazil, Caruaru, 1996	sand filtration, carbon filtration, cation/anion exchange filtration, microfiltration	*Microcystis, Anabaena, Cylindrospermopsis,* variety of unknown picoplanktonic cyanobacteria	LD, NSE,GI after hemodialysis by 98% of patients, 76 deaths	(*263-266*)
Brazil, Itaparica Dam, 1988	copper sulphate	*Anabaena, Microcystis*	GI	(*267*)
Canada, Saskatchewan	swimming	*Microcystis spp., Anabaena circinalis*	GI, NSE	(*247*)
China, Haining city	NR	SND	Colorectal cancer	(*255, 268*)
China, south-east, 1972-1990	NR	*Microcystis*	PLC	(*253, 269*)
India, Udaipur	NR	*Microcystis aeruginosa*	GI	reported in (*270*)
Former East Prussia, Koenigsberg Haff	NR	*Anabaena*	GI, NSE	(*271*)
Finland, Tampere	NR	SND	HF	(*272, 273*)
Philippines	NR	SND (algal toxins supposed reason for health outcomes)	GI	reported in (*270*)
Portugal, Guardian river	NR	*Aphanizomenon flos-aquae*	GI	Oliveira 1991, cited in (*274*)
Portugal, Évora	NR	*Anabaena, Aphanizomenon, Microcystis, Oscillatoria*	LD (supposed)	(*275*)
Sweden, Scania, 1994	contamination of treated drinking water with untreated river water	*Planktothrix agardhii*	GI	(*276*)
UK, Staffordshire, 1989	swimming and canoeing in reservoir water	*Microcystis aeruginosa*	GI, NSE	(*277, 278*)
USA, Allegheny Country, Pennsylvania, Sewickley reservoir, 1975	NR	*Schizothrix, Lyngbya, Plectonema, Phormidium*	GI	(*270, 279*)
USA, Pennsylvania and Nevada	water contact	*Anabaena, Aphanizomenon*	HF, GI	(*280*)
USA, Florida	different treatment systems	*Microcystis, Anabaena, Cylindrospermopsis*	PLC	(*256*)
USA, Charleston, West Virginia, Ohio and Potomac rivers, 1930-31	precipitation, filtration, chlorination	*Microcystis*	GI	(*33, 281*)
Zimbabwe, Harare, 1960-1965	NR	*Microcystis aeruginosa*	GI	(*282*)

Table 1. Examples of human health problems with cyanobacteria (GI gastrointestinal disease; HF hay fever/allergic reactions; ID intestinal damage; KD kidney damage; LD liver damage; NSE nervous system affected; NR not reported; PLC primary liver cancer; SND species not determined).

BLUE-GREEN ALGAE FOOD SUPPLEMENTS

The consumption of "blue-green algae food supplements" (*149, 283-285*) is becoming more and more common in the industrialised countries. For centuries cyanobacteria have been consumed in North Africa, Mexico and China as a protein source (*286*). The cyanobacteria which are used for the production of these supplements are harvested from specific outdoor ponds or raceways (e.g. *Spirulina*) as well as from natural lakes (e.g. *Aph. flos-aquae* from the Upper Klamath Lake, Oregon, USA). After harvesting, the cyanobacteria are freeze-dried and flash frozen or only spray-dried. The three genera *Spirulina* (e.g. *S. platensis), Chlorella* (*C. pyrenoidosa)* and *Aphanizomenon* (*Aph. flos-aquae*) are the most popular for the production of these supplements. There is no evidence that *Spirulina* and *Chlorella* produce cyanotoxins such as MCs, NODs, ANAs, PSPs or CYLs. *Aph. flos-aquae* produces PSPs (*287*) and anatoxin-a (*288*), other *Aphanizomenon*-species also synthesise cylindrospermopsin (*289*). Thus, the current knowledge suggests that some PSPs and anatoxins may be present in the supplements, but MCs should not, as, according to the harvesters' statements, MC-producing species are not harvested. *Aphanizomenon,* however*,* coexists with MC-producing *Microcystis* in many lakes (*287, 290-292*). In a French lake an *Aph. flos-aquae* bloom was shown to be associated with a MC-concentration of 279 ng/g DW (*293*). The authors suggested this to be the result of a low production of MCs by *Aph. flos-aquae*. In addition, a separation of putative "non-toxic" genera from coexisting toxic cyanobacteria genera after harvest is impossible. Four studies into the MC-concentrations of blue-green algae food supplements have been carried out. The amounts found varied from 0–78 ng/g DW (*285*), 500-35,000 ng/g DW (*283*), 0-16,400 ng/g DW (*149*) and 0-21,600 ng/g DW (S.J.H., unpublished results). These results underline the need for a worldwide guideline value and obligatory toxin testing by the provider. Only one regulatory value currently exists: the Oregon Health Division has set a guideline value of 1.0 µg MC/g DW for blue-green algae food supplements sold in Oregon/USA. To put this in a perspective, the market value for the more than 1 x 10^3 tons *Aph. flos-aquae* was about 100 $ million (1998), world production of *Spirulina* in 1995 was about 2 x 10^3 tons. The possible human health risk through these supplements is assessed in the general discussion in chapter IX.

IRRIGATION WATER AND THE FOOD CHAIN

Although plants have the possibility to absorb MCs, irrigation of field crops with cyanobacterial contaminated water and cyanobacterial blooms in rice fields are poorly investigated sources of human exposure to microcystins. MCs have been detected in tissues of *Phaseolus vulgaris* exposed to the toxin via the growth medium. In addition, the photosynthesis was shown to be inhibited in the leaves following multiple applications of 10 µg MC-LR/L via the growth medium (*294*). The same effects were shown by Abe *et al.* (*295*). After seven days of repeated spray irrigation with 2.0 µg MC-LR/L the photosynthetic rate in *P. vulgaris* was significantly reduced. Visible *Microcystis* colonies were present on *Lactuca sativa* after repeated irrigation at a commercial horticulture unit in England. After washing and methanol extraction of the lettuce leaves, up to 2.5 µg MC/g DW were detectable via an MC-immunoassay (*296*). To cite a further example, irrigation canals in Egypt often contain blooms of *Oscillatoria agardhii* with up to 2 mg MC/g DW (*60*). These toxins have been shown to be assimilated by the plants. Furthermore, the uptake of MC-LR in aquatic macrophytes has been demonstrated (*297*). This leads to the question of the aquatic and terrestrial food web with humans as the potential top predator. In the aquatic environment a risk for contamination of humans with cyanobacterial toxins exists by ingestion of crayfish, mussels and fish, especially if the liver of fish is consumed (*271*). CYL has been shown to accumulate in the Redclaw crayfish *Cherax quandricarinatus* (*298*), MCs and NODs in the mussel species *Anaconda cygnet* (*299*), *Mytilus galloprovincialis* (*300*) and *Mystiques delis* (*301*), respectively. Watanabe *et al.* (*302*) found MCs in mussels of the species *Unio douglasiae* and *Anadonta woodian* during a bloom of *M. viridis*. MC could also be detected in fish liver (*303*) and muscle (*304, 305*) in concentrations up to 280 µg/kg fish. Because MC has also been associated with livestock death and furthermore the consumption of contaminated livestock products could represent a route of human exposure, experiments with dairy cows fed with MC rich food were carried out. No carry over of detectable amounts of toxin into milk (*306, 307*) could be observed, the muscle tissue was not investigated.

DRINKING WATER

Water is the most critical natural resource, necessary for drinking, agriculture and for industrial activities. Its contamination therefore can influence not only humans but agricultural livestock and irrigated field crops as well as wildlife which drinks the water or lives in the aquatic environment. As the world's population grows, there is mounting pressure on both water supplies and quality.

In many parts of the world water is already a limited resource, although the water on the planet and the annual rainfall should be enough to exceed our needs. Only 0.1-0.3% of the total water, estimated at 1.4 x 10^9 km^3 are useable for mankind, whereby usable in this context means not salty, not trapped in polar ice caps or unavailable in deep ground waters.

On average, 110 000 km^3 rain falls on earth, whereby 65% evaporates and 25% flows into surface waters which are not usable for humans. Thus, given a world population of 6 x 10^9, 1500-2000 m^3 water are available per human and year. An estimated, 1700 m^3/y are sufficient per human (Table 2**)**.

> 1700 m^3:	sufficient water
1000-1700 m^3:	water as a limited resource
500-1000 m^3:	shortage of water
< 500 m^3:	extreme shortage of water

Table 2. Classification: What does water shortage mean (per person and year)?

Drinking water should be appetising and refreshing. Therefore it must be colourless, clear, fresh, odourless and with a good taste (*308*). Interestingly, these current quality requirements are nearly identical to those proposed by the Roman physician and engineer Vitruv (25 BC): "Drinking water should be free of deadly and harmful substances, fresh, colourless and tasty". Doubtless, one of the critical factors concerning these criteria is the presence of cyanobacteria in the raw water entering drinking water treatment plants.

Drinking water treatment

In Switzerland, the daily water use has been between 400 and 500 liters/d and inhabitant from 1955-1994. Of this, 160 liters are used in the household, the rest is needed for industry or agriculture or gets lost during transportation between water supplier and the consumer.

In Germany, every inhabitant used 128 L/d for personal use in the year 2000. Compared to 1990 this is a reduction of 12%. However, only around 3% of this water is used for drinking or cooking, the majority of "drinking water" is wasted for cleaning, hygiene or watering. The reduction in water consumption during the latter decades has paradoxically caused a growing hygienic problem, because the water remains in the water distribution system for a longer time, resulting in a higher risk of germ-growth. Before reaching the distribution system, drinking water runs through a more or less complex treatment system. The number of treatment steps depends on the raw water quality and the drinking water standards and guidelines in the different countries. Table 3 shows the drinking water sources of three different countries. The quality of spring and ground water is mostly sufficient to be used without treatment. In Switzerland, the quality of 38% of the raw water is good enough for the use as drinking water without treatment, for 33% a one-step treatment is necessary and for 29% a multi-step treatment train is required (*309*). On the other side of the world in Australia, 73% of the supplied drinking water comes from surface waters and must undergo expensive cleaning steps, because the ground water pool is small. The climatic conditions in large parts of the Australian continent lead to a contamination with cyanobacterial blooms in most of the used surface waters. The use of tap water as drinking water differs between countries and probably changes with the years. For example, in Zurich, Switzerland, 7 of 10 inhabitants are drinking tap water every day (*310*). Worldwide a consumption of 2 liters tap water per day is assumed for the calculation of drinking water guidelines.

Table 3. Where does the drinking water comes from? Values are in % total.

Source/Country		Australia	Germany		Switzerland
Spring-water (%)		9	7.8		39
Ground-water (%)		21	63.6		43
Surface-water (%)	Bank filtration	73	5.2	28.6	18
	Enriched ground water		10.2		
	River water		1.1		
	Lake water		3.1		
	Dam water		9.0		

In chapter VI, Table 15 gives an overview of the worldwide distribution of cyanobacteria in drinking water bodies and underlines the potential danger for cyanobacterial toxins in drinking water. Within the scope of this thesis a survey was carried out concerning cyanobacteria in German and Swiss water works (Table 4). Unfortunately, only a few water works provided information about the cyanobacterial contamination in raw and drinking water. Despite this, the data underline the massive presence of cyanobacteria in lakes and dams used for drinking water production.

CYANOBACTERIA AND POLITICS

Often several countries border on one water body. Diplomatic problems can occur, if one country blocks or pollutes the water body to the disadvantage of the neighbouring states. For example, rivers which have their source in Spain are used as drinking water resource in Portugal. These rivers are already highly loaded with nutrients when they pass the border and regularly show cyanobacterial blooms (*274*). Another example are the Baltic states, which blame Russia for heightened risk of toxic blooms, as the latter is responsible for 70-80% of phosphorus and nitrogen in the Gulf of Finland. This is suggested to be the reason for massive toxic cyanobacterial blooms (*311*) in this area. Thus not only battles for water but also the battles for the quality of the water could arise in the next decades.

Table 4. Survey of German and Swiss waterworks: Cyanobacteria in raw water source and raw water.

Raw water source	**Drinking water dam Weida/Germany**				**Drinking water dam Wahnbachtalsperre/Germany**			
Water treatment	microsieve (63µm) - flocculation (Al_2SO_4: 2.0-2.5 g/m^3), quartzsand (d_w: 1.55), ClO_2: 0.2 mg/L				flocculation (0.8 mg $Al_2(SO_4)_3$/L)/sedimentation, quartzsand filtration, chlorination of pipeline system (0.15-0.2 mg/L)			
Cyanobacteria: species and occurrence	species	dam	raw water	biomass (mg/L)	species	dam	raw water	max. density (cells/mL)
	Aphanizo-menon spp.	rare	some-times	-	*P. rubescens*	yes	yes	dam water 50,000 raw water 6000
	P. rubescens	yes	yes	dam: 12 raw water: 10				
Intake depth	15 m				40 m			
Intake volume (m^3/d)	~ 35,000				75,000			

Raw water source	**Drinking water dam Deesbach/Germany**			
Water treatment	flocculation (1.8 –2.0 mg $Al_2(SO_4)_3$/L)/sedimentation, two-layer filtration, ozonation with 0.5 – 2.0 g O_3/L chlorination of the pipeline system (0. -0.2 mg/L)			
Cyanobacteria: species and occurrence	species	dam	raw water	max. density/ toxin conc.
	Microcystis spp.	yes	rare	275 µg/L
	Anabaena spp.	rare	no	1475 cells/mL
Intake depth	~ 15 m			
Intake Volume (m^3/d)	16,000			

Raw water source	**Lake Luzern/Switzerland**			**Lake Sempach/Switzerland**		
Water treatment	quartzsand, filtration (1.0 m, 5m/h), ozonation			flocculation, quartzsand, ozonation, activated carbon filtration, chlorination of pipeline system		
Cyanobacteria: species and occurrence	species	lake	raw water	species	lake	raw water
	Aphanizomenon spp.	yes	yes	cyanobacteria present but not quantified.		
	Anabaena spp.	yes	yes			
	Microcystis spp.	yes	yes			
	Planktothrix spp.	yes	yes			
Intake depth	15 m			40 m		
Intake volume (m^3/d)	~ 35,000			920		

OBJECTIVES

As mentioned in the previous chapter cyanobacteria (blue-green algae) has impacted the health of human and particularly livestock for millennia. It has become clear that cyanobacteria synthesise a wide variety of compounds with unknown importance for life of these oldest organisms on earth. These molecules are thus called secondary metabolites. Some have been shown to be toxic to other organisms in very low doses. As cyanobacteria live in almost every ecological niche on earth, humans must develop mechanisms to avoid contact or ingestion with harmful compounds produced by cyanobacteria. The first step is to know by which pathway an intoxication or adverse effect on humans can occur. One important pathway for the exposure to harmful substances is by drinking water.

The aim of this study was to summarise the current knowledge concerning cyanobacteria in water treatment processes and to fill some of the detected knowledge gaps. Therefore the status of knowledge was reviewed and supplemented with new data (chapter III).

To simulate the conditions in water works in central Europe, a drinking water treatment train was developed on a laboratory-scale. Oxidative processes and different filtration materials were investigated with respect to their ability to eliminate cyanobacterial toxins during drinking water treatment in spite of high cyanobacterial densities in raw water (chapters IV + V).

An investigation into the procedures applied in waterworks is necessary for a risk assessment for humans concerning cyanobacterial toxins in drinking water. The conditions in water works which vary with changing temperature, organic load, pH and toxin concentration in raw water cannot be simulated exactly on a laboratory-scale. As *Planktothrix rubescens* has been shown to produce high amounts of microcystin and initial investigations have yielded high densities of *P. rubescens* in the raw water of several water works, two water works with *P. rubescens* species in their raw water were investigated. Not much data is available on the possibility that cyanobacterial toxins enter drinking water wells after passage through sediment during bank filtration. This possibility was investigated at Lake Hallwil/Switzerland (chapter VI).

While the investigated German and Swiss water works “only” have to deal with one kind of cyanobacterial toxins (microcystins), Australian water works often have to

eliminate paralytic shellfish poisons and/or cylindrospermopsin and/or microcystins from the raw water. Water treatment plants with flocculation and filtration steps have been examined concerning their ability both to remove intact cells and to eliminate cyanobacterial toxins from the raw water. In addition, a hypothesis has been developed concerning a possible allelopathic effect of cyanobacterial secondary metabolites (chapter VII).

One treatment step in many water works in central Europe is oxidation with ozone. Uncertainties exist about the toxicity of by-products after incomplete ozonation or degradation of microcystins. Thus MC-LR was ozonated and the by-products chemically and toxicologically characterised (chapter VIII).

For these projects different detection methods such as enzyme-linked immunosorbent assay (ELISA), radioactive protein phosphatase assay (PPA), colourimetric PPA, HPLC-DAD and HPLC-MS were carried out. Thus also the advantages and disadvantages of these different methods are discussed in this thesis.

Within the wide field of cyanobacterial toxins, ecological, toxicological, analytical as well as technical (water treatment) topics have been investigated in the studies presented here and summarised to assess the risk for human health with respect to cyanobacterial toxins.

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CYANOBACTERIAL TOXINS: REMOVAL DURING DRINKING WATER TREATMENT, AND HUMAN RISK ASSESMENT

Cyanobacteria (blue-green algae) produce toxins that may present a hazard for drinking water safety. These toxins (microcystins, nodularins, saxitoxins, anatoxin-a, anatoxin-a(s), cylindrospermopsin) are structurally diverse and their effects range from liver damage, including liver cancer, to neurotoxicity. The occurrence of cyanobacteria and their toxins in water bodies used for the production of drinking water poses a technical challenge for water utility managers. With respect to their removal in water treatment procedures, of more than 60 microcystin congeners, microcystin-LR is the best studied cyanobacterial toxin, whereas information for the other toxins is largely lacking. In response to the growing concern about non lethal acute and chronic effects of MCs, the WHO has set a provisional guideline value for MC-LR of 1.0 μg/L drinking water. This will lead to further efforts by water supplies to develop effective to remove these toxins. Of the water treatment procedures discussed in this review, chlorination, possibly micro/ultrafiltration, but especially ozonation are the most effective in destroying cyanobacteria and in removing microcystins. However, these treatment may not be sufficient during bloom situation or when high organic load is present, and toxin levels should therefore be monitored during the water treatment process. In order to perform an adequate human risk assessment of microcystin exposure via drinking water, the issue of water treatment by-products will have to be addressed in the future.

INTRODUCTION

Toxic blue-green algae in water used as drinking water or for recreational purposes pose a hazard to humans, but have long been neglected or at the most been treated on a local level. Scums of blue-green algae or cyanobacteria accumulating along the shores of ponds and lakes also present a hazard to wild and domestic animals. Providing the human population with safe drinking water is one of the most important issues in public health and will gain more importance in the coming millennium. Reports of toxic blooms and poisonings of humans and cattle range from the first report of a toxic *Nodularia* bloom in Lake Alexandrina, Australia in 1878 (*68*) to a high incidence of primary liver cancer (PLC) in China attributed to cyanobacterial toxin-contaminated drinking water (*253, 255, 312*), to the recent tragic deaths of 60 dialysis patients in Caruaru, Brazil in 1996 due to the presence of

cyanobacterial toxins in the water supply used in a hemodialysis unit (*263, 265*). The presence of cyanobacterial toxins in drinking water supplies poses a serious problem to water treatment facilities since not all technical procedures are able to effectively remove these toxins to below acceptable levels. Despite this, it is highly unlikely that lethal poisonings would occur following consumption of drinking water contaminated with cyanobacterial toxins. Of much higher concern are low level chronic exposures, since the risks associated with long-term exposure have not been adequately described. Drinking water suppliers are nevertheless confronted with a variety of questions ranging from "what levels actually occur in the drinking water sources" to the "current state of knowledge about acute and chronic effects" and "effective water treatment technologies in removing toxins" (*53*). This review aims to address these issues.

CYANOBACTERIA

Morphology and Taxonomy

Cyanobacteria are an ancient group of organisms whose habitats range from hot springs to temporarily frozen ponds in Antarctica (*313*). They occur both in freshwater and in marine environments. Cyanobacteria, like eubacteria, lack a nucleus while in contrast to their closest relatives, the purple and the green sulphur bacteria, they produce oxygen (*314*). According to the current taxonomy, 150 genera with about 2000 species, at least 40 of which are known to be toxicogenic, have been identified (*315*). Cyanobacteria grow either as single cells, as single cells in colonies or in filaments, while some filamentous genera contain special nitrogen-fixing heterocysts. Cells growing in colonies may be packed in a mucilaginous sheath, like *Microcystis sp.* or in the case of filamentous species grow as floating mats or as free-floating strands. The fact that many cyanobacterial species possess gas vacuoles which allow them to regulate their position in the water column gives them a distinct ecological advantage over other planktonic species.

Bloom formation

Prevention of bloom formation is the most efficient method for avoiding cyanobacterial toxin contamination of drinking water. Unfortunately the factors leading to cyanobacterial bloom development (cell numbers > 10^6/L), whether of toxic or non-toxic species, have not been satisfactorily identified. Factors such as

nitrogen, phosphorus, temperature, light, micronutrients (iron, molybdenum), pH and alkalinity, buoyancy, hydrologic and meteorological conditions and the morphology of the impoundment have all been implicated (for a discussion see (*11*)). More importantly, factors influencing toxin production have not been conclusively elucidated (*48*). Although these factors can be considered as being closely related to bloom formation, cell numbers and toxin levels are usually not closely related. Furthermore, few generalizations can be made from the few laboratory studies that have been conducted to date (*49-53*).

CYANOBACTERIAL TOXINS

Cyanobacteria produce a variety of toxins, subsequently called cyanotoxins, which are classified functionally into hepato-, neuro-, and cytotoxins. Additionally, cyanobacteria produce lipopolysaccharides (LPS) as well as secondary metabolites which are potentially pharmacologically useful. The former are responsible for the irritant nature of cyanobacterial material. Defined by their chemical structure, cyanotoxins fall into three groups: cyclic peptides (the hepatotoxins microcystins and nodularin), alkaloids (the neurotoxins anatoxin and saxitoxins) and LPS. The species that are most often implicated with toxicity are *Microcystis aeruginosa*, *Planktothrix* (=*Oscillatoria*) *rubescens*, *Aphanizomenon flos-aquae*, *Anabaena flos-aquae*, *Planktothrix agardhii* and *Lyngbya spp.* (Table 5).

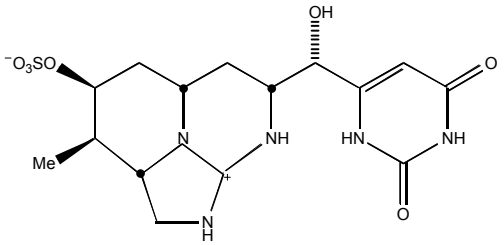
Cyclic peptides

Microcystins and nodularins are the most widespread cyanotoxins. They can be found in cyanobacterial blooms ranging from freshwater bodies to oceans. Microcystins have been described from the genera *Microcystis*, *Anabaena*, *Planktothrix*, *Nostoc*, and *Anabaenopsis*, whereas nodularin has only been found in *Nodularia* (*137, 316-319*).

TOXIN SYNTHESIS

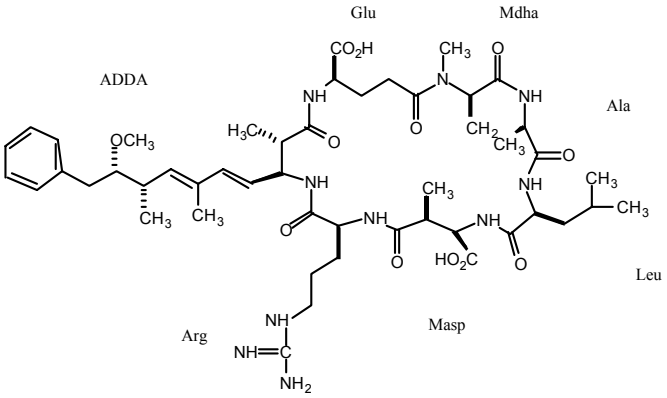
While the environmental conditions under which cyanobacteria produce toxins remain largely unknown, the way these toxins are synthesised is becoming clearer. Their small size, cyclic structure and content of unusual amino acids indicate that these peptides are synthesized non-ribosomally rather than on ribosomes (*320, 321*).

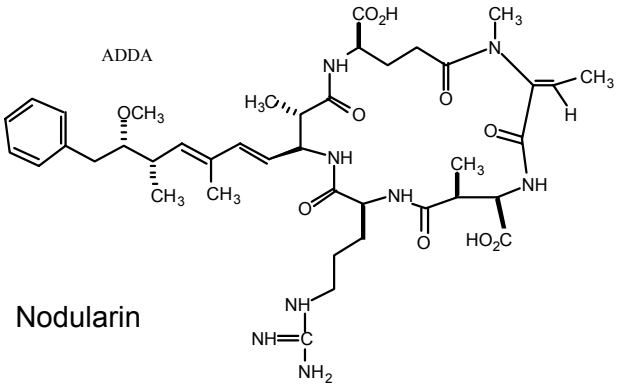
The enzymes involved in non-ribosomal peptide synthesis, peptide synthetases, have highly conserved structures. The genes coding for these peptide synthetases are modular, each module containing information for a single peptide synthetase unit. Using two conserved sequence motifs of the adenylate-forming domain of peptide synthetases to search for homologous sequences in toxic and non-toxic strains of *M. aeruginosa*, it was found that only the toxic strain contains peptide synthesis gene sequences (*321*). The ability of a cyanobacterial strain to produce toxins may thus depend primarily on the possession of these genes and on their expression under certain environmental conditions. With the emergence of a molecular genetics-based taxonomy of cyanobacteria together with the development of PCR primers and DNA probes specific for toxic strains of cyanobacteria, these toxin producing strains may be identified more rapidly in the future (*322-324*).



Cylindrospermopsin

Microcystin-LR





Nodularin

Figure 3. Structure of microcystin, nodularin, and cylindrospermopsin.

STRUCTURE AND UPTAKE

These cyclic peptides (Figure 3) are rather small molecules with a molecular weight ranging from 800-1000 Da (*80*). Most congeners are hydrophilic and generally not able to penetrate vertebrate cell membranes and therefore require uptake via an ATP-dependent transporter. One so far unidentified multispecific organic anion transporter (or bile acid transporter) has been described as the carrier of these cyclic peptides in rat liver (*95, 141, 325*). As a result of this, toxicity of microcystins and nodularins is restricted to organs expressing the organic anion transporter on their cell membranes, such as the liver. The structure of the heptapeptide microcystin was first identified in 1982 from an isolate of *M. aeruginosa*. Meanwhile about 60 congeners with the general structure cyclo-(D-alanine^{1}-X^{2}-D-MeAsp^{3}-Z^{4}-Adda^{5}-D-glutamate^{6}-Mdha^{7}) have been characterized (Table 5) (*79, 80, 316, 326, 327*). X and Z are two variable L-amino acids, D-MeAsp is D-*erythro*-ß-methylaspartic acid and Mdha is *N*-methyldehydroalanine. Adda is an unusual amino acid and unique to cyanobacterial toxins: (2S, 3S, 8S, 9S)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid. Nodularin is a pentapeptide with the general structure cyclo-(D-MeAsp^{1}-L-arginine^{2}-Adda^{3}-D-glutamate^{4}-Mdhb^{5}). Mdhb is 2-(methyl-amino)-2-dehydrobutyric acid. The most common structural variants occur in positions 2 and 4, resulting in substitutions of the L-amino acids, and demethylation of amino acids at positions 3 and/or 7. The current nomenclature names the most common structural variation, i.e. microcystin-LR (L: L-leucine, R: L-arginine) or microcystin-LW (L: L-leucine, W: L-tryptophane) (*319*). 6Z-stereoisomers of Adda have been reported for nodularin and microcystin (Table 5) and have all been reported to be less toxic in the standard mouse bioassay, with LD_{50}s of >1200 µg/kg body weight.

Cylindrospermopsin

Cylindrospermopsin (Figure 3) is a structurally distinct toxin which has been found in tropical and subtropical waters of Australia where it causes problems in water supplies (*328*). This alkaloid cyto- and hepatotoxin is produced mainly by *Cylindrospermopsis raciborskii*, but also by *Aph. ovalisporum* and *Umezakia natans*.

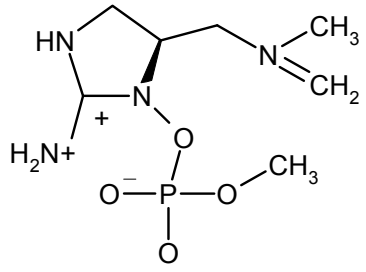
Toxin	LD_{50} (µg/kg i.p. mouse)	Organism	Reference
microcystin-LR	50	*M. aeruginosa, Aph. flos-aquae M. viridis*	(*326, 329*)
microcystin-LA	50	*M. aeruginosa, M. viridis*	(*330*)
microcystin-YR	70	*M. aeruginosa, M. viridis*	(*326*)
microcystin-RR	600	*M. aeruginosa, Anabaena sp. M. viridis*	(*331-333*)
[D-Asp3]microcystin-LR	50-300	*M. aeruginosa, Aph. flos-aquae, M. viridis, O. agardhii*	(*334, 335*)
[D-Asp3]microcystin-RR	250	*O. agardhii, M. aeruginosa, Anabaena sp*	(*137, 331*)
[Dha7]microcystin-LR	250	*M. aeruginosa, Anabaena sp., O. agardhii*	(*331, 336*)
[(6Z)-Adda]microcystin-LR	>1200	*M. viridis*	(*335*)
[(6Z)-Adda]microcystin-RR	>1200	*M. viridis*	(*335*)
nodularin	50	*N. spumigena*	(*337*)
[D-Asp1]nodularin	75	*N. spumigena*	(*338*)
Anatoxin-a	200-250	*Aph. flos-aquae, Anabaena spp. Oscillatoria sp., Aphanizomenon sp., Cylindrospermum sp.*	(*337, 339*)
Anatoxin-a(s)	20	*Aph. flos-aquae*	(*340*)
Saxitoxin	10	*Aph. flos-aquae, A. circinalis, C. raciborskii, Lyngbya wollei*	(*72, 341*)
Cylindrospermopsin	2000	*C. raciborskii, Umezakia natans, Aph. ovalisporum*	(*342*)

Table 5. Toxicity of cyanobacterial toxins.

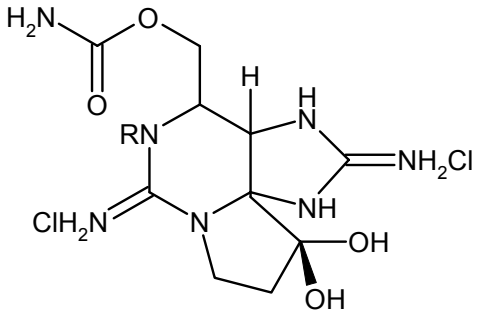
Neurotoxins

The neurotoxins (Figure 4) described in cyanobacteria can be classified into three distinct groups: 1) anatoxin-a and homoanatoxin-a, 2) anatoxin-a(s), which is structurally not related to anatoxin, and 3) saxitoxins or paralytic shellfish poisons (PSPs). Anatoxin-a has been described in *A. flos-aquae* and other *Anabaena* spp., *Planktothrix* sp. (*Oscillatoria* sp.), *Aphanizomenon* sp., *Cylindrospermum* sp. and in small amounts even in *Microcystis* sp. (*327, 343, 344*). Anatoxin-a exerts its neurotoxic effect by mimicking acetylcholine with an LD_{50} of 200-250 µg/kg body weight (*343*). Anatoxin-a(s) is the only naturally occurring organophosphate and has been isolated from *A. flos-aquae* and *A. lemmermannii* (*345*). It is a highly toxic

compound with an LD_{50} of 20 µg/kg body weight (i.p. in mouse) (*346*). Saxitoxins are better known from marine dinoflagellates ("red tide") where they are responsible for paralytic shellfish poisoning after consumption of contaminated shellfish. But saxitoxins, a group of carbamate alkaloid neurotoxins, have also been detected in relevant amounts in freshwater cyanobacteria such as *Aph. flos-aquae*, *A. circinalis*, *C. raciborskii*, and *Lyngbya wollei* (*341, 347, 348*).



Anatoxin-a (s)



R = H; saxitoxin dihydrochloride
R= OH; neosaxitoxin dihydrochloride

Anatoxin-a

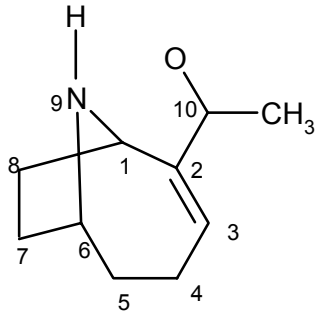


Figure 4. Structure of anatoxin-a, saxitoxins (PSPs), and anatoxin-a(s).

MICROCYSTINS

Animal Toxicity

Since the first description in 1878 of a *Nodularia spumigena* bloom in Lake Alexandrina, Australia (*68*), numerous cases of animal poisonings have been reported (Table 6). Most commonly, deaths of farm animals drinking scums of cyanobacterially contaminated ponds or poisonings of dogs swimming in cyanobacterial scum have been described (*53*). Fish kills have been reported in conjunction with cyanobacterial blooms and have often resulted in significant economic losses (*205-209*). The liver is the major target organ for microcystin toxicity, it was shown to accumulate 20-70% of a radioactively labeled toxin dose (i.v.) (*93, 349-353*). Studies in mice and pigs exposed to extracts of a toxic *M. aeruginosa* bloom demonstrated dose-dependent toxicity (*354, 355*). Increased

mortality, liver weight and plasma alanine aminotransferase levels were associated with loss of body weight. Neither other organ systems nor lactate dehydrogenase levels were affected. Death of the organism through intrahepatic haemorrhage and shock is rapid, occurring within about 3 hours in the case of mice. Pathological and ultrastructural features commonly observed in the liver are centrilobular hepatic necrosis, destruction of sinusoidal endothelium, disruption of bile canalicular function, intrahepatic haemorrhage, loss of microvilli and bleb formation in hepatocytes, and hepatocyte necrosis (*88, 141-145*).

Inhibition of protein phosphatases 1 and 2A

The toxicity of microcystins and nodularins is due to inhibition of the catalytic subunit of protein phosphatases 1 and 2A (PP1, PP2A) (*356-358*). In the case of microcystins it has been suggested that covalent binding to cysteine-273 and cysteine-266 on PP1 and PP2A, respectively, is responsible for this effect (*110, 117*). PP1 and PP2A dephosphorylate phosphoseryl- or phosphothreonyl-proteins and their inhibition leads to hyperphosphorylation of cytoskeletal proteins resulting in the deformation of hepatocytes (*136, 139, 325, 359-361*). It is not clear, however, if the covalent binding of the toxin to PP1 or PP2A is in fact responsible for the inactivation, since inactivation precedes covalent modification and nodularin does not bind covalently (*111*). It has furthermore been suggested that the Adda side chain and possibly the planar ring portion of the peptide are responsible for both recognizing and inhibiting protein phosphatases (*112, 113*).

Tumour promotion

The cyanobacterial cyclic peptides have been shown to possess tumour promoting activity by a TPA-independent pathway (*362*). Cyanobacterial extracts or microcystin-LR in drinking water induce skin tumours in rats and mice after initiation with 7, 12-dimethylbenz[a]anthrazene (DMBA) (*363, 364*). Glutathione-S-transferase placental-form (GST-P) positive foci were detected in livers of rats after i.p. injection of microcystin-LR or nodularin and initiation with diethylnitrosamine (*116, 365, 366*). It has been speculated that these toxins may be liver carcinogens, since they induce foci or small neoplastic nodules without the use of initiators (*116, 367*). Both microcystin-LR and nodularin have been shown to induce the expression of TNF-α and early response genes (c-*jun*, *jun* B, *jun* D, c-*fos*, *fos* B, *fra*-1) in rat liver

and hepatocytes (*116, 368*). In addition, mutations in the K-ras codon 12 in the RSa cell line (*369*) and DNA fragmentations have been reported after i.p. injection of cyanobacterial extract or MC-LR in mice (*370, 371*). These *in vitro* and *in vivo* data have to be viewed in the light of observations in China, where consumption of microcystin-contaminated drinking water has been associated with a high incidence of PLC (*253, 255, 312*) (see below).

Human health effects

Evidence of human poisonings by cyanobacterial toxins ranges from health effects after recreational exposure to poisonings following consumption of contaminated drinking water (Table 6).

ACUTE AND SUBCHRONIC EXPOSURES

The earliest case of gastro-enteritis from cyanobacteria was reported in 1931 in towns along the Ohio River, where low rainfall had caused the development of a large cyanobacterial bloom (*281*). The water treatment procedures employed over months to combat this bloom (pre-chlorination, sedimentation, filtration, chlorination, copper sulphate to lyse the cyanobacterial cells, aeration, activated carbon, permanganate, ammonia, and dechlorination) all proved to be ineffective in reducing taste, odour or toxin content of the drinking water. A natural *Microcystis* bloom in a water reservoir in Harare, Zimbabwe, caused gastro-enteritis in children each year at a time the bloom was decaying (*282*). A particularly extensive and toxic (microcystin-YM (*326*)) *M. aeruginosa* bloom in Malpas Dam, near Armidale, Australia, was treated with copper sulphate in 1981 after complaints of bad taste of the drinking water were received (*259*). The plant treating the water used pre-chlorination, alum flocculation, sedimentation, rapid sand filtration, post-chlorination, and fluoridation. The effect of this toxic bloom event was then monitored in a retrospective epidemiological study of liver function in the population consuming the water. It was found that the serum level of the liver enzyme γ-glutamyl transferase was elevated in that part of the population using the Malpas Dam water during the bloom and after the bloom was lysed with copper sulphate. The two most lethal poisonings attributed to cyanobacteria in drinking water occurred in Brazil. A massive *Anabaena* and *Microcystis* bloom in Itaparica Dam was responsible for 2000 gastroenteritis cases resulting in 88 deaths, mostly children (*267*). A very tragic, though relatively well

documented case occurred in a hemodialysis centre in Caruaru in 1996 (*263, 265*). The water used in the dialysis unit taken from Tabocas Reservoir, was normally sedimented with alum, filtered and chlorinated in the municipal water treatment plant prior to being supplied by truck to the clinic. At the dialysis unit, the water was further purified by passing through sand, charcoal, and an ion-exchange resin, and finally by micropore filtration. During the 1996 summer drought, the dialysis centre received water from the municipal plant which was only treated with alum, but not filtered or chlorinated. There are conflicting reports as to whether the water was chlorinated in the trucks prior to delivery to the clinic. Furthermore, the charcoal, sand and micropore filters at the clinic had not been changed in the three months prior to this episode, even though the water received from the trucks had been visibly turbid. In February, 1996 the majority (85%) of the hemodialysis patients developed a toxic illness of varying severity with a wide range of neurological symptoms as well as acute liver injury. Up to 23 patients died in the first two weeks of this episode with either neurological symptoms or from liver failure. About 37 more patients died in the following five weeks either directly from hepatotoxic effects or from complications such as sepsis, gastrointestinal bleeding or cardiovascular effects. The patients displayed cholestatic jaundice with high bilirubin and alkaline phosphatase concentrations, and increases in hepatic enzymes (aspartate and alanine aminotransferase). Liver pathology showed the presence of an “acute novel toxic hepatitis” similar to that seen in animals exposed to microcystins (*265*). Histopathology showed panlobular hepatocyte necrosis, together with cell-plate disruption and apoptosis. However, in contrast to animal models of microcystin intoxication, no intrahepatic haemorrhage could be observed. After initial uncertainties as to the causative agents of these fatal intoxications, microcystin concentrations were determined in serum and in liver tissue as well as in the water filtration columns. The latter contained intact and fragmented cyanobacterial cells as well as microcystin-LR. Levels in serum ranged from 1-10 ng/mL while concentrations in the liver were as high as 0.6 mg/kg tissue. Toxin congeners were reported to be microcystin-YR, microcystin-LR, and microcystin-AR. At the time of the outbreak, cyanobacterial counts had not been made in the reservoir, but in March 1996, it was found that the most common cyanobacterial genera present were *Aphanizomenon*, *Oscillatoria* and *Spirulina*.

CHRONIC EXPOSURE

When considering the chronic effects of long-term exposure to microcystins in drinking water, one has to take into account the high incidences of PLC in regions of China where pond and ditch water are used as drinking water supplies. In Haimen and Qidong (Jiangsu Province) pond and ditch water used as drinking water shows average microcystin concentrations of 160 pg/mL (60% of the samples analysed were positive), while microcystins could not be detected in well water (*253, 255, 312*). PLC incidences of 4.28 per 100,000 and 100.13 per 100,000 were observed in humans using well water and pond/ditch water, respectively (*253*). It has been calculated that humans living in areas with a reported high PLC incidence consume 0.19 pg microcystin per day during the four summer months of June to September over their 40-50 year life span (*255*). Co-exposure to the potent liver carcinogen aflatoxin B_1 or to HBV may result in the high incidence of PLC in this region (*312*).

EFFICACY OF WATER TREATMENT PROCEDURES

Water treatment measures should always be just one option after other techniques like selection of intake depth, offtake by bank filtration and/or use of barriers to restrict scum movement have been used. When evaluating water treatment procedures for the removal of cyanobacterial toxins, one is faced with problems regarding soluble and suspended substances. Cyanotoxins are produced within the cyanobacterial cells and thus toxin removal involves measures to destroy or avoid the cells. The cyanotoxins also are all water soluble, thus remediation measures involve chemical procedures reducing the toxicity or completely removing the toxins from the drinking water.

Most, especially early, studies had to rely on relatively crude measurements of acute toxicity, since more specific analytical methods were not available. The cyclic heptapeptides have been the focus of most of these studies, but some studies on removal of saxitoxins and anatoxin-a also exist.

Coagulation/flocculation, dissolved air flotation, and activated carbon adsorption

Coagulation or flocculation involves the aggregation of smaller particles into larger particles using chemicals such as ferric chloride or aluminium sulphate. Coagulation can be an efficient method for eliminating cyanobacterial cells from water, whereas

soluble cyanotoxins are not very efficiently removed by this method (*372, 373*). The efficiency of cyanobacterial removal is dependent on an optimisation of chemical doses and coagulation pH (*374*). Coagulation may cause additional problems such as lysis of cyanobacterial cells leading to release of toxins (*372*). When employing dissolved air flotation (DAF), it is important to consider that different cyanobacterial species behave differently depending on their physical properties: in a Belgian DAF plant *Microcystis* was removed by 40-80%, *Anabaena* by 90-100%, but *Planktothrix* only by 30% (*375*). Since conventional water treatment usually involves a combination of these methods, most of the research has focused on the effect of coagulation/flocculation in combination with other measures. In one of the earliest studies, toxins isolated from algal material were subjected to: 1) activated carbon filtration; 2) pre-chlorination, flocculation with $FeCl_3$, sedimentation, sand filtration, and activated carbon filtration; 3) lime pre-treatment, flocculation with $FeCl_3$, chlorination, and activated carbon filtration (*376*). The toxicity of these samples was then tested in the mouse bioassay. Chlorination, flocculation, or sand filtration were unable to destroy the toxins, only the last step, powdered activated carbon (PAC) at a ratio of 1:10 to 1:100 (toxin: activated carbon), removed a toxin concentration of 3 µg/mL to below toxic levels. Studies using 50 mg lyophilised cyanobacteria also show that conventional flocculation, filtration, and chlorination are not efficient in destroying the toxins: HPLC analysis shows a toxin reduction of up to only 34% (*377, 378*). Only the inclusion of a treatment step with activated carbon resulted in 100% removal of the toxins from water. Based on these laboratory studies, pilot-scale experiments were performed to study the feasibility of predicting the behaviour of cyanobacterial toxins in water treatment practice (*379*). Both fresh and freeze-dried cyanobacteria were subjected to the following processes: 1) flocculation with $Al_2(SO_4)$ plus sedimentation plus filtration; 2) PAC plus flocculation with $Al_2(SO_4)$ plus sedimentation plus filtration. Toxicity was measured using the mouse bioassay and HPLC. As was to be expected from the laboratory scale studies, only the inclusion of PAC significantly reduced toxicity. Activated carbon is, however, not always a very efficient method. A study aimed at the removal of cyanobacterial cells with rapid sand filtration and activated carbon found a reduction of cyanobacteria of only 42% (*380*). More detailed studies with activated carbon show that both PAC as well as granular activated carbon (GAC) effectively and quickly (contact times of 30 minutes are sufficient) eliminate cyanotoxins from water (*381-383*). In the case of PAC, dosing is

an important parameter (10 µg toxin/L: >200 mg/PAC/L), while when using GAC the choice of the carbon source is important (coal, wood > peat, coconut), probably due to the different pore sizes relative to the size of the microcystin molecule (*384*). A major concern when using activated carbon in water treatment plants is the formation of a biofilm which can significantly impair the ability of the filter to adsorb toxins and biodegradation by the biofilm does not seem to occur (*381, 385*)(Water Works Zurich, personal communication). Furthermore, below concentrations of 0.15 µg MC-LR/L, very little microcystin will be removed by activated carbon in the presence of a biofilm or natural organic matter (*385*). This finding has consequences for the risk assessment of a chronic exposure to low microcystin concentrations.

Rapid filtration and slow sand filtration

The performance of rapid filtration, a method usually employed after coagulation to remove the floc, does not effectively remove cyanobacterial cells (*380, 386*). Conventional water treatment requires regular backwashing of the filters, but if this washing process is performed inadequately, lysis of cyanobacterial cells on the filters can lead to release of toxins into the water (*11, 387*). Furthermore, sand filtration alone does not lead to substantial reduction of toxicity (*381*) and blocking due to overloading should be avoided (*11*).

Chlorination

In general, chlorination is not an effective process in destroying cyanotoxins (*376-378, 388*). The efficiency of chlorination seems to depend largely on the chloride compounds and the concentration used. Aqueous chlorine and calcium hypochlorite at ≥1 mg/L remove more than 95% of microcystins or nodularin while sodium hypochlorite at the same dose or chloramine achieve at most 40-80% removal (*373, 389, 390*). A chlorine residual of at least 0.5 mg/L should be present after 30 minutes contact time, in order to destroy cyclic peptides completely (*391*). It should be noted, however, that even when acute toxicity, as measured by the mouse bioassay, was removed by this process, progressive liver damage could still be detected in the animals. This subacute toxicity may be due to incomplete toxin removal or to the formation of chlorination by-products, which have been implicated in toxicity (*392*). Anatoxin-a or saxitoxins could neither be destroyed with chlorine doses exceeding a 30 minute chlorine demand, nor by changes in pH (*388, 389*). Cylindrospermopsin,

on the other hand, was effectively oxidised by 4 mg/L chlorine at pH 7.2-7.4 (toxin concentration 20-24 µg/L) (*389*).

Light

Microcystins have been shown to be very stable under natural sunlight (*393*), whereas UV light around the absorption maxima of microcystin-LR and microcystin-RR rapidly decomposed the toxins (*394*). A photocatalytic process using a TiO_2 catalyst and UV radiation also quickly decomposed microcystin-LR, -YR, and –YA with half-lives of less than 5 minutes (*395*). The efficiency of this process was largely dependent on the organic load of the water (*395*).

Membrane processes

Microfiltration (MF) and ultrafiltration (UF) are technologies that have emerged in recent years and have therefore not been thoroughly investigated as to their efficiency in removing cyanobacterial cells or toxins (*53*). One study showed that both UF and MF can be very efficient (>98%) in removing whole cells of toxic *M. aeruginosa* (*396*). An important point when considering filtration, is the lysis of cells. In the case of the above cited study, some damage to cells could be observed, but toxin was not detected in the filtrate. UF was also effective in reducing microcystin and nodularin levels in the filtrate, this may be expected from a membrane with a very low molecular weight cut-off pore size (nanofiltration membrane) (*383, 397*).

Ozonation

In Europe and North America, ozonation has primarily been used for disinfection purposes or to remove colour and/or odour (*398*). Ozone was initially used at the beginning of the water treatment train mainly to inactivate viruses and bacteria. In recent years, though, many water treatment plants have included a two-stage ozonation treatment, either with pre- and inter-ozonation, inter- and post-ozonation, or with pre- and post-ozonation.

In water, two pathways for the oxidation of organic pollutants by ozone have been described (*398, 399*): direct attack by molecular ozone via cyclo-addition or electrophilic reaction and indirect attack by free radicals (primarily ·OH) formed by the decomposition of ozone. The mechanism involving cycloaddition in water usually results in the formation of aldehydes, carboxylic acids, ketones, and/or carbon

dioxide. The electrophilic attack by molecular ozone probably occurs on atoms carrying negative charge, like N, P, O, or nucleophilic C. An indirect attack by free radicals generally occurs via one of three pathways: hydrogen abstraction, electron transfer, or radical addition.

MICROCYSTINS AND NODULARIN

Ozone is one of the most powerful oxidizing agents and its potential to destroy cyanobacterial toxins has been investigated in the last 10 years (see Table 7 for a summary).

In one of the earliest studies looking at the effect of ozone on cyanotoxins, researchers at the British Foundation for Water Research ozonated microcystin-LR purified from *M. aeruginosa* and assessed toxicity using a mouse bioassay (*372, 400*). After ozonation, the toxicity of the cyanotoxin is reduced, which could be shown by a prolongation of mouse survival time, but the results can not be quantified since the authors omitted to detail ozone or toxin concentrations. HPLC and FAB-MS analysis also show a reduction in the microcystin peak after a 2 second ozonation. Interestingly, several new peaks appeared, but were not tested separately for toxicity. This very fast destruction of microcystin was corroborated in Australian studies quantifying the effect of ozone on different microcystin-LR concentrations (*373, 389, 401*) and in our own work (*402*). These studies showed that up to 800 µg/L MC-LR can be oxidized to below the HPLC detection limit by less than 0.2 mg/L ozone within seconds to minutes. The reaction of ozone with nodularin also occurs very rapidly: when reacting 88 µg/L nodularin with 0.05 mg/O_3, there was zero toxin recovery after 15 seconds (*401*). With these studies it was also demonstrated that the removal of microcystins is proportional to the ozone dose when the microcystin concentration is below the ozone demand (*373*). Complete removal of microcystin is achieved and an ozone residual is detected when the ozone demand of the water has been met.

Location	Year	Case	Organism	Toxin	Ref.
Lake Alexandrina, Australia	1878	livestock, stupor	*N. spumigena*	NOD	(*68*)
Ohio River, USA	1931	humans, gastro-enteritis	unspec. cyanobacteria	unknown	(*281*)
Zimbabwe	1966	humans, gastro-enteritis	*M. aeruginosa*	unknown	(*282*)
Alpine Lakes, Switzerland	1974-1994	cattle deaths, liver damage	*O. limosa, O. tenuis*, *Phormidium* sp., *Tychonema* sp., *Pseudoanabaena catenata, P. autumnale*	protein phosphatase inhibition	(*403*)
Armidale, Australia	1983	Humans, liver damage	*M. aeruginosa*	unknown	(*259*)
Palm Island, Australia	1983	Humans, hepato-enteritis	*C. raciborskii*	CYL	(*191*)
Richmond Lake, USA	1988	livestock, dogs, fish, birds	*A. flos-aquae, Aph. flos-aquae, M. aeruginosa*	ANA-a(s)	(*236*)
Rudyard Reservoir, UK	1989	humans: pneumonia, diarrhoea	*M. aeruginosa*	MC-LR	(*277*)
Darling River, Australia	1990-1991	livestock, deaths	*A. circinalis*	PSPs	(*341*)
Loch Insh Scotland, UK	1992	dog, death	*Oscillatoria* sp.	ANA	(*233*)
Itaparica Dam, Brazil	1993	humans, 88 deaths, gastro-enteritis	*Anabaena* sp. *Microcystis* sp	unknown	(*267*)
Loch Leven Scotland, UK	1994	fish, deaths	*A flos-aquae*	MC	*(233)*
Nandong District Jiangsu Province Nanhui/Shanghai Fusui China	1994-1995	humans, PLC 100 of 10^5 (toxins act w/ HbsAg & aflatoxin)	*M. aeruginosa, P. agardhii, O. tenuis*, *Lyngbya* sp., *Anabaena* sp.	MC	(*253, 255, 319*)
Caruaru, Brazil	1996	hemodialysis patients, 60 death	*Aphanizomenon sp , Oscillatoria spec*.	MC	(*263, 265*)

Table 6. Examples for toxic cyanobacteria episodes.

CYANOBACTERIAL EXTRACTS, CELLS AND ORGANIC LOAD

Obviously, a more realistic way to test the efficiency of ozonation would be to use either cyanobacterial extracts or whole cells (Table 8). Oxidation reactions of ozone with cyanobacterial toxins are always in competition with other organic compounds in the water. As a result naturally occurring organic matter is one of the most important factors to consider in terms of toxin dynamics. In a study designed to model continuous operation, ozone doses from 1-10 mg/L were tested over 5-10 minutes for their ability to degrade 10 µg/L microcystin added to different water sources (*383*). Two mg/L ozone added to raw water leads to a 60% removal of

microcystin, while the same dose added to treated water removes toxins by 98%. A similar ozone demand was measured in a study: 500 µg/L MC-LR was oxidised with 0.2 mg O_3/L over four minutes in organic-free water (*404*). The author calculated an ozone demand of 0.6 mg/L with almost complete microcystin removal. However, only 50% of the same microcystin concentration was removed when filtered Seine River (France) water was oxidized with 0.5 mg O_3/L over 10 minutes. This lead to a much higher ozone demand of 1.6 mg/L. Our own results show that cyanobacterial extracts (*M. aeruginosa* or *P. rubescens*) containing 50-100 µg/L microcystin-LR-equivalents need to be oxidized with at least 1.0 mg O_3/L to effectively destroy the toxins present, while ozone residuals were undetectable after 10 minutes (*387*). These results show that ozone consumption by natural organic matter still occurs at the pre-ozonation stage. During post-ozonation, 1 mg O_3/L removed 38% of the microcystin, whereas >2 mg O_3/L removed toxin related toxicity below the limit of detection. The importance of organic load and ozone concentration was also demonstrated in Australian studies: cyanobacterial extracts containing 135-220 µg/L MC-LR required 1.0 mg/L ozone over five minutes for complete toxin destruction (*389, 401*). After this treatment, the ozone residual was zero, reflecting the higher organic load and resultant high ozone demand. The critical importance of ozone dose, especially with respect to the organic load of the water, was also shown in several Finnish studies (*377-379*). Fresh and freeze-dried natural bloom material (*M. aeruginosa, M. wesenbergii, M. viridis*) from a Finnish lake (LD_{50} 60-75 mg/kg BW, mouse i.p.) as well as a laboratory culture of *P. agardhii* (NIVA-CYA 126; LD_{50} 190 mg/kg BW, mouse i.p.) were used. In a pilot plant setup, 35 mg/L fresh (50 µg/L toxin) or 24 mg/L (15 µg/L toxin) freeze-dried cyanobacteria were subjected to pre-ozonation at a dose of 1.0-1.5 mg O_3/L. This treatment resulted in a reduction of toxicity by 50% (freeze-dried) and 90% (fresh). As can be seen, toxin reduction from fresh cyanobacteria was better than from freeze-dried material. This may be explained by the improved coagulation due to pre-ozonation. Pre-ozonation has been widely used to assist coagulation (*398*). The major problem associated with this method is the danger of cell lysis and toxin release. A second post-ozonation step using an ozone concentration high enough to oxidize the remaining organic matter and toxin, would then be essential. The experiment with freeze-dried material corresponds to a situation where the bloom disintegrates and cells lyse due to chemical treatment or as a result of natural causes. Preozonation with 0.5 - 1.0 mg O_3/L in that case is not the

most effective treatment. Postozonation is a preferred method since more of the oxidation capacity could be used on toxins instead of on other organic material. A common problem with these early studies is, however, that toxicity was determined by the mouse bioassay (detection in the µg range) or by HPLC (detection the ng-µg range), two relatively insensitive assays (*405*).

Ozonation of intact cells during pre-ozonation steps poses the risk of cyanobacterial lysis and increased ozone demand. If cyanobacteria are not monitored at the water intake level and thus enter the water treatment process, the treatment plant may not be prepared to meet the increased ozone demand. This leads to either an increase in the soluble toxin concentration in the water and/or to incomplete degradation of the cyanotoxins. Simulating bloom situations, studies were performed with *M. aeruginosa* concentrations from 1 x 10^4 - 2 x 10^6 cells/mL (*388, 401*) (Table 8). Depending on cell number and organic load of the spiked water, an ozone demand between 2-3 mg/L (over five minutes) and 29 mg/L (over 12 minutes) was calculated. This ozone demand is relatively high considering that water treatment plants regularly employ a concentration of 0.5 mg/L at pre-ozonation and 1.0 mg/L at post-ozonation stages, respectively. This can also be seen in our own study where ozonation with 1.0 mg O_3/L did not completely destroy the toxins present in 1 x 10^5 cells/mL (*387*) (Table 8). Furthermore, when a culture of 1.63 x 10^6 *M. aeruginosa* cells/mL was ozonated with a maximum of 3.7 mg O_3/L, only 36% of total toxin was removed after five minutes (*401*).

ANATOXIN-A, ANATOXIN-A(S), AND SAXITOXINS

The efficiency of oxidation with ozone with respect to anatoxin-a, anatoxin-a(s) or the saxitoxins (PSPs) has not been well characterized (*377, 379*). Using raw and filtered waters, the British Foundation for Water Research determined that anatoxin-a is more resistant to removal by ozone than microcystin-LR (*388*). The maximal ozone dose applied (4.5 mg/L) in raw water reduces the anatoxin-a concentration from 2.4 µg/L to 0.6 µg/L, while no ozone residual could be detected. In filtered water, without competition from natural organic material, 2.2 mg O_3/L destroys an anatoxin-a concentration to below the limit of detection (0.3 µg/L). But again, no ozone residual could be detected. The PSPs require even higher O_3 doses. Ozonation over 15 minutes at 4.2 mg/min was necessary to reduce the neurotoxicity of an *A. circinalis* extract to near the lethal threshold concentration (*401*). After 30 minutes ozonation, the mice

survived the doses. There are indications that other PSP toxins, like GTX2, dcGTX2, dcGTX3, C1 and C2, may also be effectively oxidised by ozone (*391*). These studies stress the need for more detailed and quantifiable studies regarding the efficiency of ozone in destroying the neurotoxins.

pH

A very important parameter in the oxidation efficiency of ozone is pH: at pH values >7.5, toxins can still be detected in the samples. This is due to the lower oxidation potential of ozone under alkaline conditions (1.24V) compared to acidic conditions (2.07V).

MC-LR (µg/L)	O_3 dose (mg/L)	Duration (min)	OM present	Destruction (%)	O_3 demand (mg/L)	O_3 residual (mg/L)	Reference
21	1.2	5	-	73	ND	0.13	(*383*)
9	1.0	5	+	50	ND	0	(*383*)
500	0.2	4	-	99	0.6		(*404*)
500	0.5	10	+	50	1.6		(*404*)
≤200	1.0	5	-	100	ND	0	(*389, 401*)
15	1.0-1.5	30	+	50	ND	ND	(*377-379*)
50	1.0-1.5	30	+	90	ND	ND	(*377-379*)
10	0.5-1.5	9	-	90-100	ND	0.4-1.2	(*387*)
50-100	0.5-1.5	9	+	0-100	ND	0.1-0.6	(*387*)

Table 7. Effect of ozone on destruction of cyanobacterial toxins in the presence or absence of organic matter. ND not determined, OM organic matter.

M. aeruginosa (cells/mL)	O_3 dose (mg/L)	Duration (min)	Destruction (%)	O_3 demand (mg/L)	O_3 residual (mg/L)	Reference
1.63×10^6	3.7	5	36	ND	0	(*401*)
2.05×10^6	2.5	12	100	29	ND	(*401*)
1×10^4	0.8	10	60	ND	0.01	(*388*)
1×10^5	1.3	10	65	ND	0	(*388*)
1×10^5	1.0-1.5	9	50-100	ND	0.25-1.4	(*387*)
5×10^5	1.0-1.5	9	30-75	ND	0.4-0.8	(*387*)

Table 8. Effect of ozone on destruction of cyanobacterial toxins from cells in the presence or absence of organic matter.

OZONATION BY-PRODUCTS

In contrast to chlorination by-products, the issue of ozonation by-products has not been properly addressed. One has to keep in mind though, that the amount of ozone applied is always less than what would be required to oxidise all the organic material to CO_2 and H_2O, especially in water with high organic content. One can therefore expect semi-oxidation products to form (*406*). Such oxidation products were found by HPLC when cyanobacteria were pre-ozonated. Their toxicity was, however, not investigated (*379*). An indication of the effect of ozone on microcystins stems from chemical characterisation studies, since ozonolysis has been widely employed for structural characterisation of organic compounds by cleavage of carbon-carbon double bonds (*329, 407*). In the case of microcystins and nodularin, ozonolysis has been applied in the determination of the absolute configurations of the Adda moiety (*408*). It has been described that the double bond between C-6 and C-7 of the Adda side chain is easily cleaved by ozone to give 3-methoxy-2-methyl-4-phenylbutyric acid. In order to realistically assess the consequences of ozonation on cyanobacterial toxins, the ozonation by-products have to be identified and their toxicity tested not only in acute tests, but also in subacute tests such as the phosphatase inhibition assay as well as in chronic situations.

RISK ASSESSMENT

The health risk posed by exposure to cyanotoxins is difficult to quantify, since the actual exposure and resulting effects have still not been conclusively determined, especially for the human situation. The most likely route for human exposure is the oral route via drinking water (*170, 171*), from the recreational use of lakes and rivers (*248*), and/or due to the consumption of algal health food tablets (*149, 283, 284*). The dermal route may play a role during the recreational use of water bodies (swimming, canoeing etc.) (*170, 171*).

Due to the growing concern about health effects of cyanotoxins especially via drinking water, WHO has adopted a provisional guideline value for microcystin-LR of 1.0 µg/L in 1998 (*1*). This guideline value is based on a tolerable daily intake (TDI) value derived from two animal studies (*355, 409*). The first study is a 13-week mouse oral study which determined a no-observable adverse effect level (NOAEL) of 40 µg/kg BW per day based on serum enzyme levels and liver histopathology (*409*). Applying a

total uncertainty factor of 1000 (10 for intra- and interspecies variability respectively, and 10 for limitations in the database, especially lack of data on chronic toxicity and carcinogenicity), a provisional TDI of 0.04 µg/kg BW per day has been derived for microcystin-LR. This TDI was supported by a 44-day pig oral study which determined a lowest observable adverse effect level (LOAEL) of 100 µg microcystin-LR equivalents/kg BW per day (*355*). In this study, the cyanobacterial material fed to the pigs contained several microcystin congeners, but only microcystin-YR was tentatively identified. To this LOAEL, a total uncertainty factor of 1500 was applied (10 for intraspecies variability, 3 for interspecies variability, 5 for extrapolating from a LOAEL to a NOAEL, and 10 for the less-than-lifetime exposure). This resulted in a provisional TDI of 0.067 µg/kg BW per day. WHO used the lower of these two values for establishing the provisional guideline value. This value is calculated by applying the TDI (0.04 µg/kg BW) to a typical daily water intake in liters (L = 2 liters) by an individual of a given body weight (BW = 60 kg) and a proportion (P = 0.8) of the total daily intake to the intake by drinking water:

Guideline value = TDI x BW x P/L

The resulting value of 0.96 µg/L was rounded to 1.0 µg/L and should be applied to cyanobacterial cell-bound and extracellular microcystins. This provisional guideline value is applicable only for microcystin-LR, since the database for other microcystin congeners or even other cyanotoxins such as the saxitoxins is too small to derive a TDI. Health Canada is applying an uncertainty factor of 3000 to the NOAEL of 40 µg/kg BW per day from the 13-week mouse study by adding a factor of 3 for evidence of tumour promotion and weak evidence of a potential for carcinogenicity in humans (*410*). They thus derive a TDI of 0.013 µg/kg BW per day and conclude that the consumption of 1.5 L drinking water containing <0.5 µg MC-LR/L by a 60 kg person would not exceed this TDI. This discussion of the WHO guideline value opens many questions for operators of water treatment plants. Since the guideline value is really only valid for microcystin-LR, in situations where it is not the most dominant congener or not even present, the evaluation of quantitative measurements with respect to the guideline may be problematic. This is true for HPLC analysis as well as for the mouse bioassay and the protein phosphatase inhibition assay (*11*). Results should thus always be reported with these points in mind and should, if possible, be reported for microcystin-LR “concentration equivalents” or “toxicity equivalents”.

The next question which obviously arises, is, which water treatment procedures are adequate to reduce cyanotoxin levels to at least below the WHO guideline value of 1.0 µg/L?

Assessment of water treatment procedures has shown that most methods would result in a reduction of cyanobacterial toxins concentrations to below acutely toxic levels as well as below the new WHO guideline value of 1.0 µg/L drinking water. A completely different situation may arise, however, during a bloom and when water treatment procedures such as chlorination and/or activated carbon are not used together. Even when using ozonation, the specific situation during a bloom has to be observed. Parameters such as organic load of the water has to be determined and toxin levels during the treatment steps have to be monitored. Until issues such as ozonolysis by-products have been resolved, even a very efficient method like ozonation has to be treated with caution. These by-products, which may especially be formed when an insufficient ozone dose has been used, have been detected in several studies but neither their structure nor their toxicity has been determined. Binding to and inhibition of protein phosphatases is considered to be a key mechanism by which microcystins and nodularins exhibit their toxicity. It is therefore critical to know which structural modification to the toxin molecule changes the affinity to and inhibition of the phosphatase. So far, it has been shown that neither the Adda residue alone (*113, 411*) nor linear nodularin- or microcystin-precursor peptides (*114*) bind protein phosphatase or bind with high IC_{50}s of 0.5-1.0 mM and show no toxicity in the mouse bioassay. However, it has also been shown that substitution of the Adda side chain with an L-Cys residue, still leads to interaction of the toxin with the hydrophobic groove of the catalytic subunit of the phosphatase (*114*). The mechanism of tumour promotion by microcystins and nodularins as well as the quantitative relationships have not been satisfactorily elucidated. It is therefore not clear if the inhibition of protein phosphatase constitutes the only or major pathways for toxicity and/or tumour promotion. Our work has shown that in hepatocytes, microcystin binds to proteins other than the phosphatases (*412, 413*). A chronic exposure to cyanobacterial toxins and/or to the ozonolysis by-products should therefore be avoided. The situation for the saxitoxins, anatoxin-a, anatoxin-a(s), and cylindrospermopsin is even less resolved. A broader scientific background on which risk assessment and management steps are based should be developed. This can lead

to sound process-based risk assessment and to the development of effective procedures for water treatment strategies aimed at specific situations.

ENVIRONMENTAL HEALTH PERSPECTIVES • VOLUME 110 | NUMBER 11 | NOVEMBER 2002

EFFECT OF OZONATION ON THE REMOVAL OF CYANOBACTERIAL TOXINS DURING DRINKING WATER TREATMENT

Water treatment plants faced with toxic cyanobacteria have to be able to remove cyanotoxins from raw water. In this study we investigated the efficacy of ozonation coupled with various filtration steps under different cyanobacterial bloom conditions. Cyanobacteria were ozonated in a laboratory-scale batch reactor modelled on a system used by a modern waterworks, with subsequent activated carbon and sand filtration steps. The presence of cyanobacterial toxins (microcystins) was determined using the protein phosphatase inhibition assay. We found that ozone concentrations of at least 1.5 mg/L were required to provide enough oxidation potential to destroy the toxin present in 5 x 10^5 *Microcystis aeruginosa* cells/mL [total organic carbon (TOC), 1.56 mg/L]. High raw water TOC was shown to reduce the efficiency of free toxin oxidation and destruction. In addition, ozonation of raw waters containing high cyanobacteria cell densities will result in cell lysis and liberation of intracellular toxins. Thus, we emphasised that only regular and simultaneous monitoring of TOC/dissolved organic carbon and cyanobacterial cell densities, in conjunction with online residual O3 concentration determination and efficient filtration steps, can ensure the provision of safe drinking water from surface waters contaminated with toxic cyanobacterial blooms.

INTRODUCTION

The presence of toxic cyanobacterial blooms occurring in bodies of water used either as drinking water reservoirs or for recreational purposes may represent serious health risks for the human population. A large number of intoxications not only of cattle (*219, 414, 415*), dogs (*232, 238*), and waterfowl (*228, 229*) but also of humans has been reported. A high incidence of primary liver cancer in China has been attributed to drinking water contaminated with cyanobacterial toxins (*253, 255, 312*), and the tragic deaths of 60 patients in a hemodialysis clinic in Brazil in 1996 was connected to the presence of cyanobacterial toxins in the water supply (*263, 265*). The latter event highlights the importance of adequate water treatment techniques: inadequate bloom monitoring and water treatment by the city's water utility, in combination with insufficient maintenance of the clinic's filters, led to this disastrous event.

Cyanobacteria produce a variety of toxins that are usually defined by their chemical structure and fall into three groups: cyclic peptides (the hepatotoxic microcystins and nodularins), alkaloids (the neurotoxic saxitoxins and anatoxins, and the protein-synthesis–inhibiting cylindrospermopsin), and lipopolysaccharides. The cyanobacterial genera most often associated with toxicity are *Anabaena*, *Aphanizomenon*, *Cylindrospermopsis*, *Lyngbya*, *Microcystis*, *Nodularia*, and *Planktothrix* (*165, 416*). The cyclic peptide toxins are the most widespread freshwater cyanobacterial toxins and are important with respect to treatment of drinking water. Acute intoxications with microcystins result in fulminant liver damage (*141*). Microcystins and nodularins have also been shown to be tumour promoters (*362*).
Concern regarding these potential health risks has prompted the World Health Organization (WHO) to adopt a provisional guideline value for microcystin-LR (MC-LR), the most common microcystin congener, of 1.0 µg/L drinking water (*1*). The development of methods to effectively reduce the concentrations of the toxins as well as their potential breakdown products to below acceptable levels in drinking water has thus become an important focus of current research efforts (*417*). The destruction of cyanobacterial cells by chemical or mechanical means (coagulation/flocculation) is not only insufficient but may also enhance the release of toxins contained within the cells and thus mandates further treatment of the water for drinking purposes. Rapid filtration and slow sand filtration are also not efficient in removing cyanobacterial cells and, in the event of cell lysis on the filter, may even lead to release of toxins into the water. Adsorption via granular activated carbon (GAC) or powdered activated carbon (PAC) adsorption can be an efficient method, provided that an effective carbon type is employed, the carbon is unused, and the dosing of carbon is adequate. Chlorination has been shown in several studies to be inefficient in removing cyanobacterial toxins. Chlorination also has the inherent disadvantage that chlorination by-products are generated, which have been implicated in the subacute toxicity (progressive liver damage) seen in mice after intraperitoneal injection of chlorinated microcystin (*392*). Only if a residual of ≥ 0. 5 mg Cl_2 /L is present after 30 min of contact time is a destruction of cyclic peptides guaranteed. The combination of titanium dioxide, ultraviolet (UV) light, and hydrogen peroxide has been demonstrated to be a potentially viable technique for waterworks faced with microcystin contamination in raw water (*418*). Micro- or ultrafiltration has so far revealed promising results in removing cyanobacterial cells and toxins.

Ozonation has been shown to be a very effective method for destroying microcystins and nodularins. Generally, ozonation is used as a single or multiple application in the water treatment train as an early phase (preozonation) or late phase (intermediate ozonation) of the water treatment process. Pure MC-LR and nodularin can be oxidised within seconds to minutes (*401*). However, because of the competition between the toxins and organic material in the raw water, the ozone present may be rapidly depleted, resulting in incomplete oxidation of the toxins (*419*). Thus, a single ozonation step may not be sufficient, and additional ozonation (intermediate ozonation) is advised. Even if the water treatment train involves both pre-ozonation and intermediate ozonation steps, major problems with toxin contamination may arise if the cyanobacterial biomass is not monitored at the level of water intake. In the case of a large cyanobacterial bloom (high cell numbers), treatment plants could be ill-prepared to meet the increased O_3 demand due to the high organic load. This could lead either to an increase in the soluble toxin concentration and/or to incomplete destruction of the cyanobacterial toxins. Furthermore, the issue of ozonation by-products of cyanobacterial toxins has so far not been adequately addressed. Indeed, one has to be aware of the fact that waterworks do not aim at complete oxidation of organic material to carbon dioxide and water. One can therefore expect semioxidation products to form. Such ozonation by-products have been found by high-performance liquid chromatography (HPLC) when cyanobacteria were ozonated, but neither their structure nor their toxicity has been characterised (*372*).

An additional confounding factor in the analysis of water treatment efficiency in cyanobacterial toxin destruction is the fact that the toxins were either “quantified” via the mouse bioassay, detecting only acutely toxic doses of microcystins or other cyanobacterial toxins, or by HPLC, which clearly does not detect all of the toxins (> 70 microcystin congeners, > 5 nodularin congeners and others) potentially present.

The objective of this study was to determine the potential limitation of a water treatment system having ozonation coupled with several filtration steps, when faced with varying bloom conditions. Toxin analyses were carried out via a biochemical detection technique [protein phosphatase inhibition assay (PPA)]. HPLC-UV was used to identify the predominant microcystin congeners in the cyanobacterial samples. To address these questions, an ozonation batch reactor was modelled according to the technically advanced system in the Lengg waterworks on Lake Zurich, Switzerland. MC-LR, an extract of *Planktothrix rubescens*, a cyanobacterium

blooming regularly in Lake Zurich, and a culture of *Microcystis aeruginosa* PCC 7806 were oxidised using different O_3 concentrations. Filtration steps using fast filtration, activated carbon, and slow sand filtration were included to study the retention of toxins at every step.

MATERIAL AND METHODS

Culture and extraction of cyanobacteria.

M. aeruginosa (PCC 7806) was cultured in 10-L flasks using BG 11 medium (*420*) (26°C, 0.003% CO_2, 24 hr light). Cells were harvested when a density of 10^7 cells/mL was reached. *P. rubescens* was collected from a depth of 10 m from a bloom in Lake Zurich, Switzerland (August 1997), using a net sampler (45 μm) and concentrated via centrifugation (10,000 g, 15 min). The concentrated material was lyophilised and stored at −20°C until extraction. Extracts were obtained from 1 g (dry weight) of cyanobacteria samples. These samples, suspended in 25 mL 75% methanol, were sonicated for 60 min and centrifuged at 48,000 *g* for 60 min, and the supernatants were collected. This procedure was repeated three times. The methanol was removed via rotary evaporation and the final extract resuspended in a defined volume of MilliQ water (MQ-H_2O). Biomass of *M. aeruginosa* was determined via cell counting using a Casy 1 (model TTC; Schaerfe System, Germany) as well as in a Neubauer counting chamber.

Dissolved (DOC)/Total Organic Carbon (TOC) Determination.

Cell culture (100 mL) and extract (100 mL of a 1:1000 dilution) samples were filtered through glass microfiber filters (GF/F; Whatman, USA). Dissolved organic carbon (DOC) in the filtrate was tested using a NCS 2500 elemental analyser (CE Instruments). Particulate organic carbon (POC) was determined in the filter using a TOC-5000 A analyser (Shimadzu). Total organic carbon (TOC) was calculated by adding DOC and POC (precision ± 1%). The detection limit was approximately 0.2 μg carbon.

Determination and quantification of the toxins.

PROTEIN PHOSPHATASE ASSAY (PPA)

PPA was performed as described previously (*421*) using a phosphatase extracted from rape seed (*Brassicus napus*), ^{32}P-adenosine triphosphate (kindly provided by Prof. Werner Hofer, University of Constance), and with MC-LR (Calbiochem, USA) as a standard. In short, samples were preincubated with rape seed phosphatase (10 min, 30°C) and then incubated with ^{32}P -phosphorylase A (5 min, 30°C). The reaction was stopped with ice-cold 20% trichloroacetic acid and centrifugation. Free ^{32}P in the supernatant was extracted with acid molybdate scintillation cocktail (Ready Safe; Beckman, Germany) was added and radioactivity was counted in a liquid scintillation counter (LS 6500, Beckman). The degree of protein phosphatase inhibition (PP-inhibiting capacity) was calculated as a percentage of the phosphatase activity of the control. The PPA was used to approximate the toxin contents of *P. rubescens* extracts and the *M. aeruginosa* cultures. The PP-inhibiting capacity of the latter samples was compared with a MC-LR standard curve and expressed as MC-LR equivalents. The detection limit of the radioactive PPA that we use in our laboratory is 0.05 µg MC-LR/L with a derived IC_{50} of 0.25 µg MC-LR/L. Because of the PPA-determined

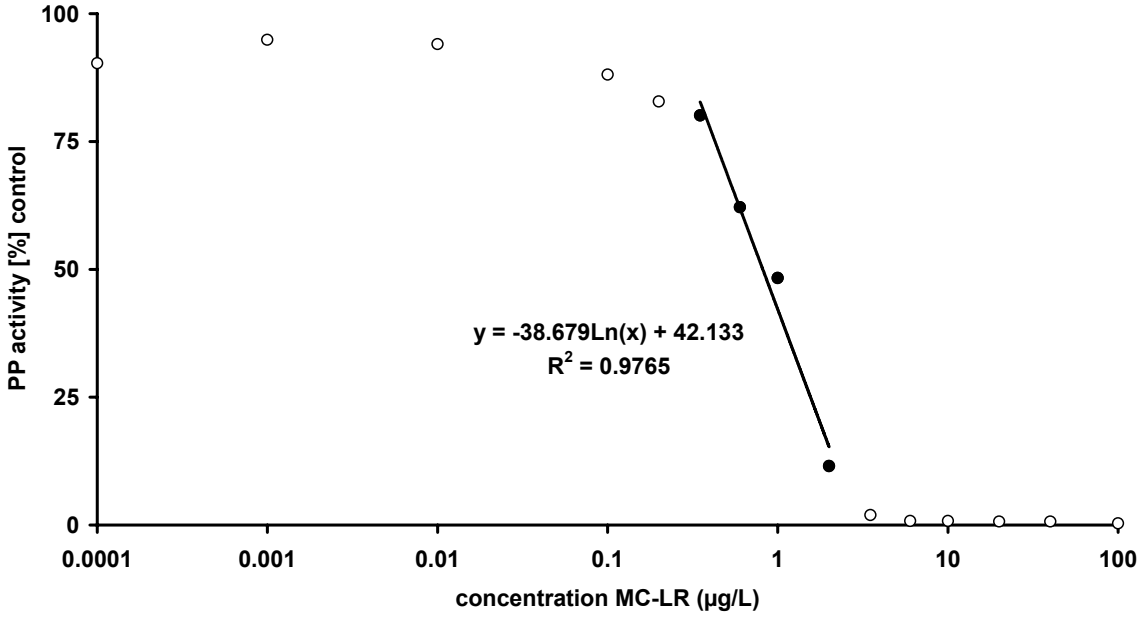


Figure 5. Inhibition of protein phosphatases by MC-LR (diluted 1:4). The IC_{50} extrapolated to 0.25 µg MC-LR/L. y = -38.679 Ln(x) + 42.133. R^2 = 0.9765

dilution factor (1:4), the WHO (*1*) guideline of 1.0 µg/L corresponds to 50% inhibition in the assay (Figure 5).

HPLC

HPLC was used for determination of microcystin congeners in *M. aeruginosa* and *P. rubescens* samples. Toxins were analysed according to the method B described by Meriluoto *et al.* (*422*), with slight variations. Briefly, external standards were prepared for MC-LR (Calbiochem, USA) microcystin-RR (MC-RR) (Sigma, Germany), desmethyl MC-LR and desmethyl MC-RR (kindly provided by J. Meriluoto, Turku, Finland). Acetonitrile/0.0135 M ammonium acetate (27:73 v/v) was used as the mobile phase at a flow rate of 1 mL/min. Solid-phase extraction (SPE) of cyanobacterial extracts (1 mL each) was performed using Isolute C18 end-capped SPE cartridges (International Sorbent Technology, UK) conditioned with 10 mL methanol and subsequently washed with 10 mL MQ-H_2O. Cyanobacterial extracts were applied to the cartridge and, after washing with 10 mL MQ-H_2O, eluted with 10 mL of 100% methanol. The eluent was dried under a nitrogen atmosphere and resuspended in 1 mL of the mobile phase (acetonitrile/0.0135 M ammonium acetate). If necessary, extracts were filtered through a 0.22 µm Millex-GV filter. Extracts were injected into the HPLC (Beckman Autosampler 507e, Solvent Module 125, Programmable Detector Module 166, Beckman Ultrasphere ODS Column, 250 x 4.6 mm, 5 µm) and peaks were compared with standards.

OZONATION

O_3 was produced in an O_3 generator (type LN 103 AT, kindly provided by Ozonia, Duebendorf, Switzerland) by regulation of voltage (25–50 mA) and gas flow (166–208 cm^3/min) with oxygen as substrate. MC-LR purified standard and extracts of *P. rubescens* and *M. aeruginosa* culture material were ozonated with different concentrations for 9 min of contact time (O_3 on) and 60 min of reaction time (O_3 off) in a 3.2-L batch reaction vessel equipped with a fritted glass sparger. Culture and extract were diluted in artificial lake water (625 mg/L NaCl, 962 mg/L $NaHCO_3$, 30 mg/L KCl, 20 mg/L $CaCl_2$ x H_2O, 60 mg/L $CaSO_4$ x $2H_2O$, 154 mg/L $MgSO_4$ x 7H2O), ozonated at constant pH (8.0) and constant temperature (6–8°C). Artificial lake water was used to obtain a standardised medium, which tap water cannot provide, and to reflect lake conditions. MC-LR was ozonated in MQ-H_2O. The final

concentrations were 0.5, 1.0, and 1.5 mg diluted O_3/L. The O_3 concentrations could not be adjusted exactly after 9 min of contact time and varied therefore around the aim concentrations of 0.5, 1.0, and 1.5 mg diluted O_3/L. Samples for toxicity determination via PPA were collected before ozonation, after 9 min of contact time, and after 60 min of reaction time and stored at −20°C. O_3 concentration was determined at times t_9 (at 9 minutes after O_3 contact time), t_{14}, t_{19}, t_{29}, … , t_{69} by the Indigo method (*423*).

FILTRATION

To test the efficiency of the filtration steps typically used in a technically advanced water treatment system to remove or retain cyanobacterial toxins and/or their ozonation by-products, a laboratory-scale model filter system was set up that was composed of three columns. The columns used for filtration were 80 cm long with an internal diameter of 9 cm: *a*) Column 1: rapid sand filtration with 40% (1,780 cm^3) pumice/60% (2,800 cm^3) quartz sand, average flow of rate 425 mL/min; *b*) Column 2: activated carbon filtration with 75% (3,436 cm^3) GAC/25% (1,145 cm^3) quartz sand, average flow rate of 475 mL/min; and *c*) Column 3: slow sand filtration with 100% slow sand (4,580 cm^3), average flow rate of 125 mL/min.

The efficiency of the filtration steps was tested with extract of a *P. rubescens* bloom and with a toxic *M. aeruginosa* culture. Previously ozonated (0.5 mg O_3/L) samples (2.5 L), which still had shown phosphatase inhibitory activity after ozonation, were filtered successively through these three columns. After each filtration step, samples were collected and stored at −20°C for PPA. In experiments with *P. rubescens* extract (100 µg MC-RR and desmethyl MC-RR/L), the filtration materials were changed after every filtration step. In the second experiment with the *M. aeruginosa* culture (MC-LR equivalents between 25 and 250 µg/L), filtration materials were reused to determine the effects of cyanobacterial preloading on the removal/retention capabilities of the filtration materials.

RESULTS

To mimic different cyanobacterial bloom situations resulting either in high cell concentrations and high TOC concentrations or in high concentration of free toxin in the water, pure MC-LR (in MQ-H_2O), extract of a *P. rubescens* bloom, and *M. aeruginosa* cells at different cell densities (both in artificial lake water) were

ozonated in a batch reactor system. TOC concentrations ranged from < 0.14 mg/L for MQ-H_2O to 3.54 mg/L for *P. rubescens* extract in MQ-H_2O (Table 9). Initial toxin concentrations in the samples (*M. aeruginosa* cells and *P. rubescens* extract), determined using the PPA, were between 12 and 100 µg/L, expressed as MC-LR equivalents. The concurrent analysis of these samples via HPLC demonstrated the presence of MC-LR in the *M. aeruginosa* cells and MC-RR and desmethyl MC-RR in the *P. rubescens* extract. Ozonation of these samples with 0.5, 1.0, and 1.5 mg O_3/L led to a decrease in the PP-inhibiting capacity of the samples (Table 10). This reduction appeared largely dependent on the respective TOC of the samples as well as on the presence of intact cells and the respective cell numbers present.

Ozonation of MC-LR

Ozonation of 10 µg/L MC-LR with 0.5 mg O_3/L quickly destroyed the toxin within 9 min contact time (t_0–t_9; Table 10). No oxidation capacity (O_3 residual) was detectable 30 min minutes after active ozonation (t_{39}, Figure 6A), whereas 50% of the initial O_3 was still detectable in the corresponding controls. In contrast, ozonation with either 1.0 or 1.5 mg/L left an O_3 residual of 0.27 ± 0.16 and 0.72 ± 0.03 mg/L, respectively, at 30 min after active ozonation (t_{39}; Figure 6B,C).

Ozonation of *P. rubescens* extract

One of the main problems associated with oxidation reactions is that numerous particles and organic and inorganic compounds can compete for the oxidative capacity during ozonation. This is exemplified with extract from a toxic *P. rubescens* bloom, which has high concentrations of TOC and toxin (Table 10, Figure 6A). As shown in Table 10, 0.5 mg O_3/L was not sufficient to significantly reduce the PP-inhibiting capacity of this extract. Indeed, all the O_3 was consumed within 10 min after initial ozonation (t_{19}; Figure 6A). Using higher O_3 concentrations, the PPA-inhibiting capacity could be reduced (Table 10). However, all of the O_3 was consumed to < 0.2 mg/L within 10 min (t_{19}), and no O_3 residual could be detected after 20 min (t_{29}; Figure 6B,C).

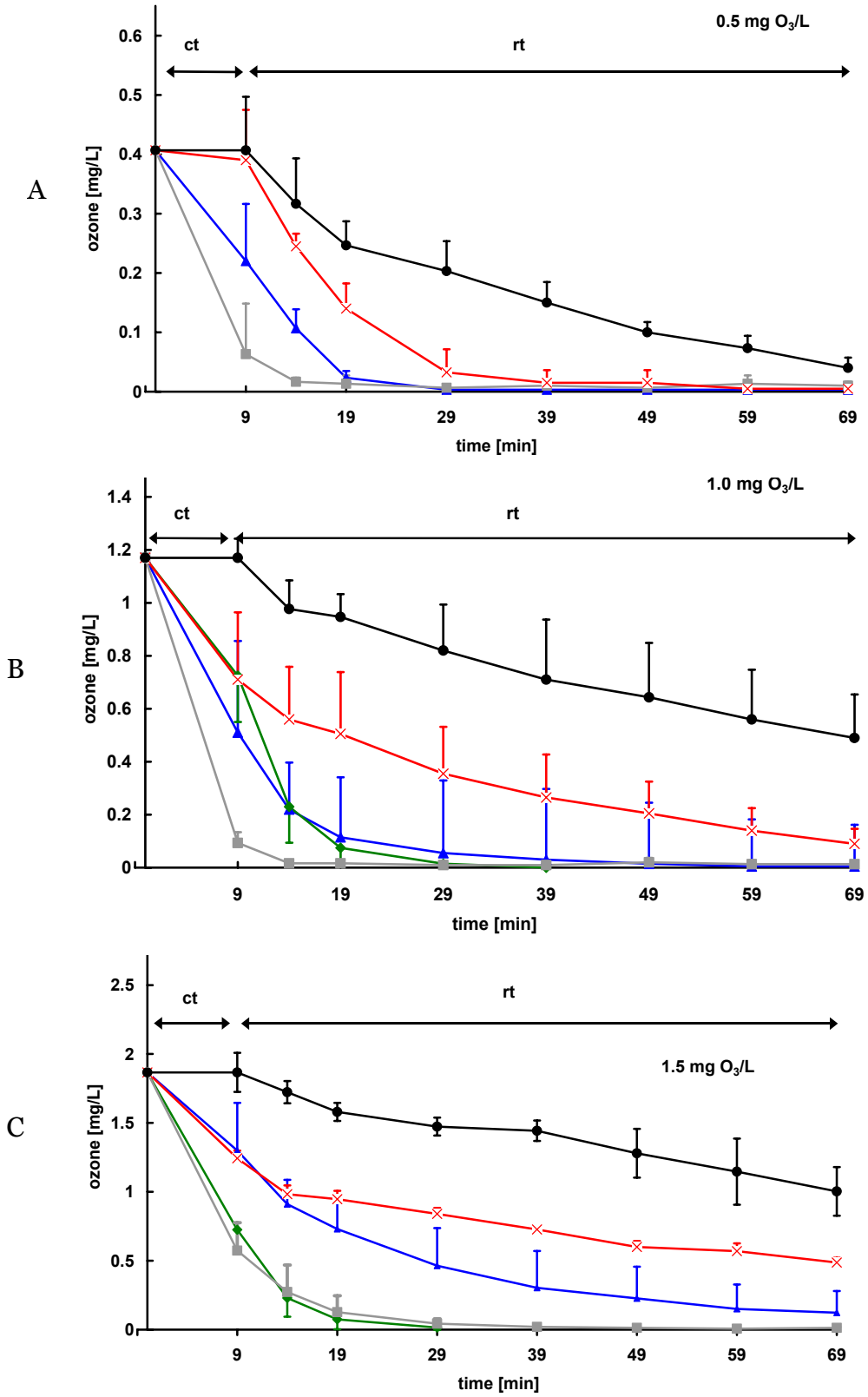


Figure 6. Effects of ozonation on MC-LR (✕, 10 µg/l), 1x10^5 cells/ml *M. aeruginosa* (▲), 5x10^5 cells/ml *M. aeruginosa* (◆), and extract of *P. rubescens* (■) on ozone consumption compared with control (●). A) 0.5 mg O_3/l ; B) 1.0 mg O_3/l ; C) 1.5 mg O_3/l . ct: contact time, rt: reaction time (n=3). Error bars indicate SD

Ozonation of *M. aeruginosa* cells

Oxidation of intact cyanobacteria most closely mimics the situation when a cyanobacterial bloom enters the water intake of a water treatment plant. Ozonation of intact cells, while consuming a large proportion of the oxidation capacity, will induce cell lysis and thus could provide increased toxin concentrations in the treated water, this being largely dependent on the cell density of the respective bloom. Therefore, different cell densities of toxic *M. aeruginosa* were subjected to ozonation at three different O_3 concentrations. As demonstrated in Table 10, ozonation of 1 x 10^5 cells *M. aeruginosa*/mL with 0.5 mg O_3/L was not sufficient to completely destroy the PP-inhibiting capacity after 9 min (t_9) and even after 69 min (t_{69}) of contact time. Indeed, ozonation with 0.5 mg/L and a cyanobacteria density of 1 x 10^5 cells *M. aeruginosa*/mL also consumed almost all of the O_3 within 19 min (t_{19}), while the toxin content was still high (Figure 6A, Table 10). Although 1.0 mg/L O_3 was sufficient to cope with a "bloom" containing 1 x 10^5 cells *M. aeruginosa*/mL, this was not the case when cell densities were higher (i.e., 5 x 10^5 cells *M. aeruginosa*/mL). When 1 x 10^5 or 5 x 10^5 cells *M. aeruginosa*/mL were ozonated with 1.0 mg O_3/L, also nearly all of the O_3 was consumed (< 0.2 mg/L) within 19 min of contact time (t19; Figure 6B, Table 10). However, while although the amount of O_3 ozone was sufficient to reduce the toxin in the sample with 1 x 10^5 cells/mL (Table 10), a PP-inhibiting capacity of > 20% (~0.37 μg MC-LR/L) remained after 69 min minutes of contact time (t_{69}) in the sample containing 5 x 10^5 cells/mL. In the latter case, a minimum of 1.5 mg O_3/L was required to significantly reduce the PP-inhibiting capacity of the cyanobacteria (Table 10).

Effects of filtration

Modern water treatment plants routinely employ different filtration steps after ozonation. For example, the Lengg waterworks uses quartz sand/pumice, activated carbon/quartz sand, and then slow sand. To study the effect of the filtration steps on the reduction of the PP-inhibiting capacity, *P. rubescens* extract still displaying toxic activity after ozonation with 0.5 mg O_3/L was filtered in the laboratory-scale model filter system. The filtration medium was changed after each experiment. Although quartzsand/pumice filtration removed most of the PP-inhibiting capacity, activated carbon/quartzsand and slow sand filtration was necessary to completely reduce the

remaining PP-inhibiting capacity (Figure 7). However, water treatment plants normally do not change the filter materials for several years (10-15 years on average at the Zurich Water Works Zurich/Switzerland). Therefore, the danger of repeated overloading of the filtration materials resulting from bloom events theoretically exists. During back-washing of filter material, release of toxic material may also occur. To mimic the latter situation, a *M. aeruginosa* sample (2 x 10^6 cells/mL) still displaying PP-inhibiting capacity following after previous ozonation with 1.0 mg/L O_3 was filtered through the filter columns of the lab-scale model filter system. As expected, activated carbon/quartzsand filtration removed most of the PP-inhibiting capacity (Figure 8). However, when an ozonated (1.0 mg O_3/L) *M. aeruginosa* sample (2 x 10^5 cells/mL) was filtered with the same columns (i.e., without changing the filter materials) an increased PP-inhibiting capacity was observed (Figure 8).

Sample	Toxin concentration (PPA)	Dominant toxin congener(s) (HPLC)	TOC [mg C/L]
MQ-H_2O	-	-	0.14
Artificial lake water	-	-	0.36
MC-LR (in MQ-H_2O)	10 µg/L	MC-LR	0.146
***P. rubescens* extract (in MQ-H_2O)**	100 µg MC-LR equiv./L	MC-RR, desmethyl - MC-RR (>80%)	3.54
***M. aeruginosa* 10^5 cells/mL (in artificial lake water)**	12 µg MC-LR equiv./L	MC-LR (>90%)	0.6
***M. aeruginosa* 5×10^5 cells/mL (in artificial lake water)**	60 µg MC-LR equiv./L	MC-LR (>90%)	1.54

Table 9. TOC and concentration and congener composition of toxins and of ozonated samples. 0.14 for MQ-H_2O + 0.006 for microcystin (calculated, not measured).

Because the PP-inhibiting capacity of the second sample had been almost completely removed by ozonation, this increased inhibiting capacity could not stem from this sample. Most likely, this inhibiting capacity resulted from toxins retained in the filters during the first filtration experiment and then released from the quartz sand/pumice filters during the second filtration experiment. Subsequent activated carbon/quartz sand filtration, however, was able to remove the majority of the observed PP-inhibiting capacity (Figure 8).

	MC-LR (10 µg/L)			*P. rubescens* extract		
Ozone (mg/L)	**0.5**	**1.0**	**1.5**	**0.5**	**1.0**	**1.5**
PP activity t_0 [% control]	62.5 (±1.9)	69.3 (± 4.4)	62.7 (± 4.2)	3.7 (± 1.6)	3.4 (± 2.6)	3.3 (± 1.1)
PP activity t_9 [% control]	105.3 (± 2.0)	87.6 (± 7.3)	91.3 (± 5.1)	6.2 (± 6.1)	110.7 (± 12.3)	87.7 (± 7.4)
PP activity t_{69} [% control]	103.2 (± 14.9)	94 (± 14.8)	103.4 (± 10.4)	8.8 (± 2.8)	99.4 (± 13.9)	95.5 (± 6.6)

Table 10. Protein phosphatase activity before (t_0), after ozonation (t_9) and after 60 min reaction time (ozone off, t_{69}) with different concentrations (± sd, n=3, ND not determined). Shown are results after ozonation of MC-LR, *P. rubescens* extract (above) and different cell densities of *M. aeruginosa* (below).

	M. aeruginosa					
	1 x 10^5 cells/mL			**5×10^5 cells/mL**		
Ozone (mg/L)	**0.5**	**1.0**	**1.5**	**0.5**	**1.0**	**1.5**
PP activity t_0 [% control]	35.7 (± 11.9)	36.8 (± 0.4)	34.6 (± 7.8)	ND	0.9 (± 1.6)	0.5 (± 0.2)
PP activity t_9 [% control]	67.1 (± 4.1)	98.3 (± 0.5)	85.8 (± 6.0)	ND	39.2 (± 20.7)	81.8 (± 13.9)
PP activity t_{69} [% control]	88.9 (± 13.7)	101.9 (± 9.4)	92.3 (± 2.9)	ND	77.9 (± 20.9)	92.6 (± 2.9)

DISCUSSION

Ozonation has previously been shown to be an effective method to reduce the cyclic peptide toxin concentration of waterborne cyanobacteria (*401, 419*). This study confirms those findings. The data presented here, however, emphasise that it is essential to investigate the conditions under which toxin destruction is optimal.

The importance of free toxin regarding drinking water contamination is moderated by the fact that, in most cases, the free toxin levels rarely exceed 10 µg/L or < 10% of the total toxin present in the actual cyanobacteria bloom (*424-426*). Microcystin levels > 70 µg dissolved MC/L have only been measured in a case of a thick surface scum, which lysed and rotted (*196*). Free MC-LR is oxidised rapidly by O_3 and has a half-life of 1 sec at 0.1–2.0 mg O_3/L (*419*). More emphasis must therefore be placed on the efficacy of ozonation of raw water contaminated with high densities of cyanobacterial cells. *Microcystis* and *Planktothrix* can reach very high cell densities (10^3–10^6 cells/mL) in water reservoirs (*54, 305, 400, 427-431*). Treatment of raw

water from such contaminated bodies of water is often the only choice because alternative water sources may not be available or the depth of the water body may be too low to use alternative water intake levels. The aim of water treatment plants is to eliminate colour, bad taste, infectious organisms, and known toxic compounds such as pesticides. The intention is usually not the destruction of cyanobacterial cells by ozonation (*432*), but rather the removal of intact cells by flocculation and filtration. Destruction of cells may lead to an increase of macromolecular compounds (disinfection by-products), which are difficult to remove during water treatment (*433*). However, on a daily basis, it is not always possible to apply an effective O_3 concentration that does not lead to destruction of cyanobacterial cells. Microscopic observations during the experiments in this study showed that the majority of *Microcystis* cells lyse at concentrations of 1 mg O_3/L, a concentration routinely used in water treatment plants. This contradicts the observations of Plummer and Edzwald (*433*), who reported lysis of cyanobacterial cells only at concentrations > 3 mg O_3/L. Consequently, oxidation of intact cyanobacterial cells often leads to cell lysis and

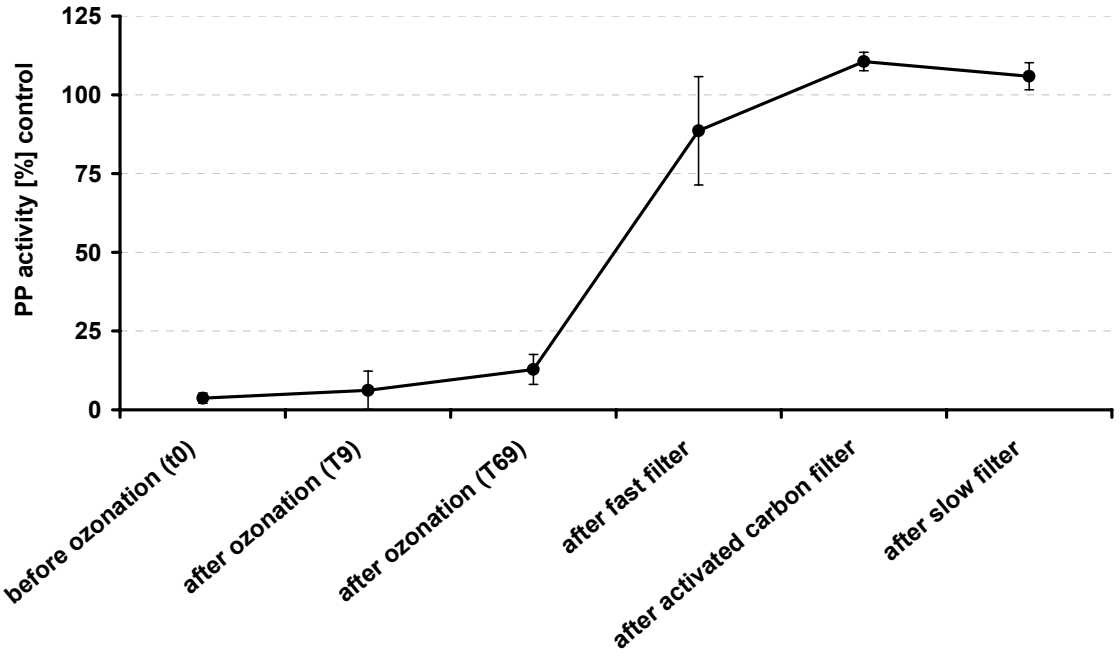


Figure 7. Filtration of a toxic *P. rubescens* extract through quartz sand/pumice (fast filter), activated carbon/quartz sand (activated carbon filter) and then slow sand after the ozonation step (0.5 mg O_3/l). PP activity is shown as percentage of control (n=3). Error bars indicate SD.

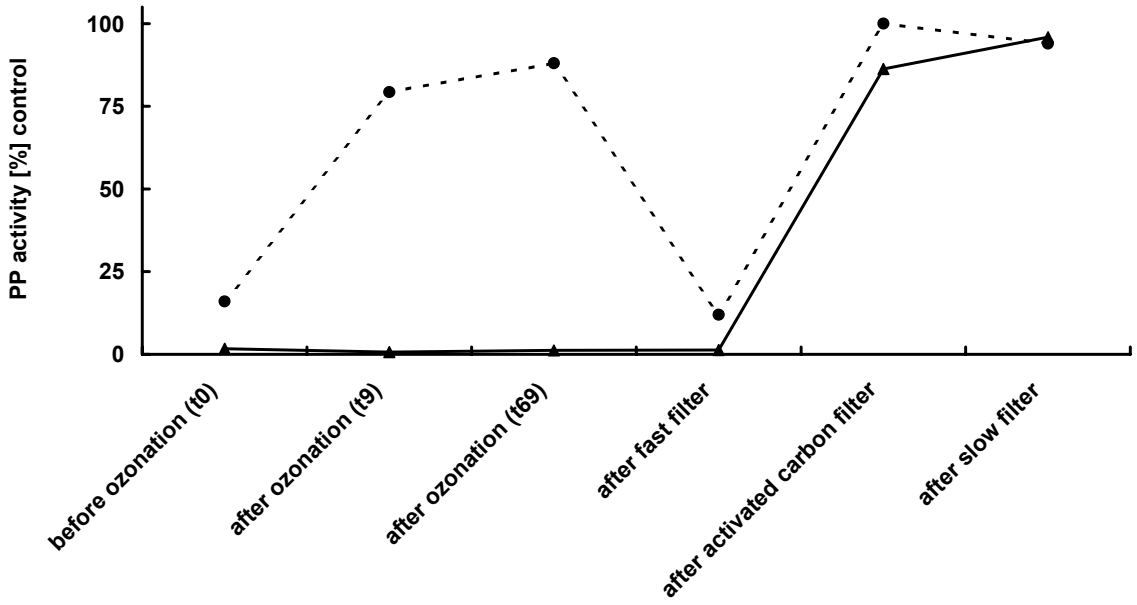


Figure 8. Result of repeated use of filtration material [quartzsand/ pumice(fast filter), activated carbon/quartzsand (activated carbon filter), and slow sand]. In the first filtration (▲) *M. aeruginosa* cells (2 x 10^6/mL; PPA still displaying PP-activity after ozonation with 1.0 mg/L) were filtered through the three filters after the ozonation step. In the second filtration (•) 2x10^5 cells *M. aeruginosa*/mL (no toxicity after ozonation with 1.0 mg/L) were filtered through the same material. A breakthrough of PP-inhibiting compounds are present after the first filtration. PP activity is shown as percentage of control of a representative experiment.

subsequent release of toxins. Therefore, sufficient oxidation capacity has to be provided to destroy both the cells and the toxins. Indeed, as shown in this study in cases where cell numbers exceed 10^5 cells/mL, an average of 0.5 mg O_3/L will not suffice to guarantee complete toxin destruction (Table 10). Only single pulse (9 min of contact time) of ozonation with 1.5 O_3 mg/L provides enough oxidation capacity to ensure the destruction of the PP-inhibiting toxins after 60 min of reaction time (Table 10). As an alternative to average O_3 concentrations, water treatment plants treat water with 1.0 mg/L O_3, monitor O_3 concentrations, and automatically add O_3 to the reaction basin when the residual O_3 concentrations fall below 0.5 mg/L. This, however, may not ensure complete destruction of cyanobacterial toxins because of variations in TOC composition and concentrations in raw water, resulting in O_3 concentrations falling below 0.5 mg/L for certain time periods and thus allowing cyanobacterial cells to enter the next filtration step.

One of the most important factors influencing ozonation capacity is TOC or DOC (*383, 419, 434*). The extent and rate of TOC removal typically increase as ozone dose

increases (*435*), but at routinely employed O_3 ozone concentrations of > 1.2 mg O_3/mg C, TOC is not decisively reduced (~10–20%) (*433, 436, 437*). Nevertheless, if the TOC is high in raw water, the efficacy of O_3 in destroying free cyanobacterial toxins is dramatically reduced, as exemplified by free MC-RR/desmethyl MC-RR in this study. Existing data suggest that the cyanobacterial toxins, a subset of the natural organic compounds in raw water, are oxidised proportionally to their fraction of the TOC. Consequently, it appears vital that the intra- and extracellular toxin concentrations as well as the TOC are known before raw water is treated with O_3. The results presented here are important in consideration of the fact that raw water may contain varying and often higher TOC values than the ones employed in the present study (Lengg water treatment plant, Lengg 1996–2000, DOC 1.1–1.4 mg C/L). Because of variation of TOC and therefore variation of substances that can be oxidised (e.g. compound structures containing double bonds), a generalised statement cannot be made. In contrast to the situation in waterworks, in this study cyanobacteria were the exclusive source of the organic material. However, Shawwa and Smith (*419*) show that TOC generally influences the kinetics of MC-LR oxidation by O_3. During ozonation of extracts of toxic *P. rubescens*, a predominant phytoplankton species in many European lakes that regularly gives rise to toxic blooms (*438, 439*), O_3 concentrations < 1.0 mg/L are insufficient to completely destroy the toxins when TOC levels rise above 3.0 mg/L (Tables 9 and 10). A comparison of the experiments using *P. rubescens* extracts with those using *M. aeruginosa* at a density of 5 x 10^5 cells/mL demonstrates the difference between free toxin in TOC-rich water and cell-bound toxin. Despite a lower TOC content and lower toxin concentrations, phosphatase inhibition could still be detected in the experiments using *M. aeruginosa* cells but not in the *P. rubescens* extracts. Similar results were obtained in the only comparable study, where during a 10-min ozonation of 10^4 and 10^5 *M. aeruginosa* cells with 0.8 and 1.3 mg O_3/L, respectively, only about 60% of the toxin was destroyed and the O_3 was completely consumed (*388*). Even ozonation with very high O_3 levels (3.7 mg/L, 5 min) is not sufficient to completely eliminate microcystin levels when high cell numbers (2 x 10^6 cells/mL) are present and when dealing with high TOC levels (8–11.4 mg/L) (*401*). Ozonation of toxin-free cyanobacteria was not performed in the present study or, to our knowledge, by other investigators. Therefore, we cannot exclude the possibility that other cyanobacterial compounds show a PP-inhibiting activity after ozonation of whole cells. Our experiments were carried out at 6–8°C

and at pH 8. O_3 capacity of water decreases with increasing temperature [this investigation (data not shown) and Langlais *et al.* (*398*)] and decreasing pH (*398, 401*)]. These parameters have to be kept in mind when different waterworks (with different raw waters) and different investigations are compared with each other.

The Lengg water treatment plant at Lake Zurich, Switzerland, employs one filtration step between preozonation and the main intermediate ozonation step and two filtration steps before drinking water release. This situation was modelled in the present study and was shown to be very efficient in removing remaining phosphatase inhibitory activity of *P. rubescens* extracts when using fresh filter materials (Figure 7). In water treatment plants, however, such filter materials may be in use for several years and are used as a substrate for a microbial biofilm rather than for filtration/adsorption purposes. This can therefore significantly impair the ability of the activated carbon filter to adsorb toxins (*382, 385*), whereas biodegradation on the biofilm does not seem to occur (*385*). Frequent changes of filter materials are obviously also very costly and thus rarely carried out. As the present study suggests, there could potentially be a danger of a breakthrough of cyanobacterial toxins even when quartz sand/pumice filter materials are used only once (Figure 8). Therefore, only the efficient destruction of the cells and toxins via high concentrations of O_3 potentially offers the necessary protection from microcystin contamination of drinking water.

In light of the tumour-promoting activity of microcystins, an exposure of the general public even to very low microcystin concentrations should be avoided. However, because ozonolysis by-products have not been characterized regarding their structure and toxicity, the reliability of even a very efficient method such as ozonation during cyanobacterial bloom events should be treated with caution. In conclusion, despite the uncertainties described above, a modern water treatment plant using pre- and intermediate ozonation steps in conjunction with online O_3 measurements and different filtration steps, and regular monitoring of TOC/DOC and cyanobacterial cell densities in the raw water, should be able to provide safe drinking water.

EFFICACY OF OZONATION AND DIFFERENT FILTRATION STEPS IN A LABORATORY IMITATION OF DRINKING WATER TREATMENT PROCESSES

Drinking water for human consumption is taken from ground, spring or surface waters. Because many surface waters in Europe are predominated by the filamentous species *P. rubescens*, drinking water treatment plants must eliminate these cyanobacteria to avoid bad taste and odour in drinking water. But *P. rubescens* is also a known producer of several congeners of microcystins, a family of heptapeptides with primarily hepatotoxic effects. To mimic the drinking water treatment in European water works, cell densities of 15,000 cells *P. rubescens*/mL were ozonated with 0.8 mg ozone/L and subsequently filtered through different filter materials. After ozonation, fifty percent of the cells were still present as recorded by an image processing system (IPS). The toxin concentration measured by Adda-ELISA increased four- to six-fold after ozonation and reached a microcystin (MC) concentration of more than the WHO provisional guideline value of 1.0 µg MC-LR equivalents/L. However, the concentration decreased in the subsequent filtration steps to below 10 ng MC-LR equiv./L. The presented results show the efficacy and ability of relatively low ozone concentrations to break up cells of filamentous cyanobacteria followed by a release of the formerly intracellular toxin into the water and the removal of these toxins by a three-step filtration method.

INTRODUCTION

Red cyanobacterial blooms in surface waters caused by *Planktothrix rubescens* are a known phenomenon in anecdotic reports (*62, 440*). Blooms of cyanobacteria (blue-green algae) are also called water blooms, which are cyanobacterial cells of such density as to render it visible to the human eye (*8*). Blooms of *P. rubescens* are untypical with respect to this definition, because they occur in deeper layers of the surface waters and are thus invisible for the human eye (*4, 303, 441*). Only seldomly high densities of *P. rubescens* appear at the water surface to build a visible red bloom. The particular behaviour of *P. rubescens* results in a frequent occurrence of this cyanobacterial species in raw water of water works ((*4, 442*), chapters VI, VII). Water works try to avoid raw water with high organic load to minimize the costs for drinking water treatment. Because most phyto- and zooplankton exits in the epilimnion of surface water, the water intake of most of the water works is set in

water depths between 15 and 40 m. However, *P. rubescens* is found either stratified in the metalimnion of the water bodies or, after spring and/or autumn storms, distributed over the whole water column (*27*). The gas vacuole apparatus of these cyanobacteria allows the cyanobacterial population to stratify in layers with optimised conditions with respect to light and nutrients, but a minimal density gradient in the water column is necessary to stay in a distinct layer (*443*). The vacuoles are very stable and according to Walsby *et al.* (*439*) guarantee a survival of half of the population even if they are distributed in deep layers of the lake (70-90m). However, the stability of the gas vacuoles differs between subpopulations of *P. rubescens (439)*. In contrast, Gammeter *et al.* (*25*) mentioned a dying off under light limitation in the hypolimnion and suggested, that *P. rubescens* only remain vital in the euphotic zone. In addition, *P. rubescens* prefers higher temperatures in the lake and is oligophotic (*444*). Therefore this cyanobacterial species has an effective light harvesting system including phycoerythrin and carotenoids. As well as this ecological characteristic, *P. rubescens* produces several microcystin congeners (*115, 445, 446*) and other biologically active secondary metabolites (*447*). Microcystins have been shown to promote liver tumours (*367, 448*) and evidence exists that they can also initiate tumours (*449*). Thus it is likely, that microcystins can act as carcinogens, which underlines the importance of eliminating microcystins during drinking water treatment.

Only one study is available in which *P. rubescens* filaments have been used for drinking water treatment experiments (*450*). An ozone concentration of 0.3 mg/L has been shown to reduce the intracellular toxicity by one third and the extracellular concentration of microcystin by half, both measured by HPLC-MS-MS. Subsequent flocculation and filtration reduced the intracellular toxin concentration to below 0.1 µg/L, but the extracellular toxin concentration increased to an amount higher than the concentration in raw water. The researchers explained this increase by the mechanical pressure during the treatment process, which results in increased lysis of the *P. rubescens* filaments.

Own investigations in a drinking water treatment plant in Switzerland with *P. rubescens* in raw water (chapter VI) and lab-scale experiments with the colony-building cyanobacterium *Microcystis aeruginosa* have shown, that ozonation can result in higher extracellular toxin concentrations (chapter IV,(*445*)).

To investigate the possible lysis of the filamentous cyanobacterium *P. rubescens* during ozonation and the elimination efficacy with respect to cells and free microcystins during ozonation and filtration processes, typical treatment steps were modelled at the laboratory scale and the extracellular toxin concentrations measured after every treatment step.

MATERIAL AND METHODS

The cell density of a culture [in BG11 medium (*420*)] of the *P. rubescens* strain A7 (kindly provided by J. Blom, Institute of Plant Biology, University of Zurich, Switzerland), isolated from Lake Zurich, was determined using IPS (Visiometrics, Constance, Germany) (*451*). Subsequently the culture was diluted with artificial lake water (described in detail in chapter IV or (*445*)) to reach cell densities of approximately 15,000 cells/mL. Experiment 1 (E1) and 2 (E2) were carried out with almost identical cell densities (Table 11). Fifty mL of the *P. rubescens* solutions were combined with 200 mL of water with a concentration of approximately 1.2 mg O_3/L. The ozone concentration was measured in the samples with the *P. rubescens* culture and in a control sample, in which 50 ml BG11 was added instead of the *P. rubescens* culture. Subsequently, the ozonated samples were filtered through the following filtration system (kindly provided from Water Works Zurich, Switzerland):

Fast filter system (FFS): pumice: 78 mL + quartz sand: 122 mL

Activated carbon filter system (ACFS): peat-based granulated activated carbon (Norit Row 0.8 Supra; Norit, Amersfoort, Netherlands): 150 mL + quartz sand: 50 mL

Slow sand filter (SSF; graining: 0.2-2 mm): 200 mL

Before use, the filters were washed with 2 L MQ-H_2O water to clean the filter material. Then the filters were preloaded with 2 L water from Lake Constance to mimic used filter materials. The lake water was tested as negative for microcystins before and after the filtration.

After ozonation and each filtration step 30 mL of the sample were filtered through 8 µm filter (AE 99, Schleicher & Schuell, Dassel, Germany). The lengths of the *P. rubescens* filaments were measured by IPS and converted to cell densities. The filtrate was concentrated via solid phase extraction (SPE, C18 columns, Macherey & Nagel, Germany) and resuspended in 20% MeOH.

The ozonated samples E1 and E2 were filtered in succession without change of the filter material. After filtration of the two samples (E1 and E2), the filters were washed

with 500 ml lake water (Lake Constance). After passage through FFS, ACFS and slow sand filtration, 30 mL were concentrated via SPE. After that, the filter material was extracted with 20% MeOH (12 hours) and subsequently concentrated via SPE. The toxin concentrations of the pre-concentrated samples were determined by Adda-ELISA. The ELISA method was performed as described in chapter IV. The samples were analysed three times in duplicates using a different 96 well plate for each assay. The mean values of each give the three values for calculation of the standard deviation (n=3). The main microcystin congener produced by the applied *P. rubescens* strain A7 has been identified as [D-Asp^3, (*E*)-Dhb^7]microcystin-RR (*115*).

RESULTS AND DISCUSSION

Ozonation

The control measurement of the ozone concentration showed a concentration of 0.8 mg O_3/L. Because the dilution ratio was identical, it can be assumed that the initial concentration was the same in the control sample and in the samples with *P. rubescens*. The first ozone measurement of the *P. rubescens* sample after 30 seconds resulted in a concentration of 0.06 mg/l for E1 (Figure 9), while no ozone residual could be detected in E2. The ozone decrease in the control sample followed a first order kinetic model as previously published by Elovitz and von Gunten (*452*).

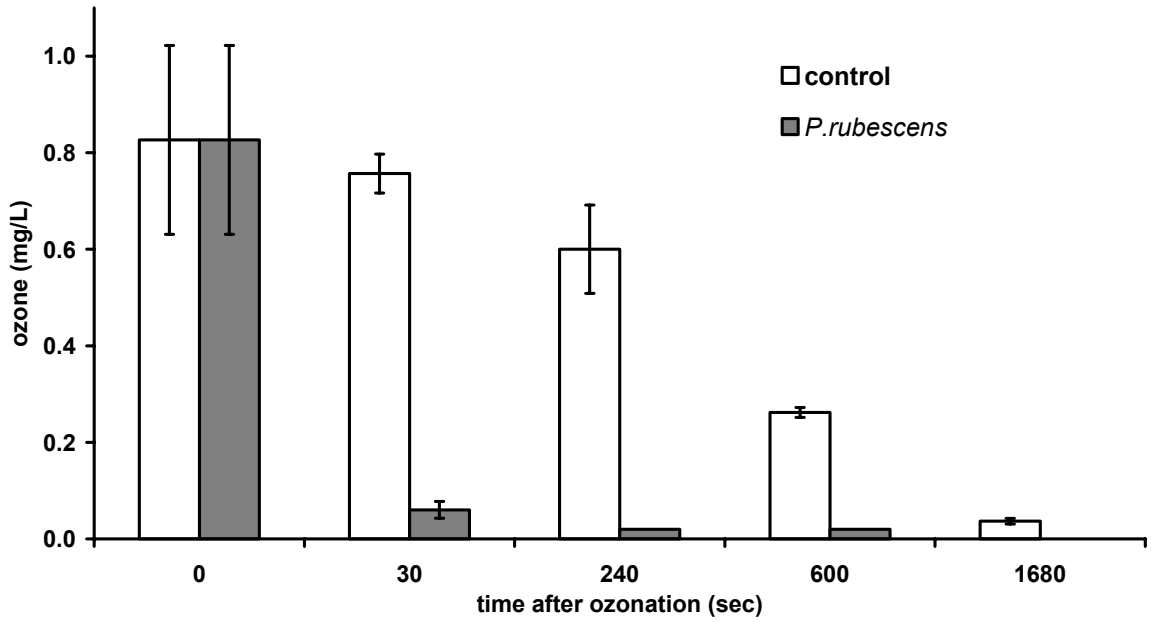


Figure 9. Ozone concentration during oxidation of a *P. rubescens* culture with 15,000 cells/mL (E1) and the control experiment (w/o cells). No ozone could be measured in E2.

The ozone concentration of 0.8 mg/l was responsible for lysis of approximately 50% of the cyanobacterial cells in both experiments (Table 11). The extracellular toxin concentration increased 6.1 and 4.4 -fold after ozonation in E1 and E2, respectively. This increase can be explained with lysis of half of the cells as detected via the image processing system (IPS, Visiometrics). The toxin content per cell can be estimated at 0.1-0.2 pg/cell, which results in an overall toxin concentration of 1.5 to 3.0 µg microcystin/L. If half of the cells were destroyed, the extracellular microcystin content increased to 0.75 to 1.5 µg/L. As shown in Figure 10, the detected concentration increased to 1.1-1.4 µg/L and is therefore in the expected range. Additionally, a slight damaging of the filaments through oxidation is possible, which may have been followed by an increased release of toxin. If the cells were pre-damaged by oxidation, the pressure on the cyanobacterial filaments during the filtration process could result in an increased release of toxins from damaged cells. Because uncertainties exist if the used IPS can differentiate between completely intact and porus cells, no statement can be made about the condition of the cyanobacterial filaments. No toxin release could be observed by Pietsch *et al.* (*450*) in a study with a natural *P. rubescens* population oxidised with 0.3 mg O_3/L. The intra- and the extracellular toxin amounts decreased by 1/3 and 1/2, respectively in this study. The lower ozone concentration used could explain why 2/3 of the filaments remained stable and did not release their toxins. In the same study a culture of *Microcystis aeruginosa* was oxidised with 0.1 and 0.2 mg O_3/L, which resulted in an

	***P. rubescens* (cells/mL)**	
	E1	**E2**
before ozonation	14686	15330
after ozonation	6339	7982
after pumice	390	807
after quartz sand I	0	0

Table 11. Cell densities of *Planktothrix rubescens* before and during drinking water.

increase of extracellular toxin from an initial concentration of 0.32 to 1.4 and 1.5 µg/L, respectively, while the intracellular toxin was reduced from 7.1 to 4.3 and 2.3 µg/L, respectively. Although the ozone concentration was low in the studies by Pietsch *et al.* (*450*), the increase in extracellular toxin is similar to the results in the study presented here (Figure 10). Another study was also carried out with *M. aeruginosa*, in which cyanobacterial cells were ozonated with 0.9 mg/L followed by an increase of the extracellular toxin from 0.1 to 0.45 µg/L and a simultaneous decrease of intracellular toxin from 0.9 to 0.5 µg/L.

In summary, the results presented in this study with *P. rubescens* are comparable with studies in which *M. aeruginosa* was ozonated. These studies underline the potential risk of an increased toxin release after ozonation, if the ozone concentration is not sufficient to break up the cells and degrade the toxins totally or if the ozone concentration is to high and a damage of the filaments cannot be prevented. This prevention is the aim of water works (*432*), however, it must be assumed that it is very difficult to regulate the ozone flow proportional to the natural organic mater in the raw water to prevent any cyanobacterial cell lysis.

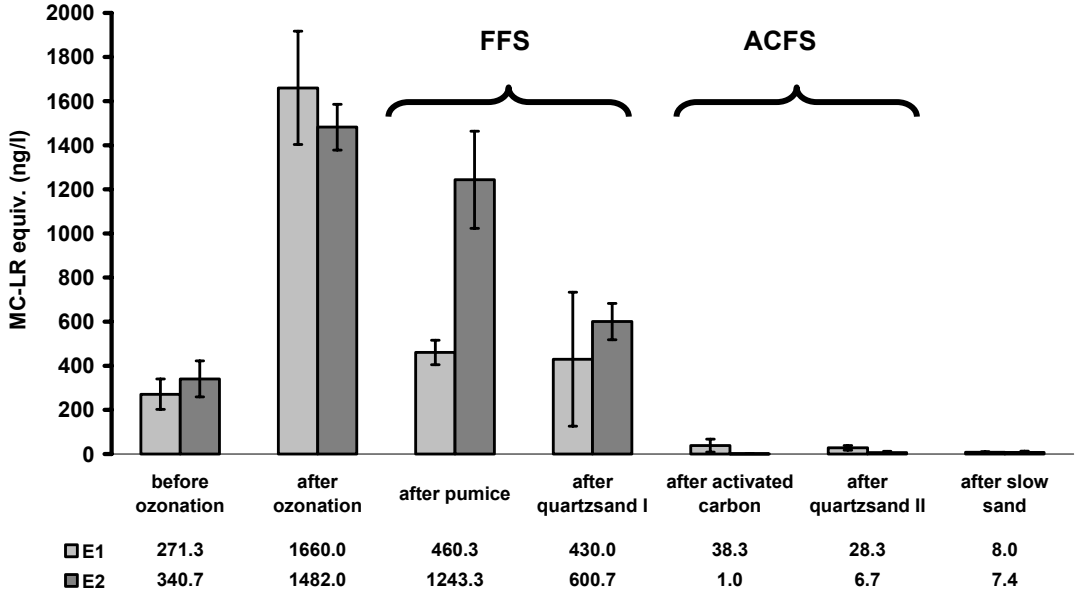


Figure 10. Extracellular toxin concentration during the water treatment process detected by Adda-ELISA. Shown are MC-LR equivalents of E1 and E2. E2 was filtered through the same filter material as E1; standard deviations result from toxicity test by ELISA (n=3); FFS fast filtration system, ACFS activated carbon filtration system.

Filtration

After ozonation, the *P. rubescens* samples were cleaned by filtration systems. To determine the efficacy of each filtration layer within the filtration systems (FFS, ACFS) the individual materials (pumice/quartzsand for FFS, activated carbon/quartzsand for ACFS) were filled into different columns and the efficacy of each analysed separately (Figure 10).

After filtration with pumice the cell counts/filaments determined by IPS were reduced by 94% and 90% in E1 and E2, respectively (Table 11). No cells or filaments could be identified after quartzsand filtration or the further filtration steps. After filtration through pumice the extracellular toxin concentration in E1 was reduced by 73% (Figure 10). While quartzsand could not remove microcystin from the sample, the activated-carbon-filter reduced the toxin content to values below 50 ng/L. However, the results of the three ELISA tests of the quartz sand filter sample have shown values between 0.2 and 0.8 µg MC-LR equiv./L (see standard deviation in Figure 10), thus an assessment of this value is problematic. After the following quartz sand filter and slow sand filter, the Adda-Elisa gave a low signal in only one of the triplicates, thus a complete removal of microcystin can be assumed. In the subsequent filtration of the sample E2 through the same filter materials, a reduction of only 16% could be determined after pumice filtration. Either the filtration (adsorption) capacity of the material was exhausted or microcystins from the previous filtration were washed out by this filtration step. Both reasons are possible and point out a fast reduction in the filtration efficacy of pumice. After the following quartz sand filtration the microcystin concentration was reduced to 600 ng/L, which means 40% of the microcystin concentration after ozonation. The activated carbon filter removed the microcystins almost completely. Thus, only traces of toxins could be detected after the subsequent filtration steps (quarzsand/slow sand, Figure 10).

Table 12. Toxin concentration in lake water after filtration through microcystin-preloaded filter system.

	FFS (pumice/quartzsand)	ACFS (activated carbon/quartzsand)	slow sand filter
MC-LR equivalents (ng/L)	83 (41.5 ng)	< 1	<1

After filtration of the two *P. rubescens* samples, the filters were washed with water from Lake Constance to mimic the process in water works with a period of *P. rubescens* contaminated water followed by MC-free raw water. The lake water was tested as MC - negative before filtration. After filtration with 500 ml lake water, 83 ng MC/L could be detected after the FFS, while no toxin could be demonstrated after the ACFS and slow sand filter (Table 12). This means that less than 1% of the toxin remaining in the FFS was washed out by 500 mL lake water. None of the approximately 158 ng of toxin (Table 13) was dissociated from the ACFS by the lake water.

After E1, E2 and the washing step with lake water filter materials were extracted with 20% MeOH. The results of the toxin determination are shown in Table 13. The values are calculated from the ELISA results presented in Figure 10, whereby the toxin concentration in the sample taken after each filtration step (30 mL) was subtracted. Of the calculated toxin amount remaining in the filter between 21% and 30% could be extracted and detected (Table 13). The greater adsorptive capacity of pumice and activated carbon compared to quartz sand seems to interfere with the extraction with 20% MeOH. This result underlines the ability of the filter material to retain the toxins.

The efficacy of the filter material to remove microcystins depends on the age/use of the filter material, the ratio toxin/filter material and the pretreatment of the sample.

Table 13. Toxin amounts in the filtration materials. The applied and remaining toxin amounts are calculated from the ELISA results presented in Figure 10. The toxin amounts after filter extraction were determined directly via ELISA.

	FFS		ACFS		
	pumice	**quartz-sand I**	**activated carbon**	**quartz-sand II**	**slow sand**
Applied toxin amount (ng MC-LR equiv.)	691	325	165	<7	<5
Remaining toxin in filter (ng MC-LR equiv.)	315	195	158	<4	<4
Extracted toxin amount (ng MC-LR equiv.)	72	57	39	<1	<1
Extraction efficiency (remained toxin = 100%)	23%	30%	21%	-	-

Other studies with cyanobacteria and cyanobacterial toxins have shown that the efficacy of filter systems decrease after repeated use (*417, 445, 450*). In the study presented here the pumice filter showed a distinctly lower capacity to adsorb microcystins which corresponds with the data from literature mentioned latter (*417, 445, 450*). In water works the fast filter (e.g. FFS in water work Lengg/Zurich/Switzerland) is backwashed after a defined time period to free the filter from clogging material (pers. communication with water works Lengg/Zurich/Switzerland). In addition, short term experiments cannot investigate the effect of a biofilm on the degradation of cyanobacterial toxins. The biofilms in the filter systems of water works are known to contain bacteria which can degrade microcystins (*3, 55, 198, 453*). Thus, the minor adsorptive capacity of the filter could be compensated by the capacity of the biofilm to detoxify microcystins by degradation. In water works the activated carbon and slow sand filter are in use for years, thus the experiments presented here can only provide evidence for the efficacy of relatively new filter materials. Nevertheless, if the toxins reach the filter system after ozonation and the filter systems have not been changed over a period of years, a bacterial degradation is required as no other possibility of removal or detoxification exists. Because microcystins are very stable, after exhaustion of the adsorptive capacity of the filter systems, the microcystins are washed out or the microcystins from new water charges are no longer removed by the filter systems.

CONCLUSION

The applied density of 15,000 cells /mL routinely occur in water works which take their raw water from *P. rubescens* habitats. It could be shown, that a combination of ozonation with approximately 0.8 mg/L followed by a filtration system with fast filtration, activated carbon filtration and slow sand filtration can not only eliminate *P. rubescens* filaments with a density of ~ 15,000 cells/mL, but also the toxins produced by these cyanobacteria. The increase of the extracellular toxin concentration was similar between the two experiments. But the ozonation of raw water with 15,000 cells/mL without additional natural organic mater in the raw water resulted in a massive release of toxin into the drinking water. Thus, to ensure an oxidation of the released toxin, a higher ct-value is necessary (higher ozone concentrations or a longer incubation with a stabile ozone concentration, $ct = \int [O3] dt$).

The applied filtration system was efficient in removing extracellular microcystin. However, as previously shown in chapter IV (*445*), a FFS preloaded with intra- or extracellular toxins may release toxins in the subsequent charge of water. The different qualities of raw water and the cyanobacteria and cyanobacterial toxins therein as well as the variety of applied treatment systems complicate general statements with respect to the "best" method to remove cyanobacteria and cyanobacterial toxins. In this case, lab-scale experiments can only give rough recommendations, but every water works with cyanobacteria in raw water should be obliged to routinely test the raw water, the drinking water but also the efficacy of the treatment steps to avoid a breakthrough of liver tumour promoting cyanobacterial toxins.

OCCURRENCE AND ELIMINATION OF CYANOBACTERIAL TOXINS IN GERMAN AND SWISS WATER TREATMENT PLANTS

Cyanobacteria (blue-green algae) are ubiquitous in surface waters and many species including *Microcystis*, *Nodularia*, *Cylindrospermopsis*, *Anabaena* and *Aphanizomenon* are known to produce toxins such as microcystins, cylindrospermopsins and saxitoxins. Toxin-producing species are abundant in surface waters used for recreational purposes, as a drinking water resource, as irrigation water for agriculture or as growing media for cyanobacterial health foods. In addition, fish and shellfish exposed to or feeding on toxic cyanobacteria directly or indirectly through accumulation in the food chain are also possible sources for human cyanobacterial toxin exposure. The toxicity of microcystins and their presence in surface waters used for drinking water production has prompted the WHO to publish a provisional guideline value of 1.0 µg microcystin (MC)-LR/L drinking water. To verify the efficiency of two different water treatment systems and bank filtration with respect to reduction of cyanobacterial toxins, the concentrations of microcystins in water samples from surface waters and their associated water works in Switzerland and Germany were investigated. A main part of the study presented here shows the abundance of toxic *P. rubescens* in a Swiss lake used for the preparation of drinking water via bank filtration and the consequences of the presence of this cyanobacterium for the quality of drinking water. Toxin concentrations in samples from drinking water treatment plants ranged from below 1.0 µg MC-LR equiv./L to more than 30 µg/L in raw water and were below 1.0 µg/L in drinking water.

INTRODUCTION

The majority of the populations in industrialised countries are dependent on drinking water from public or private water suppliers. These water works have to guarantee the drinking water quality according to the national drinking water guidelines. The guidelines include microbial (e.g. *E. coli*, coliforme bacteria) and chemical (e.g. cyanides, pesticides) parameters as health-relevant points, and indicator parameters (smell, taste, conductivity) as important factors for the efficient and smooth running of the water works (*454*). These parameters must be controlled by the water works to avoid contamination of drinking water with harmful substances. Guidelines for cyanobacterial toxins exist in several countries worldwide (Table 14). Most of these countries have a history of problems with cyanobacterial contamination in drinking

water reservoirs and they may serve as examples for the rest of the world. In Europe, these biotoxins are not yet clearly regulated. However, in the European Water Framework Directive (2000/60/EC, (*455*)), which characterises high priority water pollutants, toxin-producing cyanobacteria (blue-green algae) have been specifically highlighted as potential key hazardous pollutants. The harmful potential of cyanobacterial toxins for the population is appreciated in many European countries and has been described in many publications (*305, 417, 456, 457*).

The fact that cyanobacteria are able to exist even in hot springs in volcanic regions (*464*) and in cold and hot deserts such as Antarctica (*313, 465*) or the Atacama desert (*465*), underlines the omnipresence of these organisms. Cyanobacteria are ubiquitous in surface waters worldwide and many species including *Microcystis*, *Nodularia*, *Cylindrospermopsis*, *Anabaena* and *Aphanizomenon* are known to produce toxins such as microcystins (MC), cylindrospermopsins (CYL), anatoxins and paralytic

Table 14. Guideline values for toxic cyanobacterial secondary metabolites.

	Microcystins	PSPs	Anatoxin-a	Cylindrospermopsin	
Australia	lifetime exposure: 1.3 µg/L brief period: 10 µg/L	3.0 µg/L (suggested for brief period)	mentioned in guideline (w/o value)	mentioned in guideline (w/o value)	(*458, 459*)
Brazil	1.0 µg/L	3.0 µg/L (suggested)	-	15 µg/L (suggested)	(*460*)
Canada	1.5 µg/L	-	-	-	(*461*)
France	1.0 µg/L	-	-	-	(*462*)
European Drinking Water Directive, 1998	0.1 µg/L (default value)*	0.1 µg/L (default value)*	0.1 µg/L (default value)*	0.1 µg/L (default value)*	
New Zealand	1.0 µg/L	1.0 µg/l	3.0 µg/L (1.0 µg/L for anatoxin-a(s) + homoanatoxin)	3.0 µg/L	(*463*)
Oregon (USA)	1.0 µg/g (health food)	-	-	-	(*149*)
WHO	1.0 µg/L (provisional)	-	-	-	(*1*)

* the value of 0.1 µg/L was originally used for pesticides, but nowadays it is applied as a provisional value for other toxins whose character is unknown or as a quality target designed to be well below the concentration derived from health criteria (*4*).

shellfish poisons (saxitoxins, PSPs) (*289*). However, due to a lack of toxicity data for other toxins, the World Health Organization (*1*) has only set a provisional guideline for MC-LR (L: lysine, R: arginine). This guideline value is 1.0 µg/L drinking water (Table 14). Microcystins and nodularins have a high acute toxicity with LD50s from 36 to 122 µg/kg in mice and rats i.p. or i.v. (*337, 466*), but have also been implicated in tumour promotion in both liver (*367*) and colon (*448*). Nodularins and MCs are also suspected to induce liver carcinogenesis (*116, 449*). The inhibition of protein phosphatase enzymes seems to be responsible for the toxicity of both substances, but additional mechanisms are likely.

However, the mechanisms and target organs of cyanobacterial toxins vary. PSPs and anatoxins (anatoxin-a, anatoxin-a(s), homoanatoxin) block signal transmission in the nervous system by inhibiting the axonal sodium-channel and by disturbing the neuromuscular transmission, respectively. Although these substances have shown high acute toxicity with LD_{50} values of 10 µg/kg BW (mice, i.p.) (*341*), the risk of an acute intoxication through drinking water is low, because humans avoid the unpleasant smelling water, although the odours are caused by other secondary metabolites such as geosmin (*467*). There is no indication that chronic exposure to low levels of anatoxin-a lead to an impairment of health (*468*). No data are available concerning chronic exposure to PSPs.

CYL inhibits protein synthesis. Moreover, the investigations of Falconer and Humpage (*448, 469*) point to a possible tumour initiating activity of CYL via induction of strand breaks at the DNA level and induction of aneuploidy by disturbing the kinetochore/spindle function. In recent years, the former tropical *C. raciborskii*, the main producer of CYL, has reached more temperate zones (*470*). Thus, water works in central Europe need to take into account, that toxic *Cylindrospermopsis* may also occur in their raw water sources.

Humans may come into contact with cyanobacterial toxins through ingestion or dermal contact with cyanobacteria and their respective toxins. Possible pathways for exposure are during recreational activities (*248, 471*), by ingestion of contaminated agricultural products (*294-296*), cyanobacterial health foods (*149*) or contaminated shellfish (*299*) or fish (*303*). It is becoming increasingly clear, that almost every part of the world depending on drinking water from surface waters, has or will encounter problems with toxic cyanobacteria in its drinking water system (Table 15), due to the ubiquitous presence in raw water feeding into water works. Thus, water treatment

systems must eliminate cyanobacteria and their toxins from the water. In the case of microcystin producing cyanobacteria, about 80-90% of the toxins are cell-bound (*52*).

Conventional water treatment with only a filtration step (*472*) or with an additional flocculation step has been shown to be ineffective in removing dissolved microcystins from water (*385*). Flocculation with an appropriate concentration of flocculent is only suitable for removing cyanobacterial cells from water. However, the possibility of a partial cell lysis exists. This would lead to an increase in extracellular toxin concentration which cannot be eliminated by the methods mentioned. Furthermore, intact cells have been observed in final water after the whole treatment train (*380*).

In ozonation water treatment processes both ozone and OH radicals work as oxidising agents, whereby ozone acts specifically and the OH radicals react unspecifically and quickly with all organic compounds (*473*). Preozonation with ~0.5-1.5 mg/L should inactivate bacteria (*474*), viruses and protozoa and detoxify harmful compounds such as phenols, polycyclic aromatics and microcystins. Undesirable taste-and-odour substances are also eliminated. Furthermore, other natural organic matter is modified to products which are more easily adsorbed and filtered (*475*). At concentrations below 2.5 mg O_3/L a desired flocculation effect is observed, above this concentration, the effect is reversed (*476*). Subsequent to ozonation two-layer-filters remove the majority of the organic substances (e.g. cyanobacterial cells) and thus act as a mechanical rough cleaning step. This function makes a backwashing at regular intervals necessary. Intermediate ozonation with ~0.5 mg/L is necessary to guarantee the elimination of harmful substances including cyanobacterial toxins, which "survive" the preceding treatment steps. In addition, intermediate ozonation improves particle removal in the subsequent filter system (*477*). Activated carbon eliminates the surplus ozone, adsorbs hydrophobic compounds, and acts as substrate for bacteria, which mineralise most of the organic by-products (ketones, aldehydes, acids) produced by the ozonation step (*309, 478*). Thus, activated carbon filters act as biofilms, that potentially metabolise organic compounds, however also show a significantly impaired ability to adsorb toxins. Moreover, biodegradation of microcystins by the biofilm does not seem to occur (*382, 385*). The slow sand filter also functions as a substrate for bacteria, which detoxify and clean the treated water similarly to natural water cleaning conditions.

Bank filtration is a concept widely applied in Germany. The water is collected along the shoreline of rivers (*479*) and lakes (*290, 480*). This water is partly used as drinking water without further treatment. Results from Finland (*481*) and Germany (*290*) have demonstrated the possibility of a breakthrough of cyanobacterial toxins into the drinking water wells.

To verify the efficiency of water treatment and bank filtration with respect to reduction of cyanobacterial toxins, the concentrations of microcystins in water samples from surface waters and their associated water works in Switzerland and Germany have been investigated. A central focus of this manuscript is the abundance of toxic *P. rubescens* over several years in a Swiss lake used for sediment filtration and the consequences of this for the quality of drinking water.

Table 15 (I-IV) Cyanobacteria and/or cyanobacterial toxins in drinking water treatment worldwide; if not named in detail, toxins in raw and final water are microcystins (MCs); NR not reported, *An. Anabaena, Aph. Aphanizomenon, C. Cylindrospermopsis, M. Microcystis, O. Oscillatoria, P. Planktothrix* (*O. agardhii* = *P. agardhii*).

I of IV	Water treatment	Cyanobacteria	Raw water	Final water	Removal	Additional information	
Bahía Blanca, Argentina	NR	*An. circinalis* *M. aeruginosa*	48,320-84,032 cells/mL	276-2,472 cells/mL	NR	-	(*482*)
San Roque Dam, Argentina	NR	*M. aeruginosa*	LD_{50}(mice): 500 µg/kg	MC detectable via HPLC	NR	drinking water supply for Cordoba	(*483*)
Solomon Dam, Australia	copper sulphate	*Cylindro-spermopsis*	NR	NR	NR	effects on human health reported	(*191, 257, 258*)
Malpas Dam, Australia	copper sulphate	*Microcystis*	NR	NR	NR	effects on human health reported	(*259*)
Lakes, ponds, reservoirs, Bangladesh	NR	*M. aeruginosa*	many samples MC positive	tap water samples MC positive	NR	-	(*484*)
Itaparica Dam, Brazil	copper sulphate	*Anabaena, Microcystis*	NR	NR	NR	effects on human health reported	(*267*)
Flemish water reservoirs, Belgium	NR	*M. aeruginosa, Aph. flos-aquae, An. flos-aquae, P. agardhii*	toxic by mouse bioassay	NR	NR	No dermal effects observed	(*485*)
Camrose plant, Alberta, Canada	coagulation-sedimentation, filtration, chlorination, PAC	NR	0.15-0.87 µg/L (n=14)	0.09-0.18 µg/L (n=14)	59-97%	-	(*385*)
Ferintosh plant, Alberta, Canada	coagulation-sedimentation, filtration, chlorination, GAC	NR	0.27-2.28 µg/L (n=6)	0.05-0.12 µg/L (n=6)	7-90%	-	(*385*)
North and Central Alberta, Canada	NR	*M. aeruginosa, Aph. flos-aquae, An. flos-aquae, Gomphosphaeria*	≤ 605 µg/g DW	NR	NR	eight lakes, six farm dugouts	(*291*)

II of IV	Water treatment	Cyanobacteria	Raw water	Final water	Removal	Additional information	
Water works, Czech Republic	NR	NR	≤8.7 μg/L (n=12)	0.0-7.79 μg/L (n=12)	11-100%	-	(*486*)
Yellow River, Zhengzhou City, China	NR	NR	≤47,620 cells/mL	NR	NR	~70% of samples with MCs	(*487*)
Haimen City, China	comparison between different water sources (well, tap-water, river and pond)	NR	NR	≤1.9 μg/L	NR	MCs may be associated with incidence of colorectal cancer	(*268*)
China	NR	NR	0.28-35.3 μg/L	≤1.4 μg/L	78-100%	-	(*488*)
River Nile, Egypt	flocculation, sedimentation, sand filtration, chlorination	*O. tenuis*	0.3 mg/g DW	NR	NR	-	(*61*)
Lake Uelemiste, Estonia	NR	max biomass: 40 mg/L	NR	NR	NR	drinking water for Tallinn	(*489*)
Bank filtration plants, Finland	bank filtration	most abundant: *P. agardhii*	0.1-1.9 μg/L (n=8)	0.01-0.1 μg/L (n=6)	>90%	coccoid cells and fragments of filamentous cyanobacteria observed in bank filtered drinking water; MCs in 9/58 samples from treated water	(*481*)
Aland Islands, Finland	NR	red and green variant of *O. agardhii*	≤37 μg/L	ND	ND	observed fish and bird kills, Lake has been abandoned as drinking water supply since August 1987	(*490-492*)
Lake Haukkajärvi, Finland	NR	predominated by *O. agardhii* *An.lemmermanii* *M. aeruginosa*	toxic in mouse bioassay	NR	NR	-	(*493*)
Water treatment plants, Finland	rapid sand filtration	NR	NR	NR	14%	trichomes of *O. agardhii* passed through treatment process in spite of high reduction efficiency in water work	(*380*)
	rapid sand filtration, activated carbon filtration, chlorination	*Gonyostonum semen*			42%		
	contact filtration with $Al_2((SO)_4)_3$, activated carbon filtration, chlorination	*An.lemmermanii* *Acanthoceras zachariasii,* *Aph. flos-aquae*			99%		
	activated carbon filtration, flocculation with $Al_2((SO)_4)_3$, sedimentation, sand filtration, chlorination	*M. wesenbergii,* *O. agardhii*			99.9%		
Charente, France	NR	NR	NR	0.22 μg	NR		(*494*)
Water reservoirs, Bretagne, France	NR	*Microcystis sp.* *Aphanizomenon sp.* *Anabaena sp.* *Oscillatoria sp.*	toxic in several bioassays	NR	NR	-	(*495*)
Saint-Caprais reservoir, France	ozonation (0.07 mg/L) PAC (20 mg/L)	*Aph. flos-aquae*	63 μg/L	<1 ng/L	>98%	before treatment: 30% of toxin free after treatment: 100%	(*293*)
	ozonation (0.07 mg/L) PAC (40 mg/L)						
	chlorination (0.42 mg/L) PAC (20 mg/L)			33.2 ng/L ± 8.0 ng/L	45%		
	chlorination (0.42 mg/L) PAC (40 mg/L)			<1 ng/L	>98%		
Warnow River, Rostock, Germany	preozonation, flocculation/sedimentation, filtration ozonation, activated carbon	*P. agardhii,* *Microcystis spp.*	0.4 - 8.0 μg/L	0.07 - 0.11 μg/L	93-100%	-	(*496*)

III of IV	Water treatment	Cyanobacteria	Raw water	Final water	Removal	Additional information	
Weida Reservoir, Dörtendorf, Germany	microsieve, flocculation, sand filtration, chlorination and pH adjustment	*P. rubescens*	7.5-10 µg/L (HPLC) 10–28 µg/L (ELISA)	0-0.1 µg/L (HPLC) 0-0.2 µg/L (ELISA)	>98%	-	(*497*)
Radeburg Reservoir, Germany	bank filtration	*Microcystin spp. Aphanizomenon spp.*	2-19 µg/L	≤ 0.06	75-99%	removal efficiency hard to calculate due to uncertain travel times	(*290*)
Große Dhünn Reservoir, Germany	NR	*P. rubescens*	NR	NR	NR	-	(*442*)
Lake Müggelsee, Germany	bank filtration	*Microcystis. Aphanizomenon Anabaena.*	≤120 µg/L dissolved MCs	NR	NR	galleries of wells around the lake to provide drinking water for Berlin	(*292*)
Lake Tegel, Germany	bank filtration	*Microcystis*	MC-LR, MC-RR, MC-YR	NR	NR	130 vertically orientated wells of 20-60 m depth, provide drinking water for Berlin	(*480, 498*)
Östertalsperre, Germany	NR	*P. agardhii*	3.6 mg/g DW	NR	NR	-	(*499*)
Lake Kinneret, Israel	flocculation with $Al_2((SO)_4)_3$ and disinfection with chlorine, chlordioxid and chloramine	*Aph. ovalisporum*	≤150,000 cells/mL	NR	cell removal 99.9%	raw water source for the Israeli National Water Carrier	(*500, 501*)
Lake Simbirizzi, Lake Flumendosa, Lake Mulargia, Italy	Lake Flumendosa and Lake Mulargia are drinking water source for >400.000 people	*O. tenuis, O. rubescens, O. mougetii*	0.48 and 0.22 mg/g DW	NR	NR	≤6.5 x 10^6 *O. tenuis* trichomes/L ≤7 x 10^4 *O. rubescens* trichomes/L	(*502, 503*)
Florence, Italy	prechlorination (ClO_2), PAC, coagulation/ flocculation ($ALCl_3$), sedimentation, sand filtration, ozonation, postchlorination (ClO_2)	*Oscillatoria, Anabaena, Phormidium*	≤2,000 units/mL	NR	cell removal ~98%	cell removal before sand filtration: 90%	(*504*)
Drinking water reservoirs, Italy	NR	*Anabaena, Oscillatoria, Microcystis*	0.22–15.76 mg/g DW	NR	NR	-	(*505*)
Lake Tsukui, Japan	NR	*Microcystis, Anabaena*	≤3.1 µg/L	NR	NR	important water reservoirs, serving a population of ≈ 5 million	(*506, 507*)
Lake Sagami, Japan		*Microcystis, Anabaena*	0.04–482 µg/L				
Lakes and reservoirs, Korea	mostly only rapid sand filtration	*Microcystis spp. (60%), Anabaena spp. (30%), Oscillatoria spp. (10%)*	0.6-171 µg/L (n=16) 19-1229 µg/g DW (n=28)	NR	NR	ANA-a: 417-1444 µg/g DW (n=4)	(*508*)
Daechung reservoir, Korea	NR	*Microcystis spp. (47%), Anabaena spp. (39%), Oscillatoria spp. (6%)*	≤0.2 µg/L	NR	NR	MCs were continuously detectable for 6 months, 28% dissolved	(*509*)
Lake Mazais Baltezers, Latvia	sand filtration/bank filtration	*M. aeruginosa, Anabaena sp., Aph. flos-aquae*	Lake: ≤0.63 µg/L infiltration basin: ≤0.25 µg/L	≤1.47 µg/L	NR	only dissolved MC investigated, ≤ 5 x 10^6 cells/mL in lake	(*510*)
Lalla Takerkoust lake-reservoir, Morocco	NR	*M. aeruginosa, Pseudanabaena mucicola*	0.7-8.8 µg/g DW 10^2-10^6 cells/mL	NR	NR	occasionally used as drinking water reservoir, at least for lakeside residents	(*511*)

IV of IV	Water treatment	Cyanobacteria	Raw water	Final water	Removal	Additional information	
Lake Mjöse, Norway	NR	*O. bornetti fa. tenuis*	biomass: 2 g/m^3	NR	NR	water with unpleasant odour and taste could hardly be used for portable water; water supply for 200,000 persons	(*512*)
Aguieira and Monte Novo, Portugal	NR	*M. aeruginosa, Raphidiopsis mediterranea*	710-120,000 cells/mL	NR	≥24%	-	(*305, 513*)
Crestuma-Lever reservoir, Portugal	NR	*M. aeruginosa, Aph. flos-aquae*	≤12,261 cells/mL (*Microcystis*) 4.7 μg STX equiv/g DW (*Aphanizo-menon*)	NR	NR	water supply for Porto and region near Porto (2 million inhabitants)	(*287, 514*)
Minho River, Guardian River Portugal	filtration and chlorination (at Minho River)	*Microcystis spp.*(both lakes), *Aph. flos-aquae* (Guardian River)	NR	NR	NR	health problems at Guardian River	(*274*)
Monte novo and Divor reservoir, Portugal	NR	*Anabaena, Aphanizomenon, Microcystis, Oscillatoria*	NR	cyanobacterial cells in drinking water	NR	cyanobacteria(l) blooms in the reservoirs from 10/1992 – 12/1993; effects on human health supposed	(*275*)
Lakes and water reservoirs, Slovenia	NR	*An. flos-aquae, Aph. flos-aquae, P. rubescens, M. wesenbergii, M. aeruginosa*	≤18.5 mg/g TG	NR	NR	-	(*446*)
Dams and reservoirs, South Africa	NR	*An. circinalis, Aph. flos-aquae, C. raciborskii, M. aeruginosa*	NR	NR	NR	-	(*515*)
Drinking water reservoir of Seville, Spain	NR	*M. aeruginosa*	toxic	NR	NR	-	(*516*)
Water supplies, Thailand	partly without treatment	*Anabaena sp, C. raciborskii, Oscillatoria sp*	NR	<1.0 μg/L	NR	-	(*517*)
Rutland Water, Leicestershire, UK	NR	*M. aeruginosa*	surface water samples lethal in mouse bioassay	NR	NR	part of a water supply network for 1.5 million people	(*278*)
Cropston reservoir, Lower Shuatoke reservoir, Rudyard Lake, Rosthere Mere, UK	NR	*M. aeruginosa P. agardhii*	MCs, ANAs	NR	NR	-	(*225, 278, 518*)
Northern Ireland, UK	NR	*Oscillatoria sp.*	1.5 x 10^4 cells/mL	NR	NR	-	(*519*)
Water supplies, Florida, USA	different treatment systems	e.g.: *C. raciborskii, M. aeruginosa*	NR	≤90 μg/L	NR	indication of human health effects	(*256, 520*)
Sewickley reservoir, USA	NR	*Schizothrix, Lyngbya, Plectonema, Phormidium*	NR	NR	NR	effects on human health reported	(*279, 521*)
Ohio and Potomac rivers, USA	precipitation, filtration, chlorination	*Microcystis*	NR	NR	NR	effects on human health reported	(*33, 281*)
New York State waters, USA	NR	NR	45 of 191 samples positive for >0.2 μg/L	No toxin detected (>0.2 μg/L)	NR	Lake Ontario and Lake Champlain provide drinking water for >20 million people	(*522*)
Harare, Zimbabwe, 1960-1965	NR	*M. aeruginosa*	NR	NR	NR	effects on human health reported	(*282*)

MATERIAL & METHODS

Sample sites

Samples were obtained from drinking water bodies in Germany and Switzerland. The treatment systems routinely applied at the associated water works are shown in Table 16. In the water capture plant at Lake Hallwil samples were taken from a fountain containing water obtained after bank filtration and mixing with groundwater.

	Details	Water treatment steps	Predominant cyanobacteria	Analysed toxins
Lake Zurich (Switzerland)	water intake in a depth of 30m, 600m away from the shore (*523*)*8*)	preozonation, rapid sand filtration (pumice/quartzsand), intermediate ozonation, activated carbon filtration (GAC, quartzsand), slow sand filtration	*P. rubescens*	MCs
Wahnbachtal Dam (Germany)	drinking water dam and source for 780,000 people (Bonn and hinterland)	flocculation (0.8 mg $Al_2(SO_4)_3$/L)/sedimentation, quartzsand filtration, chlorination of pipeline system (0.15-0.2 mg/L)	*P. rubescens*	MCs
Lake Hallwil (Switzerland)		bank filtration	*P. rubescens*	MCs

Table 16. Detailed information about the investigated drinking water sources.

Cell counting

Cyanobacterial cell densities were only determined in the water works Lengg/Lake Zurich (Switzerland). A defined volume of sample was filtered through ammonium acetate filter (0.45 µm, Schleicher & Schuell, Germany) and the trichomes measured by using IPS-software (Visiometrics, Constance, Germany). This analysing system is presented in detail by Ernst *et al.* (*451*).

Sampling and sample preparation

Water (2L) was collected in glass bottles from the surface, from different layers in the lakes and between various treatment steps in water works. Samples from Lake Hallwil were mixed from 15m to the surface. To determine free MC in water, samples were filtered through ammonium acetate filter (0.45 µm, Schleicher & Schuell, Germany). Solid phase extraction (SPE) of the filtrate was performed using C_{18} end-capped SPE cartridges (Macherey & Nagel, Germany) conditioned with 10 mL methanol and subsequently washed with 10 mL MilliQ water (H_2O-MQ). Samples

were applied to the cartridge and, after washing with 10 mL H_2O-MQ water, eluted with 40 mL 100% methanol. The eluent was dried under a nitrogen atmosphere and resuspended in 1.5 mL H_2O-MQ.
The filter (~20 mg sample DW) was extracted three times with 1.5 mL 75% methanol in 1.8 mL tubes. Supernatants were collected, dried via vacuum centrifugation, resuspended in 10 mL H_2O-MQ and toxins concentrated by SPE (similar to that described above for the extracellular toxin), dried under a nitrogen atmosphere and resuspended in 1.5 mL H_2O-MQ.

Determination and quantification of toxins

RADIOACTIVE PROTEIN PHOSPHATASE ASSAY (rPPA)

The protein phosphatase assay was performed as described in Fischer *et al.* (*421*) using a phosphatase extracted from rape seed (*Brassicus napus*), ^{32}P-ATP (kindly provided by Prof. Werner Hofer, University of Constance), and microcystin-LR (Calbiochem, USA) as a standard. Free ^{32}P in the supernatant was extracted with acid molybdate, scintillation cocktail (Ready Safe, Beckman, Germany) was added and radioactivity counted in a scintillation counter (LS 6500, Beckman, Germany). The degree of protein phosphatase inhibition (PPA-inhibiting capacity) was calculated as % phosphatase activity of the control.
The PPA-inhibiting capacity of samples was compared to a MC-LR standard-curve. The detection limit of the radioactive PPA used is 0.05 µg MC-LR/L with a derived IC_{50} of 0.25 µg MC-LR/L. Each sample was analysed three times in triplicates. The mean values of each triplicate yielded the three values for calculation of the standard deviation (n=3).

COLOURIMETRIC PROTEIN PHOSPHATASE ASSAY (cPPA)

The colourimetric protein phosphatase assay (cPPA) was performed using the protein phosphatase 1 (PP1) instead of PP2a with microcystin-LR (Alexis, Switzerland) as a standard (*524*).
The PPA-inhibiting capacity of the samples was compared to a microcystin-LR standard-curve and expressed as microcystin-LR equivalents. The detection range (20-80% inhibition of PP1) of the colourimetric PPA used is 0.9-3.2 µg MC-LR/L with a derived IC_{50} of 1.7 µg MC-LR/L.

ADDA-ELISA

The ELISA method was performed as described by Fischer *et al.* (*525*). Briefly, ELISA plates (NUNC MaxiSorp, Denmark) were coated overnight with OVA–ADDA–hemiglutaryl (OVA–ADDA–HG) in 0.05 M sodium carbonate buffer pH 9.6 (50 µL/well, 2.5 µg/mL) at 20 °C. Unbound material was removed by aspiration. After washing with PBS, additional binding sites were blocked by incubation with OVA (1% w/v, 200 µL, >2 h, 20–25 °C). Plates were washed three times with PBS and used immediately or stored at 4 °C for up to 7 days. In the assay, sample or standard (50 µL) were added to the wells together with antiserum (AB 824 at 1/160,000). After incubation at 20–25 °C for 2 h, wells were washed twice with phosphate buffer solution containing 0.05% Tween™ 20 (PBST) and twice with PBS. Anti-sheep secondary antibody (ICN/Cappel rabbit-anti-sheep–HRP) (100 µL, dilution 1/6,000) was then added to the wells and incubated for 2 h. Subsequently, liquid in the wells was aspirated, and the wells washed twice with PBST and twice with PBS. As substrate TMB (100 µL, Sigma, Germany) was added and incubated for 30 min. The reaction was stopped by addition of H_2SO_4 (50 µL, 2 M), and absorbance determined with a microplate reader at 450 nm. The limits of quantitation and detection of the ELISA were 0.2 µg/L and 0.05 µg/L, respectively. The samples were analysed three times in duplicates using a different 96 well plate for each assay. The mean values of each plate give the three values for calculation of the standard deviation (n=3).

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

Toxins were analysed according to Lawton *et al.* (*526*). External standards were prepared for MC-LR (Alexis, Switzerland), MC-RR (Sigma, Germany), MC-YR (Sigma, Germany), and desmethyl-MC-LR and -RR, both kindly provided by J. Meriluoto, Turku, Finland. Samples (in 50% methanol) were injected into the HPLC (Diode Array Detector SPD-M10A, Auto Injector SIL-10AD, Column Oven CTO-10AC, System Controller SCL-10A, Liquid Chromatograph LC-10AT, Degasser DGU-14 A, Shimadzu; Column: Grom-Sil ODS-HE, 250 × 4,6 mm, 5 µm) and peaks were compared to standards.

RESULTS & DISCUSSION

Water works Lengg/Lake Zurich/Switzerland

The treatment train used at Zurich/Lengg is very effective in removing both cyanobacterial cells and toxins. Lake Zurich is known for having *P. rubescens* as the predominant cyanobacterial species throughout the year. Investigations of the water works at Zurich show the densities of *P. rubescens* at the depth (30 m) of raw water intake (Figure 11). Cell densities of >30,000 cells/mL (~180 trichomes/mL) are observed even at this depth. During stratification in September 1999, cell densities of up to 150,000 cells/mL were found in the metalimnion (data not shown). The toxicity of the raw water on 23/12/1999 was determined with a chemical (HPLC-DAD), a functional (rPPA) and a structural method (ELISA). The intracellular concentration (Figure 12) varied from 2450 ng desmethyl MC-RR/L (HPLC) to 4152 ng MC-LR equiv./L (rPPA) and 7050 ng MC-LR equiv./L (ELISA). Determination of the cell densities results in ~30,000 cells/mL for raw water, ~6000 cells/mL after preozonation and ~200 cells/mL after fast filtration. After ozonation the cells appeared more colourless than the cells in raw water. The cells are probably damaged

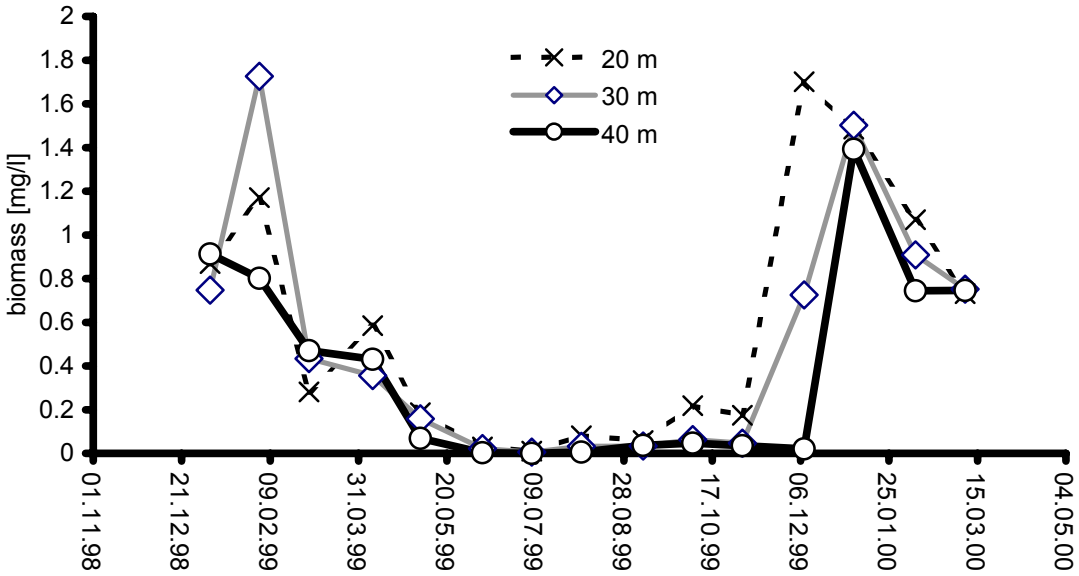


Figure 11. Densities of *P. rubescens* in raw water of the water works Lengg/ Lake Zurich (raw data provided by S. Gammeter from water works Zurich/Switzerland).

during the ozonation process and release their toxins, which then can be oxidised. This is a possible explanation for the high cell number compared to the detected toxin concentration. Less than 1% of the cells in raw water break through preozonation and fast filtration. Calculated from a density of 30,000 cells/mL in December 1999, the toxin concentration per cell was 0.088 pg (HPLC), 0.18 pg (rPPA) and 0.24 pg (Adda-ELISA). This is comparable to a concentration of 0.07-0.3 pg/cell reported for *M. aeruginosa* cells (*459*).

The Adda-ELISA system detects an extracellular concentration of ~1.0 µg MC-LR equiv./L, the value determined by HPLC-DAD und rPPA were >10 fold lower (Figure 12). The elimination of toxin was shown to be efficient during preozonation with ~1.0 mg/L. Ozonation followed by different filtration steps was effective in the elimination of MCs from drinking water during batch experiments (*401, 445*). In this water works only < 5% of the initial toxin concentrations could be detected using each of the three detection methods. Although the different detection methods resulted in slightly variable toxin concentrations, the efficacy of preozonation could be shown. After the fast filtration step the toxin concentration remained within the same range of 100-200 ng MC-LR equiv./L. However, a shift to a higher percentage of extracellular toxin seems to occur (Figure 12). The results show, that the cells did not remain intact during preozonation. Geering (*432*) suggests, that ozone must be applied so that destruction of algal cells does not occur and hence a negative impact on the remaining water treatment stages is prevented. However, this was not the case in this spot-check. Also important for the efficacy of the ozonation step is the oxidant dose:cell ratio. Plummer (*527*) found cell lysis of *Scenedesmus* (cyanobacteria) only with ratios > 0.08 mg per 1000 cells. In the case of the investigated water, the applied 1.0 mg O_3 for 30,000 cells results in a ratio of 0.033. However, this ratio is also influenced by the cyanobacterial species, TOC and temperature. Ozonation efficacy is also influenced by natural organic matter (*435*), the pH and the temperature of the raw water (*528*). Thus, these laboratory findings are difficult to transfer to the water works situation. Considering the health risk for the population it is particularly important to eliminate dissolved toxin during the following treatment steps.

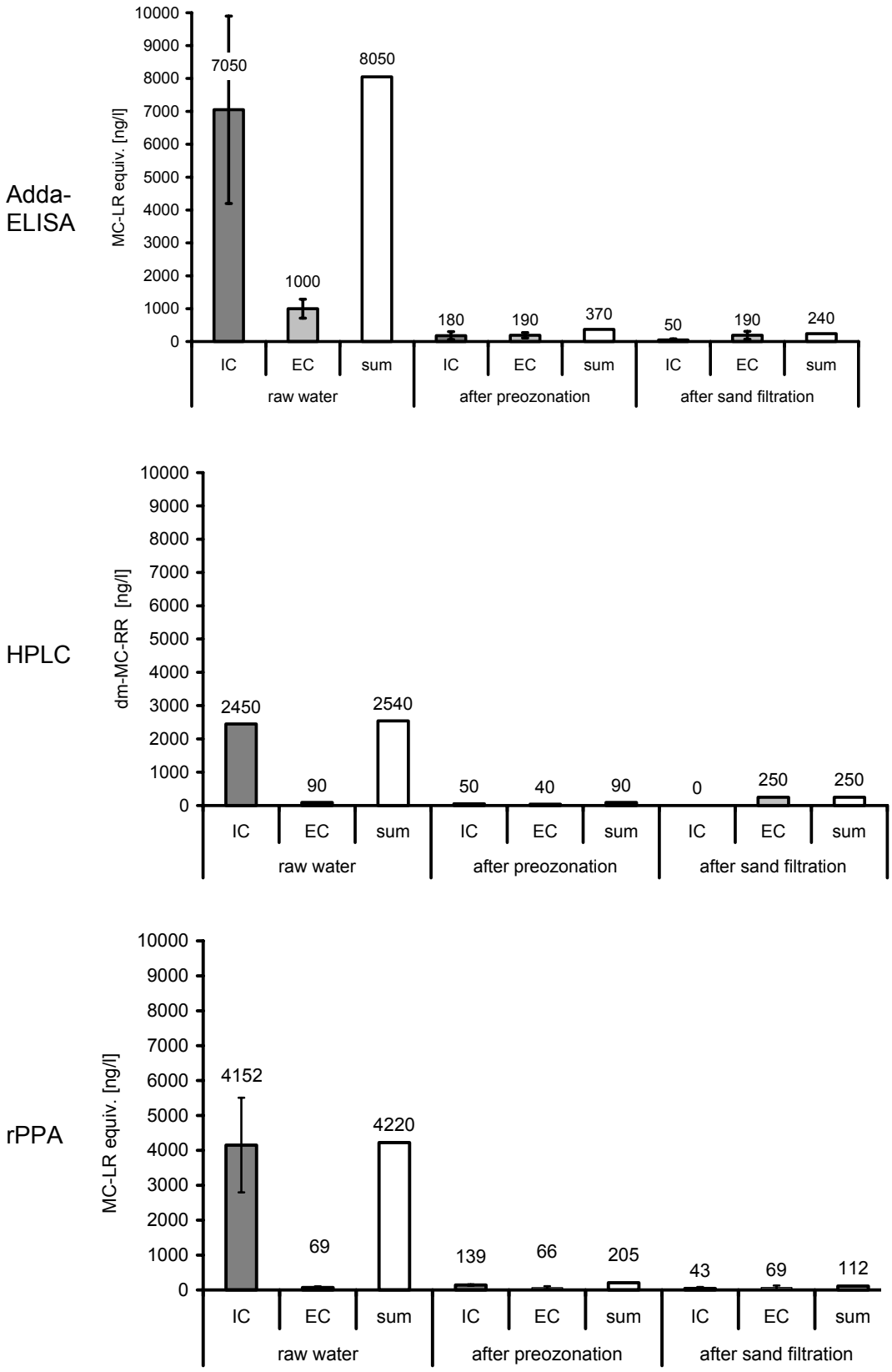


Figure 12. Intracellular (IC) and extracellular (EC) toxin concentration during the drinking water treatment process at Lake Zurich (Switzerland). Shown are the results of three different detection methods.

The ozonation in this water works seems to result not only in a lysis of the majority of the cyanobacterial cells, but also in a destruction of the dissolved toxins after lysis. While free MCs are also quickly oxidised with low ozone (0.2 mg/L) concentrations (*419*), this reaction also heavily depends on the DOC, temperature and pH present in the treated water. Thus, ozone concentrations similar to the ones applied in this water works are recommended. Low temperatures of raw water (4-8° C), pH values of 7-8 and DOC of 1-5 mg/L require high ozone doses (*419*). Rositano *et al.* (*529*) describe a relationship between the DOC and the required ozone concentration to eliminate 20 µg MC-LR/L. The concentrations rise from 0.5 mg/L for water with a DOC of 5.3 to 1.1 mg/L for a DOC of 15.5. However, these investigations were carried out at 20 °C and only with dissolved toxin. These toxins are immediately oxidised and not retained within cells. In the case of intact cells, the cells must be removed intact or the oxidation concentration and time must be high and long enough to destroy the cells and subsequently oxidise the toxins.

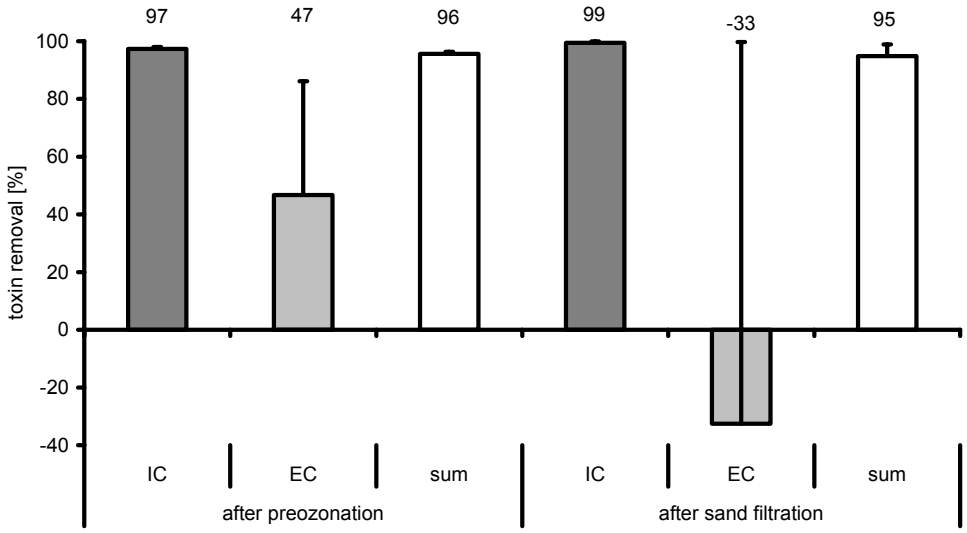


Figure 13. Efficiency of MC-elimination during the first two treatment steps at Zurich; IC intracellular, EC extracellular.

In the investigated water works, the subsequent filtration step does not further reduce the toxin concentrations (Figure 12, 13). This part of the treatment system is intended to decrease the organic load after the preozonation step. The dissolved toxins are to small to be filtered in this treatment step. No toxin degradation by bacteria seems to occur during fast filtration. It can be assumed that the following intermediate ozonation with 0.5 mg/L, the activated carbon filtration and the slow

sand filtration are able to eliminate the remaining toxin. Problems may arise if high densities of *P. rubescens* occur for a longer period in raw water. Unoxidised cyanobacterial toxins and cells could remain in the filters and be washed out with further charges (*445*). Breakthrough of toxins can occur after filtration of relatively small water volumes (*384*). Backwashing has been shown to be unable to remove large amounts of biomass from the filter media (*435*). The competitive effects of NOM and preloading of organic matter on activated carbon has been determined to cause a reduction in the capacity of activated carbon for MCs (*385*).

The Lengg water works (Zurich) employs a very effective treatment system. Because the associated lake is known for annual blooms of *P. rubescens* with cell densities of up to 1 x 10^5 cells/mL in raw water at a relevant depth, this complex water treatment procedure is necessary to guarantee safe drinking water.

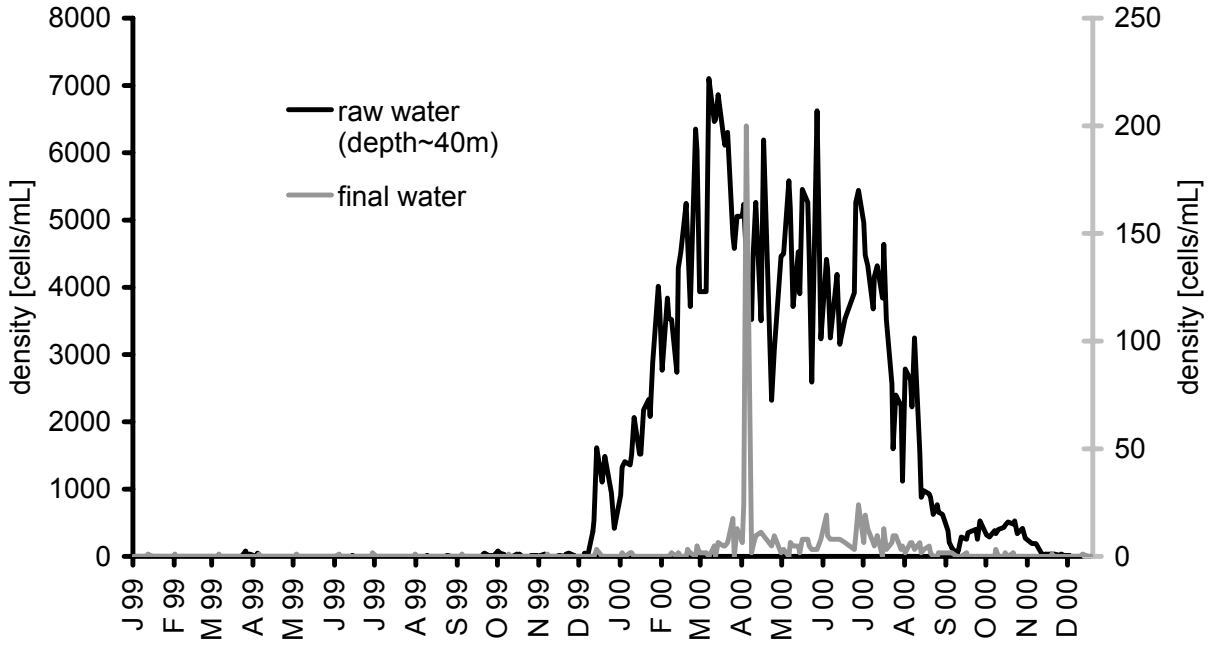


Figure 14. Density of *P. rubescens* in raw and final water in the water work of the Wahnbachtalsperre. Raw data provided by Dr. J. Clasen (water works at Wahnbachtalsperre).

Wahnbachtalsperre/Siegburg/Germany

P. rubescens is frequently observed in the Wahnbachtalsperre. Almost no *P. rubescens* trichomes were observed in 1999, whereas in 2000 *P. rubescens* predominated from January to November (Figure 14). It seems that there are "*Planktothrix*-years" in this reservoir (personal communication/Wahnbachtalsperre). Densities of up to 50,000 cell/mL were found in the metalimnion of the drinking water reservoir in 2000 (data not shown). The raw water from the water intake at a

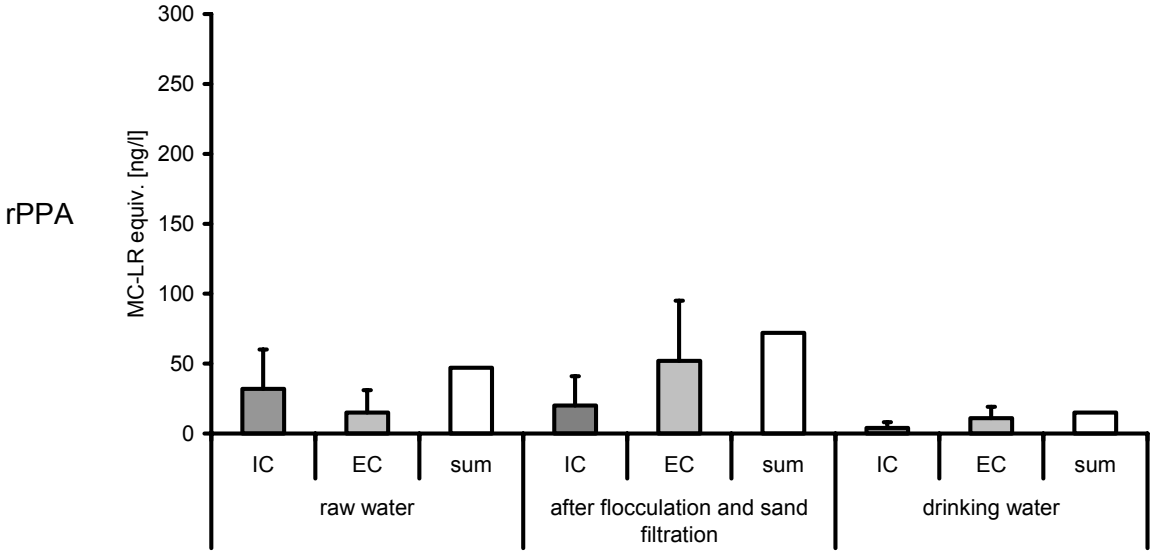
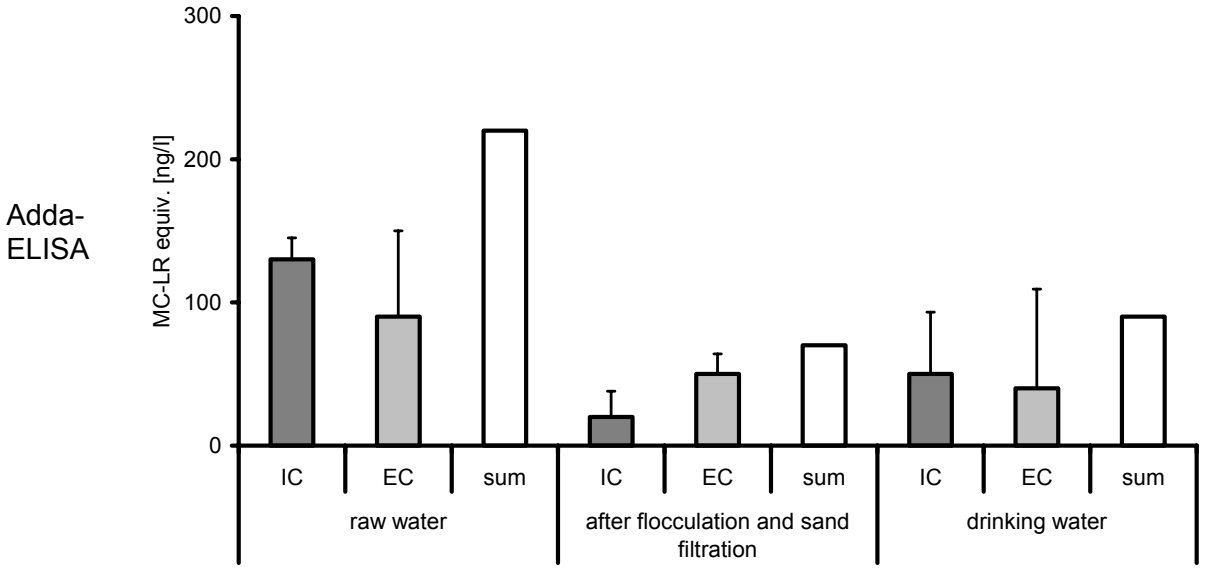


Figure 15. MC concentration in drinking water work at Wahnbachtalsperre. Shown are the results of rPPA and Adda-ELISA. Only traces could be detected via HPLC-DAD.



depth of 40 m contains up to 7000 cells/mL (Figure 14). Even in final water up to 200 cells/mL could be detected. The toxin concentration detected in raw water of this water works varied with the detection methods (Figure 15). The total toxin concentration ranged from 50 ng MC-LR equiv./L (rPPA) to 220 ng MC-LR equiv./L (Adda-ELISA). Only traces of an MC-RR variant were detectable by HPLC. Although only low amounts could be detected, toxin elimination was not efficient. Only approximately 60% of the toxicity could be removed through flocculation and filtration. At the time of this particular spot-check only low densities of *P. rubescens* occurred and lysed cells may have already released the toxin. Thus, the percentage of extracellular toxin was high. However, the pumping process could also be the reason for the detected concentration of extracellular toxin. Treatment systems employing only flocculation and filtration without an additional oxidation step or activated carbon filtration are a disadvantage with respect to the elimination of dissolved toxins. This treatment system is adequate to reduce cyanobacterial cell numbers, but is unable to remove the dissolved toxins (*373*).

The low densities of *P. rubescens* during this spot-check make a general risk assessment impossible. Since 2002, new treatment steps have been implemented in this water works (ultrasonication after flocculation and disinfection with UV). The efficacy of the new treatment to remove cyanotoxins has not yet been tested, but final disinfection with UV could be an useful tool for destroying MCs (*394*).

Lake Hallwil

Lake Hallwil was continuously “contaminated” with toxic cyanobacteria from 11/2000 until 6/2002 (Figure 16, 19). As a result, toxins were detectable in well water in spite of sediment filtration and dilution with ground water. No correlation is recognizable between toxin concentration near the lake shore and the toxin concentration in well water. However, high MC concentrations were detected by Adda-ELISA between 11/2000 and 01/2001. The high toxin concentration in the water over months, together with a possible reduced microbial activity during the cold season could be responsible for the relatively high toxin concentration of 220 ng MC-LR equiv./L in well water in 02/2001. Experiments have shown retarded biodegradation at temperatures below 4°C during slow sand filtration (*530*). In contrast, the toxin concentration in well water was unaffected by high toxin concentration (40 µg/L) at the shore near the drinking water well in 04/2001 (Figure

17). Only traces of toxins below 0.05 µg/L could be detected in five samples in spring 2001 and 2002 (Figure 17). Comparable results were obtained during a study at the Radeburg reservoir. While the reservoir water contained up to 18.5 µg MC/L, the concentration in the wells was always below 0.1 µg MC/L (*290*). MC concentrations from <0.01 to 0.1 µg/L could be detected in a study in Finnish bank filtration plants (*481*), although the toxin amounts in lake water were lower (<0.1-1.9 µg/L) than those in Lake Hallwil. No statement can be made about efficacy of bank filtration. The travel time between the trickle away of the toxin-loaded water at the shore to finding the toxin in the well depends on many factors such as temperature, pH (*531*) and the filtration material between the lake and the wells. Moreover, the filtered water is mixed with ground water. This implies even a higher concentration of MCs in the filtered water before mixing.

Jüttner (*479*) mentioned the schmutzdecke and the upper layers and not the distance between the lake and the drinking water well as the most effective components for the elimination of odorous cyanobacterial compounds. But the elimination of these compounds through bank filtration has been shown to be very effective at the river Ruhr (*479*) and several lakes around Berlin, Germany (*532*).

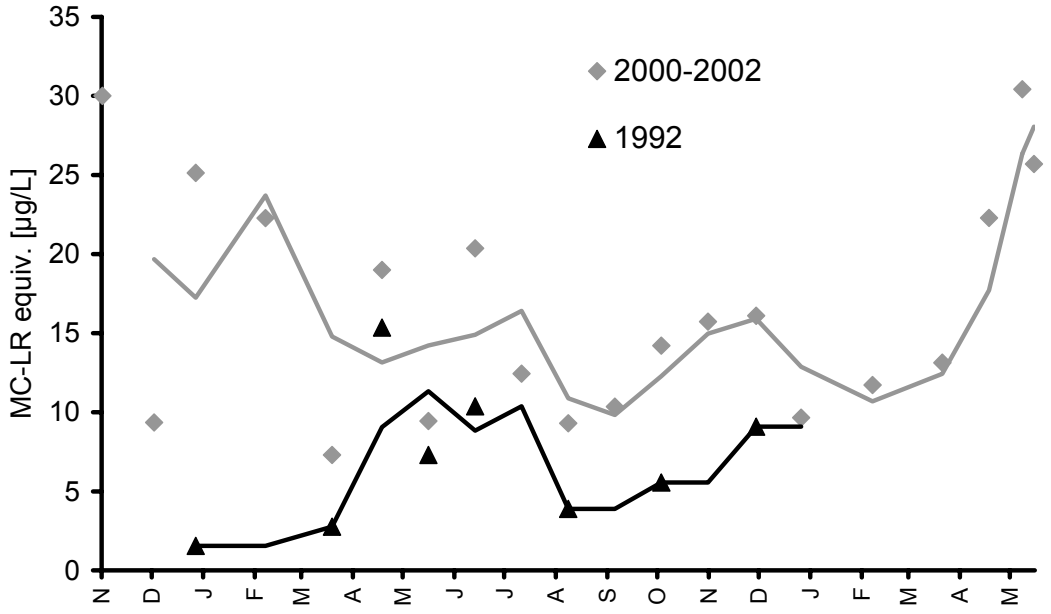


Figure 16. Toxin concentrations in Lake Hallwil (mixed samples from 1992 and 2000-2002) detected by Adda-ELISA.

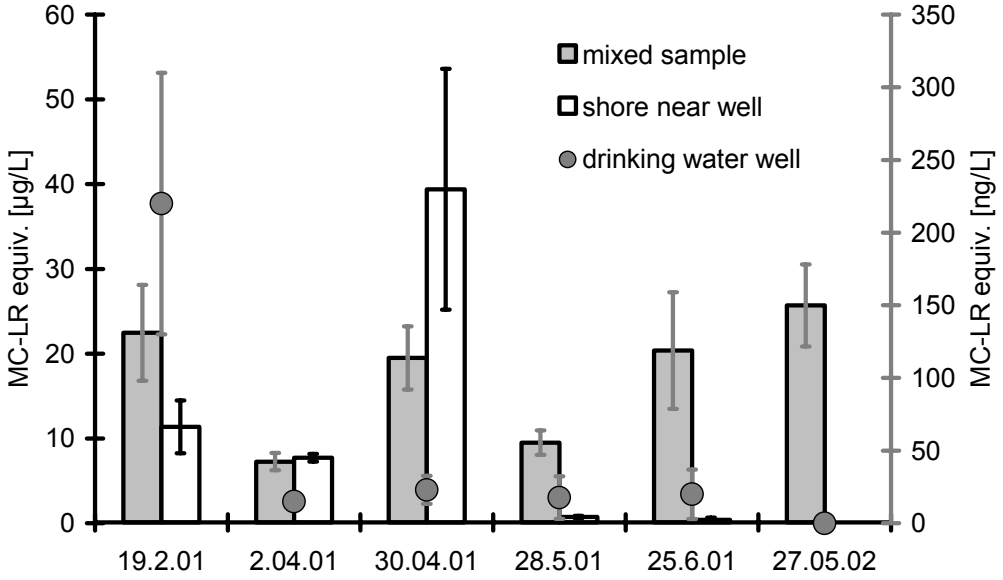


Figure 17. MC concentration (by Adda-ELISA) in Lake Hallwil (left axis) and in a drinking water well after bank filtration (right axis).

P. rubescens also occurred in 1992 in Lake Hallwil. Although the toxin concentrations were lower in 1992, the pattern of toxin concentration was similar between 1992 and 2001 (Figure 16). From May to June, an increase in toxin concentration could be recognized, followed by a decrease, possibly caused by high temperatures of the water or a shadowing of these metalimnic cyanobacteria by the abundance of other photosynthetic active organisms in the upper layers of the water column. From August to September, the toxin concentration increased again. In agreement with the findings of other researches, the toxin concentration is not always proportional to the density of the supposed toxin-producing organism (*216, 533-535*). While biomass reached its maximum in March 1992, the toxin concentration was relatively low. After the collapse of the bloom in April, the toxin amount increased. In October the bloom collapsed again but high toxin amounts could still be detected (Figure 18). The abundance of *Aph. flos-aquae* cannot explain the high MC-levels, because *Aph. flos-aquae* is not known to produce MCs. Even if this strain of *Aph. flos-aquae* is able to produce MC, this cannot explain the high MC levels in April 1992.

However, other *Oscillatoriales* which could not be exactly determined occurred during these periods in lake Hallwil. Thus, a shift in densities of phenotypically very similar species or strains is possible. These variants can be separated into toxin producers and non toxin producers. Referring to a perennial population of *P.*

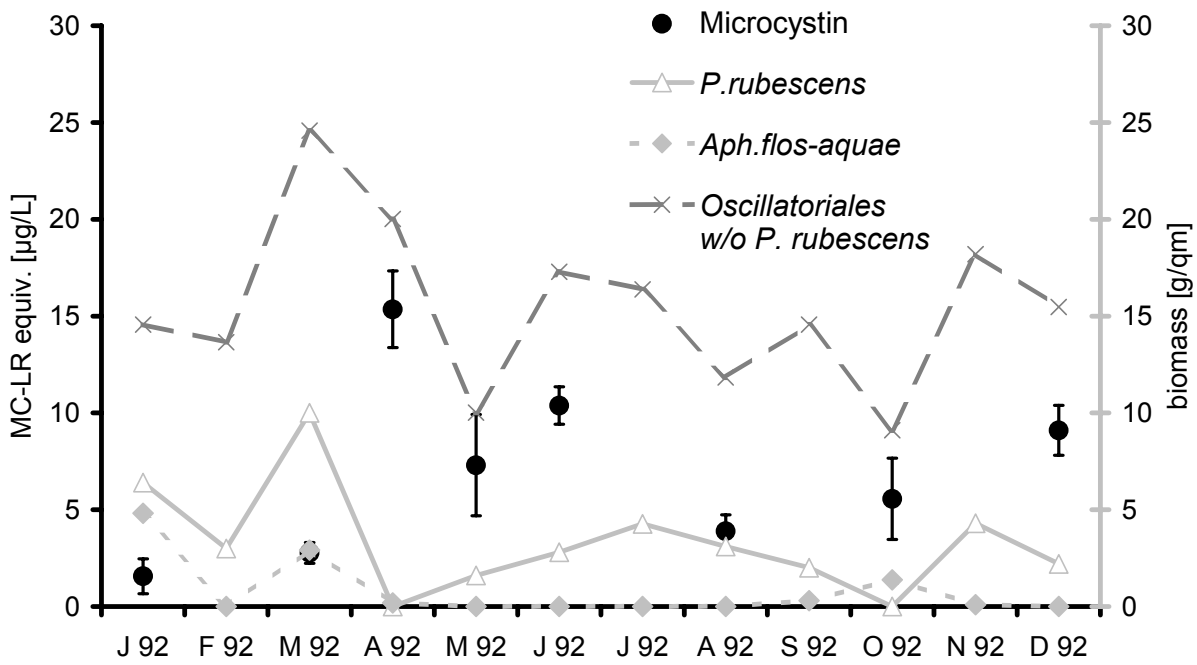


Figure 18. MC-LR concentrations and biomass of different cyanobacteria in lake Hallwil/Switzerland. Biomass data provided from Dr. Arno Stöckli, Baudepartment des Kantons Aarau, Abteilung für Umwelt, Sektion Gewässer und Betriebsabwasser, Aarau, Switzerland.

agardhii in France, Briand *et al.* (*533*) proposed, that non–toxic strains grow in winter, whereas toxic ones grow in spring and autumn. In contrast, in the Arendsee (Germany) the total MC concentration was demonstrated to correspond to the measured biovolumes of *P. rubescens* (*498*).

The toxin concentration of the weekly samples from 08/2001 till 05/2002 detected by Adda-ELISA and cPPA were compared (Figure 19). The structural detection method (Adda-ELISA) always yielded higher toxin concentrations. The differences vary from almost the same amount (02/2002) to a three-fold higher concentration 09/2001 (further discussion later in the text).

A comparison of different sample preparation methods shows the disadvantages of using one freeze – thaw cycle alone to the use of an additional extraction method with ultrasonication and solid phase extraction (Figure 20). Less than one third of the toxin was dissolved in the water phase after freeze-thawing compared to adding untrasonication and SPE.

Detection methods

In almost every case the different detection methods yielded different toxin concentrations. The Adda-ELISA detects every compound with a free Adda-amino acid in the sample. The potential biological function of this molecule is unimportant for this method. The microcystin or nodularin molecules must not be intact for this method. Detection of partly degraded products or even completely different molecules is theoretically possible. The latter, however, is unlikely. A detection method which uses the double bond on position 6 of the Adda-amino acid for a derivatisation reaction has detected no additional compounds to MCs/NODs in cyanobacteria so far (*536*). The results of the Adda-ELISA must be compared to a standard MC. The concentrations are specified as equivalents to the used congener, which was MC-LR in this study.

The rPPA and cPPA use the biological function of the phosphatases. The phosphatase inhibiting capacity of the investigated sample can be demonstrated by these test systems. The IC_{50} concentrations of the over 70 different MCs are variable and for the most part unknown. Variation within the heptapeptide was shown to influence the ability of the toxins to bind to the phosphatases (*537*). Degradation products after ozonation were shown to lose their ability to inhibit protein phosphatases. Thus the

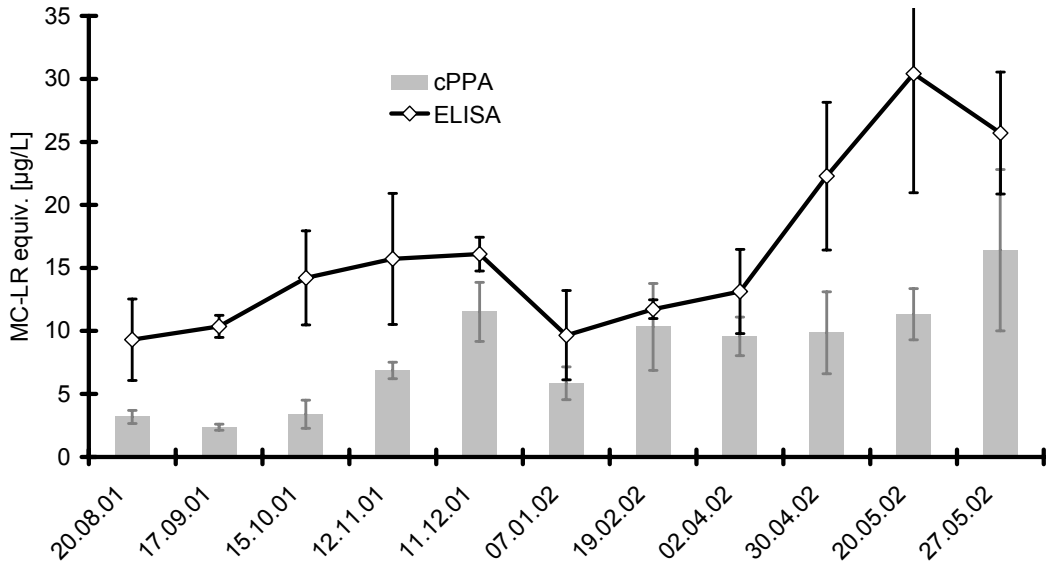


Figure 19. Toxin concentrations detected with cPPA and ELISA in Lake Hallwil.

results yield an average of every phosphatase-inhibiting compound as well as every phosphatase-activity promoting substance in the sample. The results of this assay should also be compared to a MC standard curve and are specified as MC-LR equivalent in this study.

The chemical variability in the microcystin family with more than 70 congeners is huge. It is very difficult to detect every MC within one HPLC assay. In this context every MC means detecting every MC, which is available as a standard. Six MC congeners are currently commercially available. The spectra of the congeners are different with absorption maxima between 222 and 240 nm. In addition, the peaks often co-elute with other unknown compounds. This makes the identification and quantification difficult and inaccurate. Thus, HPLC tends to underestimate the concentration of the MC-cocktail.

An international intercomparison exercise for the determination of MCs showed the difficulties involved in the analysis of the same sample with different methods and in different laboratories with different equipment (*538*). Thus, the detected concentrations must be seen as rough estimate of the real concentrations. In this study, however, exact concentrations are not necessary to estimate the risk for the population supplied with drinking water.

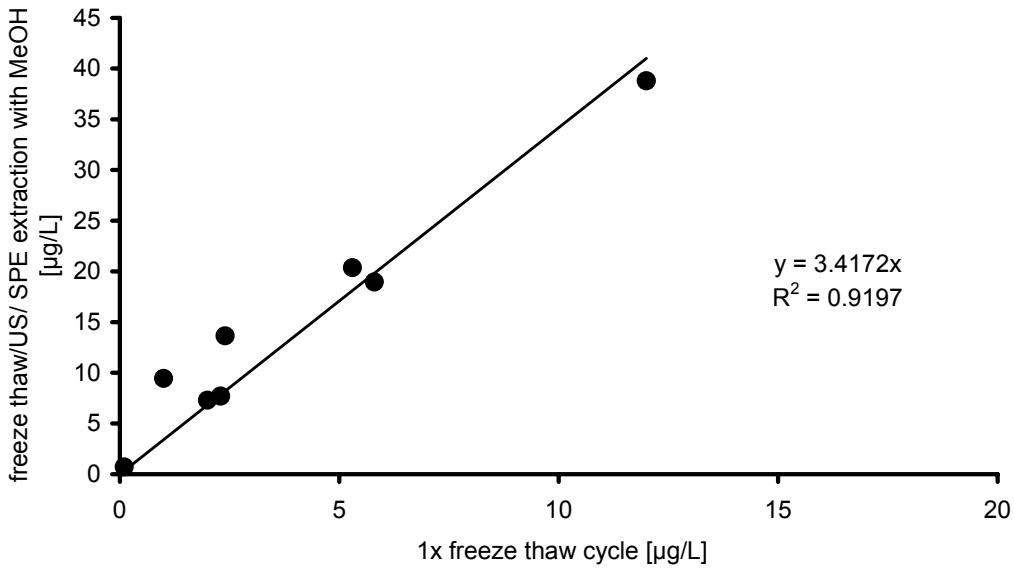


Figure 20. Comparison of two MC-extraction methods; US ultrasonication, SPE solid phase extraction.

CONCLUSION

Three completely different “cleaning“ methods to eliminate cyanobacterial toxins from raw water were investigated in this study. However, removing these secondary metabolites is not the main objective for the water works. Elimination of colour, bad taste and odour, chemicals and microorganisms should be guaranteed. Thus it is important to assess the ability of every water works to remove these harmful compounds from cyanobacterial loaded raw water.

In developed countries, a maximum of quality should be attained concerning food stuffs and drinking water. However, the raw water sources offer variable qualities of water from reservoir to reservoir and from day to day. Thus, the drinking water suppliers cannot eliminate the possibility of variations in drinking water quality. Analysis of the results of chemical and microbial investigations requires time. This means that the investigated water charge has already reached the user, when the results become available. A wise compromise could be: water should be as good as necessary not as good as possible (*523*).

Many lakes and drinking water reservoirs in Germany and Switzerland are predominated by *P. rubescens*. Due to their “behaviour” as a metalimnic inhabitant followed by mixing to deeper layers, raw water is often contaminated with high densities of *P. rubescens*. The concentration of MC did not surpass the WHO guideline of 1.0 µg/L in any of the cases presented here. However, contamination of final water during months with MC-concentrations below 0.2 µg/L can not be excluded. Nevertheless, spot-checks were taken only from final water in water works. Due to chlorination or dilution with not-contaminated water, contamination of tap water could actually be distinctly lower. With respect to the possible tumour promoting activities of MCs, chronic exposure of populations to concentrations below 0.1 µg/L should be avoided. Additionally, the presence of other routes of toxin exposure (contaminated food, algal “health” products) emphasize the need for an improved human toxin exposure assessment.

It is thus essential that the potential for cyanobacteria blooms is given careful consideration in the design and operation of drinking water reservoirs and treatment works.

OCCURRENCE AND ELIMINATION OF CYANOBACTERIAL TOXINS IN TWO AUSTRALIAN WATER TREATMENT PLANTS

In Australian freshwaters, *Anabaena circinalis* (PSPs), *Microcystis* spp. (MCs) and *Cylindrospermopsis raciborskii* (CYL) are the dominant toxic cyanobacteria. Many of these surface waters are used as drinking water sources. Therefore, the National Health and Medical Research Council of Australia set a guideline for MC-LR toxicity equivalents of 1.3 µg/L drinking water. However, due to lack of adequate data, no guideline values for PSPs (=saxitoxins) or CYL have been set. In this spot check, the concentration of MCs, saxitoxins and cylindrospermopsin were determined by ADDA-ELISA, PPA, HPLC-DAD and/or HPLC-MS/MS in two water works in Queensland/Australia and compared to phytoplankton data collected by Queensland Health, Brisbane. Depending on the predominant cyanobacterial species in a bloom, e.g. *Microcystis aeruginosa*, *Anabaena circinalis*, *Cylindrospermopsis raciborskii* concentrations of up to 8.0, 17.0 and 1.3 µg/L were found for MCs, saxitoxins and cylindrospermopsin, respectively. However, only traces (<1.0 µg/L) of these toxins were detected in final water (final product of the drinking water treatment plant) and tap water (household sample). However despite the low concentrations of toxins detected in drinking water, to guarantee safe drinking water, a further reduction of cyanobacterial toxins is recommended.

INTRODUCTION

The presence of toxic cyanobacterial blooms in water bodies used either as drinking water or for recreational purposes may present serious health risks for the human population. Safe drinking water is one of the most critical factors to guarantee long term population health. In Australia about 70% of drinking water comes from surface waters. Due to climatic conditions in many parts of this continent as well as due to the nutrient load from agriculture (*539*), the phytoplankton of reservoirs, lakes and rivers is often predominated by cyanobacteria (blue-green algae). For example, the 1000 km bloom of the Darling-Barwon river in 1991 caused a loss of one million people-days of drinking water (*539*). Water conservation and management is therefore a critical national issue in Australia. Water works not only have to reduce cyanobacterial cells, odour and colour during the water treatment process, they also have to eliminate the toxins produced by these organisms.

Cyanobacteria produce a variety of toxins, which are usually defined by their chemical structure and fall into three groups: cyclic peptides (the hepatotoxins microcystins (MC) and nodularins (NOD)), alkaloids (the neurotoxins paralytic shellfish poisons (PSPs) and anatoxins), cylindrospermopsin (CYL), and lipopolysaccharides. The cyanobacterial genera most often associated with toxicity are *Anabaena, Aphanizomenon, Cylindrospermopsis*, *Lyngbya, Microcystis*, *Nodularia* and *Planktothrix (Oscillatoria)* (*416*). The cyclic peptide toxins are the most widespread freshwater cyanobacterial toxins and are therefore very important regarding treatment of drinking water. Acute intoxications with MCs (heptamer) or NODs (pentamer) result in fulminant liver damage (*141, 540*). MCs and NODs have also been shown to be tumour promoters (*357, 362*). Additionally, NOD can act as a tumour initiator (*116*). Concerns over these health risks have prompted the World Health Organization (WHO) to adopt a provisional guideline value for MC-LR (L for leucine and R for arginine at the most variable positions of the heptapeptide) of 1.0 µg/L drinking water (*1*). The National Health and Medical Research Council of Australia have set the guideline slightly higher to 1.3 µg/L (*458*). Basis for these guideline values are the study from Fawell *et al.* (*409*). Additionally, Fitzgerald *et al.* (*459*) proposed the publication of a health alert if the concentration of 10 µg MCs/L drinking water is reached for even a brief period.

Due to the lack of data, no guideline value is set yet for concentrations of nodularin, cylindrospermopsin or saxitoxin in drinking water (*458, 541*). Acute intoxication with cylindrospermopsin (*342*) causes massive hepatocyte necrosis. Injuries in kidney, lungs and intestine have also been seen (*328*). Carcinogenic activity is caused by the ability of cylindrospermopsin to induce strand breaks at the DNA level and loss of whole chromosomes (*542*). Beside the capacity of CYL to suppress glutathione and protein synthesis, probably by inhibiting DNA transcription, other mechanisms of actions have been assumed as an explanation for the severe liver injuries followed by CYL intoxication, although no protein phosphatase inhibition could be found (*543, 544*). A further important consideration for water works is, that CYL with its zwitterionic character is highly water soluble.

PSPs are potent blockers of voltage-dependent sodium-channels and therefore restrict transmission between neurons. In relatively low doses (LD_{50} for saxitoxin i.p. in mice: 10 µg/kg (*545*)) PSPs paralyse the respiratory tract and result in rapid death (*546*). To our knowledge, Australian isolates of *Anabaena circinalis* are the only PSP-

producing cyanobacteria worldwide. In contrast, European and American strains of *A. circinalis* appear to exclusively produce anatoxins (*547*). In general, the genus *Anabaena* can produce microcystins, PSPs and anatoxin-a with toxin contents of 2 mg/g dry weight (DW) for MCs (*56*), 4.4 mg/g DW for PSPs (*548*) and a remarkable 28 mg/g DW for anatoxin-a (*56*).

Australia has a documented history of mortalities of livestock (*68, 223, 341, 549-553*) and human health impacts (*257, 259-261*). In order to minimize the risk of cyanotoxic contamination of drinking water, the development of methods to effectively reduce toxin concentrations to below acceptable levels in drinking water has thus become an important focus of current research efforts. The two water works investigated use a similar treatment process to clean the raw water: flocculation with aluminium sulphate, optional addition of powdered activated carbon (PAC), sedimentation, sand filtration and chlorination before storage. The efficiency of these methods to remove cyanobacterial cells and toxins are controversial and strongly dependent on the following factors (*417, 445, 554*)

- cyanobacterial species and density
- additional organic load
- concentration and type of flocculent and activated carbon
- pH during flocculation and chlorination
- care taken of the treatment system especially of the filter bed (backwashing etc.)
- monitoring of the water reservoir

For the investigation presented here water works were selected that obtain their raw water from surface waters in which toxic cyanobacteria are regularly found. Microcystins as well as cylindrospermopsin and different PSPs were detected in raw water of these water works. Spot checks of efficiency of the water treatment process were compared to literature data and to the phytoplankton situation in these water reservoirs over the last two years.

MATERIALS & METHODS

Characteristic of the water works

Both water works are located near Brisbane Queensland/Australia and take the raw water from water reservoirs. Detailed information is given in Table 17.

	Water treatment steps	Predominant cyanobacteria	Supplied population	Analysed toxins
Water works Gordonbrook Dam (Kingaroy)	flocculation (130 mg $Al_2(SO_4)_3$/L), sedimentation, optional PAC (12 mg/L), sand filtration	*M. aeruginosa, A. circinalis*	8000 -10000	MCs + PSPs
Water works North Pine Dam (Brisbane)	flocculation/sedimentation, optional PAC, sand filtration, chlorination before storage	*C. raciborskii*	one of the water suppliers for Brisbane (1.5 million people)	CYL + MCs

Table 17. Treatment system, predominant cyanobacteria and analysed toxins in two water works in Queensland/ Australia; PAC powdered activated carbon.

Analysis of cyanobacterial cells at Gordonbrook Dam/Kingaroy

A water sample was taken from the raw water intake in the dam and then sent to Queensland Health Scientific Services. It was fixed with lugol's iodine 7 mL/L of sample. If necessary the sample was concentrated 10 times by sedimentation in a measuring cylinder. This concentrated sample was then mixed by inverting 20 times and 1 mL transferred to a Sedgewick-Rafter counting cell. A minimum of 23 units of each alga was counted, and a minimum 3 squares in the chamber. Cells per colony were counted (or estimated for *M. aeruginosa*) for each colony.

Sample preparation for spot checks

Samples for the spot check investigation were taken at each stage from raw water to final water. Additionally, cyanobacterial blooms and sludge resulting from flocculation were sampled. The aqueous samples were stored below 10°C until filtered through Millex-GN (Millipore)-filter to separate cell-bound from free toxin within the next 48 hours. The filter and filtered water were stored at -20°C pending toxin extraction and concentration.

MICROCYSTINS + PARALYTIC SHELLFISH POISONS

The cell-bound toxin was extracted by tearing the filter into small pieces, addition of 1.5 ml of 75% methanol (MeOH) in a 2.0 ml reaction vessel, sonication for 20 min (cooled water bath) and shaking for 30 min. The methanol/sonication/shaking steps were repeated twice, the supernatants were sampled, combined, dried under nitrogen and redissolved with 10 ml H_2O. For clean-up, C_{18} solid phase extraction columns (ICT Isolute C_{18} EC, 1 g/6 ml) were conditioned with 6 ml MeOH, washed with 6 ml H_2O, loaded with sample, washed with 6 ml H_2O and the MCs were eluted with 12 ml 100% MeOH. After drying, pellets were finally dissolved in 2 ml 50% MeOH. The MCs in the filtered water (500 ml) were also concentrated by C_{18} SPE (see above). Cyanobacterial bloom or sludge material was freeze dried and stored at -20°C. For analysis, 100 mg DW of each sample was extracted with 10 ml 75% MeOH, sonicated and shaken. This procedure was repeated twice and the supernatants collected.

CYLINDROSPERMOPSINS + MICROCYSTINS

Free CYL and MCs were isolated via C_{18} carbograph SPE columns SC-103 (Lind-manufacturing) by washing the column with 10 ml H_2O followed by 100 ml of the sample (CYL), 6 ml of 10% MeOH and 6 ml of 100% MeOH (MCs). Due to the low detection limit, the CYL fraction could be directly analysed by HPLC MS/MS, whereas the 10% MeOH fraction was freeze dried and the 100% MeOH fraction dried under nitrogen. Both samples were resolved in 1 ml H_2O.
The filters were extracted twice with 1.5 ml 100% MeOH and once with 1.5 ml 75% MeOH. Sonication and shaking steps followed by centrifugation were included. The supernatants were combined, dried under nitrogen and redissolved in 1.8 ml H_2O.

Sample preparation at Queensland Health Scientific Services

In contrast to the preparation presented above, weekly samples from water works at Gordonbrook Dam were sonicated to disrupt any cells and subsequently filtered. The concentration procedure for detection of lower MC concentrations is described in the next paragraph.

Determination and quantification of the toxins

COLOURIMETRIC PROTEIN PHOSPHATASE ASSAY (CPPA)

The colorimetric protein phosphatase assay (cPPA) was performed as described by Heresztyn *et al.* (*524*) using the protein phosphatase 1 (PP1) instead of PP 2a with microcystin-LR (Alexis, Switzerland) as a standard.
The PPA-inhibiting capacity of the samples was compared to a microcystin-LR standard-curve and expressed as microcystin-LR equivalents. The detection range (20-80% inhibition of PP1) of the colorimetric PPA used is 0.9-3.2 µg MC-LR/L with a derived IC_{50} of 1.7 µg MC-LR/L.

ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA)

As a structural biological test system, an ADDA-ELISA established by Fischer *et al.* (*525*) was used. Briefly, ELISA plates (NUNC MaxiSorp, Denmark) were coated overnight with OVA–ADDA–hemiglutaryl (OVA–ADDA–HG) in 0.05 M sodium carbonate buffer pH 9.6 (50 µL/well, 2.5 µg/mL) at 20 °C. Unbound material was removed by aspiration. After washing with PBS, additional binding sites were blocked by incubation with OVA (1% w/v, 200 µL, >2 h, 20–25 °C). Plates were washed three times with PBS and used immediately or stored at 4 °C for up to 7 days. Samples or standards (50 µL) were added to the wells together with antiserum (AB824 at 1/160,000). After incubation at 20–25 °C for 2h, wells were washed twice with phosphate buffer solution containing 0.05% Tween™20 (PBST) and twice with PBS. Anti-sheep secondary antibody (ICN/Cappel rabbit-anti-sheep–HRP) (100 µL, dilution 1/6000) was then added to the wells and incubated for 2 h. Subsequently, wells were aspirated, and washed twice with PBST and twice with PBS. As substrate TMB (100 µL, Sigma, Germany) was added and incubated for 30 min. The reaction was stopped by addition of H_2SO_4 (50 µL, 2 M), and absorbance determined with a microplate reader at 450 nm. The limits of quantitation and detection of the ELISA were 0.2 µg/L and 0.05 µg/L, respectively.

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

PARALYTIC SHELLFISH POISONS

Samples were analysed by HPLC according to the method described by Lawrence *et al.* (*555*). PSPs were oxidised using hydrogen peroxide and analysed using a Shimadzu LC-10ADVp HPLC system with a Shimadzu RF-10AXL fluorescence detector set at an extinction wavelength of 330 nm and an emission wavelength of 390 nm. An Altima C18 column (150 mm x 4.6 mm, 5µm) was used with a 1 mL/min linear gradient of 1% to 8% acetonitrile/0.1M ammonium formate over 20 minutes. PSPs (if present) were identified and quantified by comparison to standards obtained from NRC, Canada. Detection limits vary for individual PSPs, however, detection limit for PSPs of 0.5 µg/L is achievable.

CYLINDROSPERMOPSIN

CYL was determined by HPLC MS/MS using a PE/Sciex API 300 mass spectrometer equipped with a turbo-ionspray interface, coupled to a Perkin Elmer series 200 HPLC system (*556*). Separation was achieved using a 5µm 150 x 4.8 mm Altima C_{18} column (Altech, Australia) run at 35°C, and a flow rate of 0.8 mL/min with a linear gradient starting at 100% A for 0.1 min, ramped to 100% B in 5 min, held for 1 min and then to 100% A in 2 min and equilibrated for 7 min.

The dead volume in the system modified the actual gradient at the column and equated to approximately 3 minutes at 100% A before the start of the gradient. (A=1% MeOH/deionised water, B=60% MeOH/deionised water, both 5 mM in ammonium acetate). Under these conditions the retention times for CYL and deoxy-CYL are 6.64 and 7.05 minutes, respectively. The column effluent was split to achieve a flow rate of 0.25 mL/min to the mass spectrometer. The mass spectrometer was operated in the multiple reaction-monitoring modes using nitrogen as the collision gas with collision energy of 44eV. The transitions m/z 416.2 $(M+H)^+$ to 194.1, 416.2/176.1 for CYL and 400.4 $(M+H)^+$ to 194.1 for deoxy-CYL were monitored with a dwell time of 300, 150 and 100 milliseconds respectively. Quantification of CYL was achieved using the 416.2/194.1 transition with other transitions monitored as confirmation ions. Using a 150 µL injection volume the limit of detection using this method is typically less than 0.2 µg/L and response is linear to at least 1000 µg/L.

MICROCYSTIN

Samples were analysed for microcystins, based on the method of Lawton *et al.* (*526*), using cell lysis by ultrasonication, a pre-concentration technique, and then HPLC. Fifty mL of bloom sample were sonicated to lyse cells using a Branson 450 watt ultrasonic probe. The solution was then filtered using a 0.45 micron disk filter. MCs were concentrated onto a C_{18} cartridge (Alltima C_{18} 7.5x4.6 mm, guard column) by pumping 25 mL of the filtered solution through the cartridge. The cartridge was then switched into the analytical HPLC system (Shimadzu, LC10A) and the eluted microcystins separated on an Alltima C_{18} column (150 x 4.6 mm, Alltech, Australia) using an 8 mM ammonium acetate/acetonitrile gradient (flow 1 ml/min, 15% acetonitrile to 35% acetonitrile in 25 min) MCs were identified by their characteristic UV spectra using a Shimadzu diode array detector. Quantitation was performed by comparing the areas of the microcystin peaks identified in the sample to the area of a standard microcystin LR of known concentration and reported as total microcystins (mg/L). A detection limit of 0.2 µg/L for individual MCs is achievable by this method.

RESULTS

Water works at Gordonbrook Dam

PHYTOPLANKTON AND TOXIN CONCENTRATIONS MONITORED OVER A THREE YEARS PERIOD

At Gordonbrook Dam (description in Table 17), extremely high cyanobacterial cell numbers occur in raw water almost continually, *M. aeruginosa* and *A. circinalis* being the predominant species. A pattern of predominance of these cyanobacterial species in different seasons over the three years period is clearly recognizable (Figure 21). Every year in February and March *M. aeruginosa* and *A. circinalis* disappear from raw water and *Planktothrix sp.* occurs for a short period (Figure 23). In 2000 and 2001 *M. aeruginosa* blooms developed after that period. Between July and September 1999 and 2001, *A. circinalis* was able to form blooms with high concentrations of PSPs, whereas no bloom occurred during the colder period 1999. In contrast, the year 2000 was predominated by *M. aeruginosa* without shift to *Anabaen*a. *M. aeruginosa* and Australian strains of *A. circinalis* are known producers of MCs and PSPs, respectively. However, MC concentrations correlate

poorly with the cell counts of *M. aeruginosa* (Figure 25, R^2 = 0.19) even without data from 5/2000 to 03/2001, when no microcystin could be detected, although a *M. aeruginosa* strain has dominated the phytoplankton with densities of up to 2.2 x 10^6 cells/mL over this period (Figure 21, Table 18). MC concentrations are highest at the beginning and the end of a *M. aeruginosa* dominated period with up to 8.0 µg/L, determined and calculated by HPLC-DAD. At two time periods (22/02 - 08/05/2000 and 10/4 - 14/08/2001) MCs could be found in raw water over months (Figure 23, 24). *A. circinalis* occurs in densities up to 3.25 x 10^5 cells/mL (Table 18, Figure 21) with R^2 = 0.51 for the PSP concentrations plotted versus *A. circinalis* densities over the three years period (Figure 25). The concentration of PSPs correlated to *A. circinalis* abundance with an estimated toxin concentration of 0.12 pg PSP/cell. In 23.3% and 22.7% of the weekly raw water samples MCs and PSPs could be detected, respectively. One of theses two toxin families could be determined in 36% of samples.

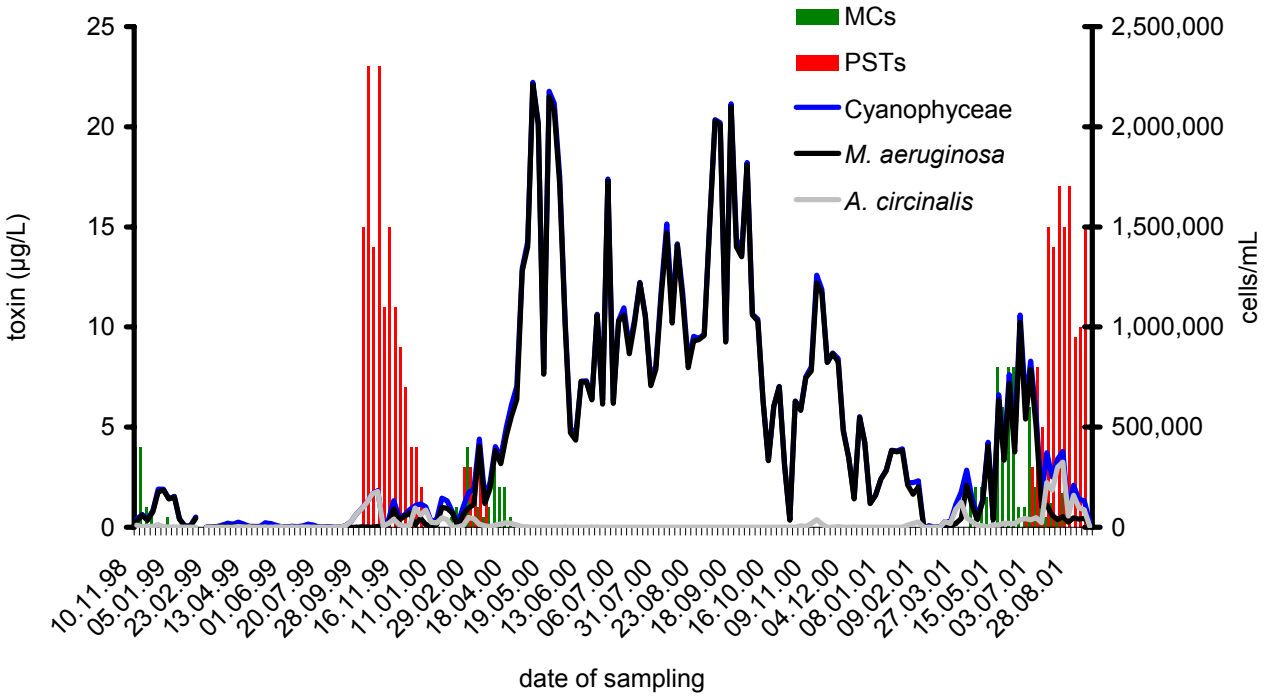


Figure 21. Cyanobacterial cell concentrations and toxin concentrations over a three years period in raw water from Gordonbrook Dam/Kingaroy. For details see **Figures 22-24** (next page).

Figure 22

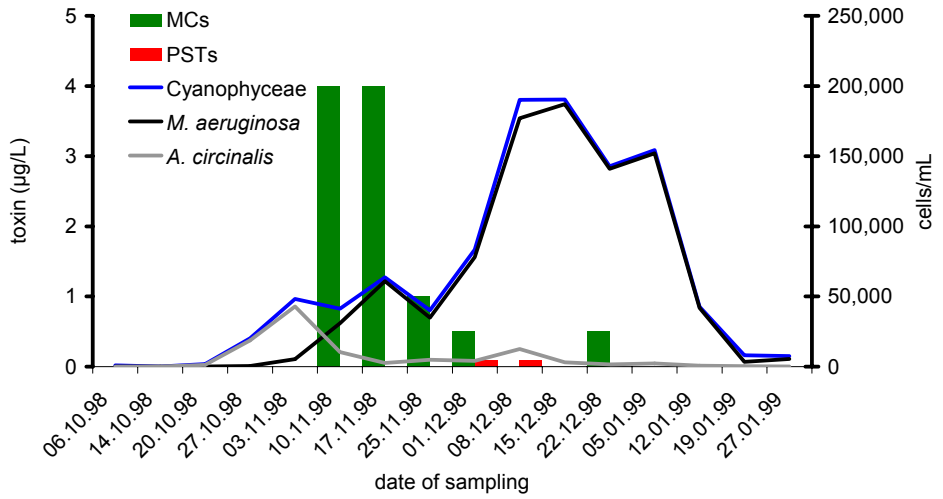


Figure 23

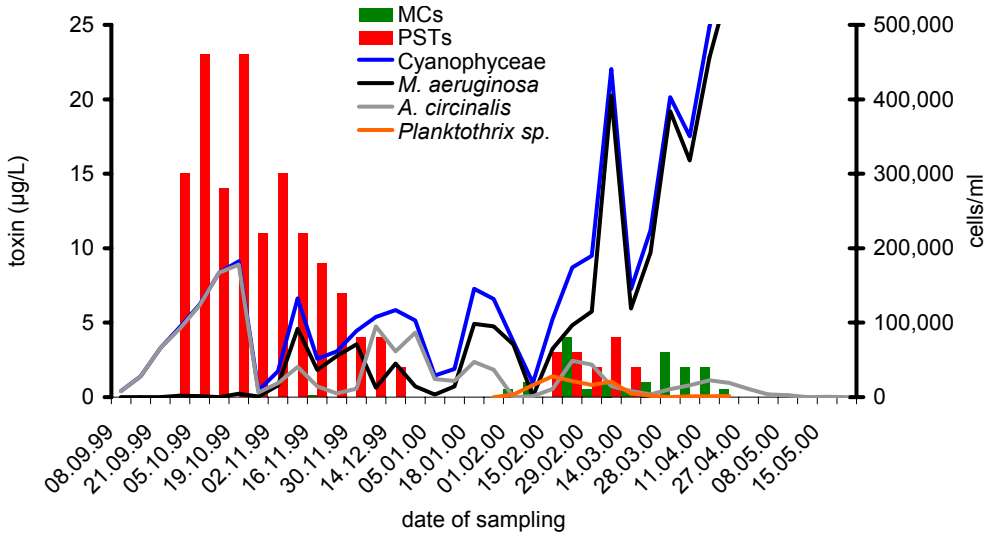


Figure 24

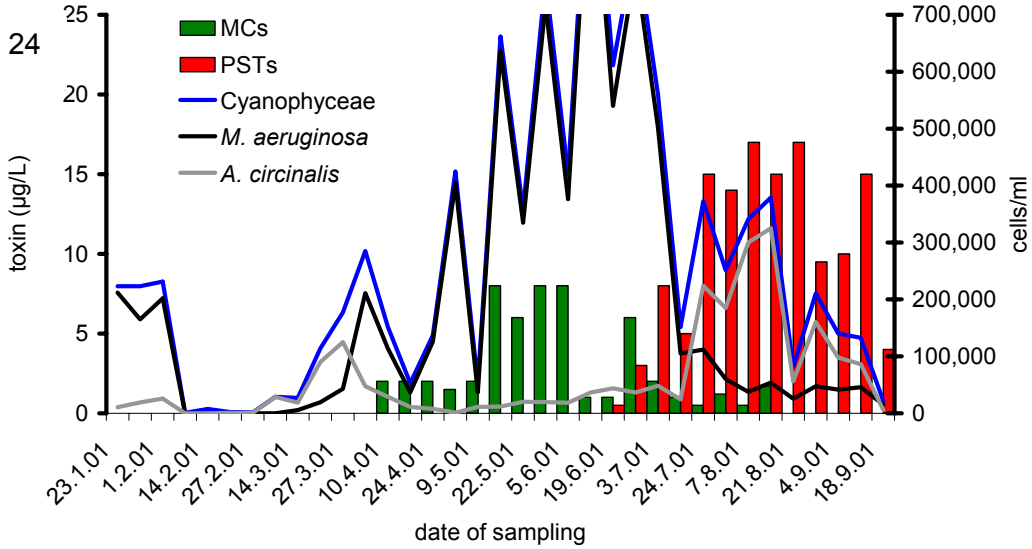


Figure 22-24. Cyanobacterial cell concentrations and toxin concentrations during periods of special interest in detail. Shown are extracts of **Figure 21**.

Table 18. Water work at Gordonbrook Dam: Cyanobacterial cell concentration in cells/mL in raw, final and tap water; others means other cyanobacteria.

		total	*M. aeruginosa*	*A. circinalis*	Others
Raw water	average	373,425	326,234	40,227	6,963
	%	100	87.4	10.8	1.9
	max	1,259,100	1,220,000	325,000	7,250
	min	500	0	0	0
Final water	average	1,433	1,389	9	35
	max	11,230	11,200	165	291
	min	45	0	0	0
Tap water	average	1,199	1,162	7	30
	max	7,178	7,165	173	290
	min	81	0	0	0

ELIMINATION OF PHYTOPLANKTON OVER A 46 WEEKS PERIOD

During the period (11/2000 – 9/2001) cyanobacterial densities reached 1.26 x 10^6 cells/mL in raw water, whereby *M. aeruginosa* was responsible for these high cell counts. On average, the water works had to eliminate 3.7 x 10^5 cyanobacterial cells/mL (Table 18). In final and tap water, the cell densities were reduced by >99% (Figure 26). Table 18 shows in detail the cell counts for total cyanophyceae and for the predominant cyanobacterial species in raw water at this water works. The percentage of cyanobacterial cell reduction is shown in Figure 27. Best removal occurs for *A. circinalis* cells. On average, only 0.04% of the cells found in raw water are still present in final and tap water. In contrast, 0.76 and 0.55% of *M. aeruginosa* cells occurred in final and tap water, respectively. Remarkable are the high standard deviations in these cases as indication for a strong variation in elimination of *Microcystis* cells. Not included in Figure 27 are data from 20/2/2001 to 27/03/2001 with low cell counts in raw water, but still high cell numbers in final water tanks (from the preceding period with higher cell densities). These situations result in higher cell densities in final and tap water than in raw water and are reported elsewhere (*380*). In 2 of 52 samples (07/00 – 06/01) PSPs in concentrations below 0.5 µg/L but no MCs were detectable in final water. No toxin reached the tap water in detectable concentrations.

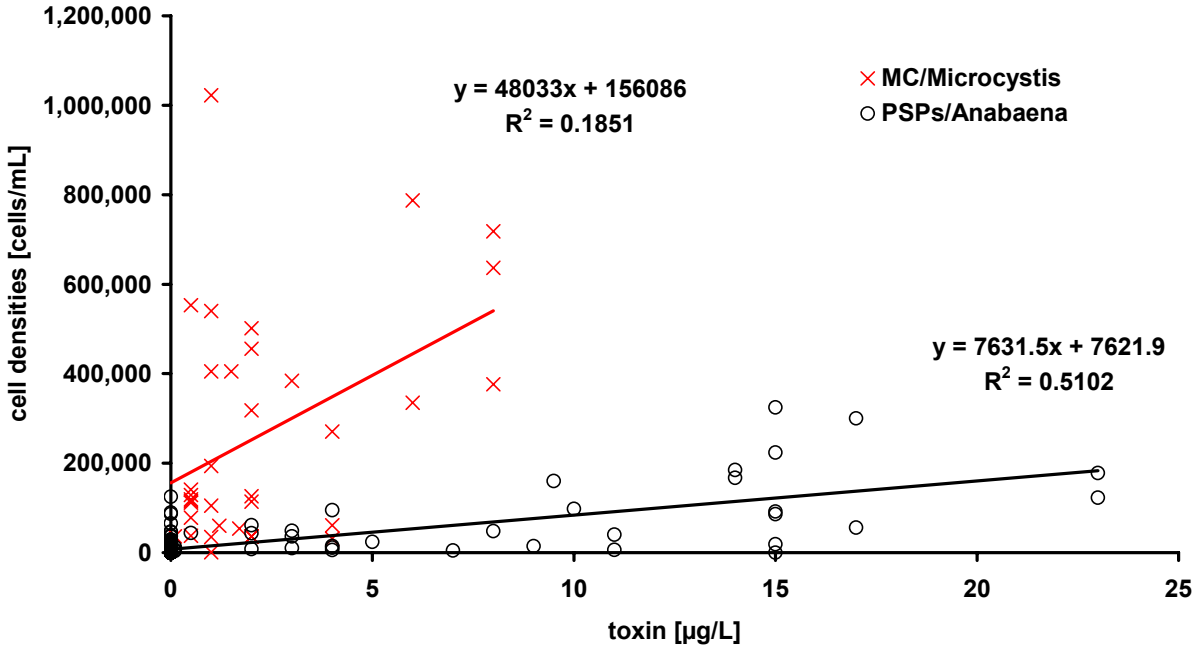


Figure 25. Relationship between cell densities and toxin concentrations in a water reservoir.

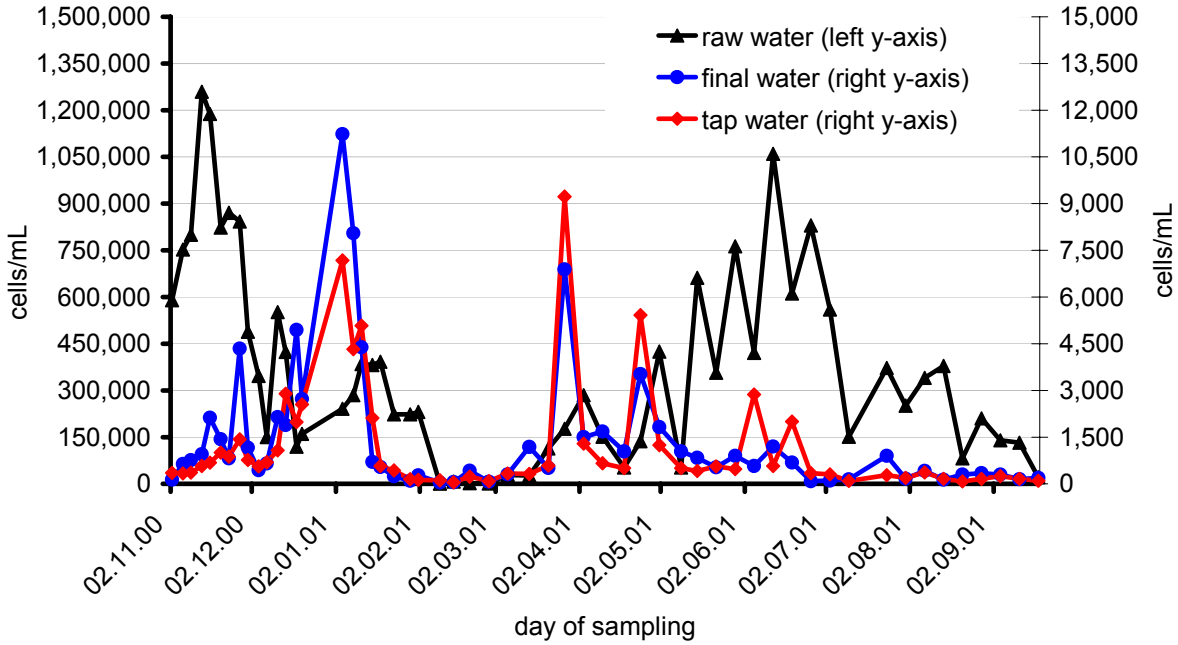


Figure 26. Reduction of cyanobacterial cells during treatment train from raw water to tap water at Gordonbrook Dam/Kingaroy/Australia.

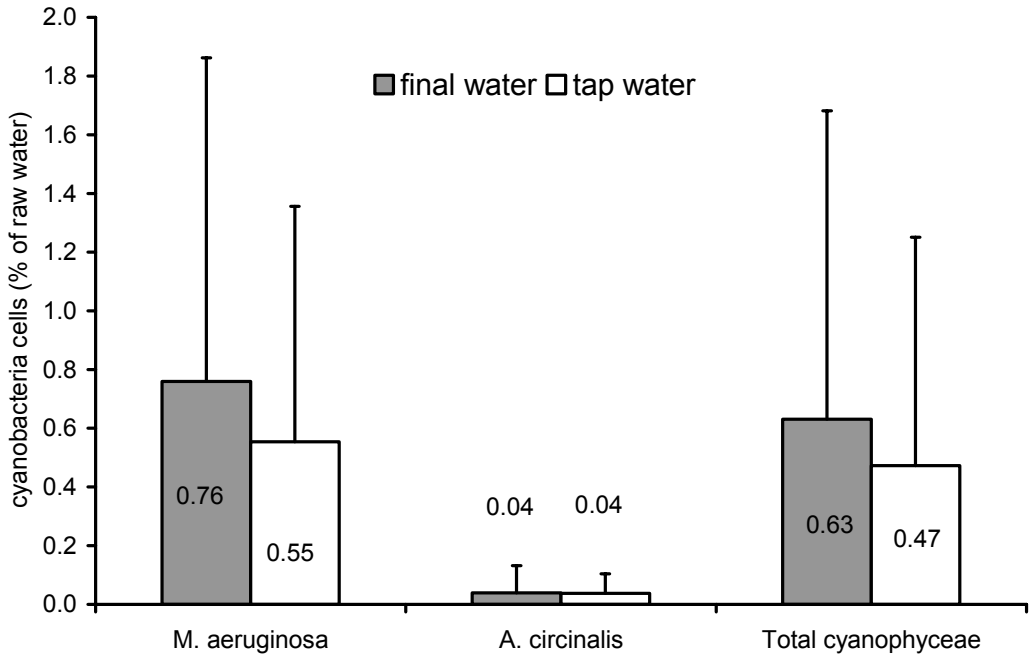


Figure 27. Cell residual after treatment as % cells compared to cells in raw water at Gordonbrook Dam.

EFFICIENCY OF WATER TREATMENT TRAIN

…….TO REMOVE CYANOBACTERIAL CELLS

During a period of high cell densities of *M. aeruginosa* and *A. circinalis* between 05/06/2001 and 01/08/2001 the efficiencies of flocculation (n=15) and, in part, of filtration (n=5) were investigated (Figure 28). Cells were reduced by ~99% through flocculation/sedimentation without differences between the cyanobacterial species (98.9% and 99.1%). However, due to high cell counts in raw water, up to 12,400 cells/mL were still present after this first treatment step. In contrast, efficiency of sand filtration for the removal of *A. circinalis* (99%) was higher compared to *M. aeruginosa* (84.8%). In summary, these investigations show an efficiency of >99.9% for flocculation combined with sand filtration for elimination of cyanobacterial cells. Nevertheless, highest cell counts during the investigated weeks were 3,056,000 cells/mL in raw water. Even a reduction by 99.9% means > 3000 cells/mL in final water. In fact, cell densities of > 3000 cells/mL were found seven times in final water during the 46 week period presented above.

…….TO REMOVE CYANOBACTERIAL TOXINS

Two spot checks were taken at time points with high cell numbers of *M. aeruginosa* and *A. circinalis* to investigate the toxin concentrations during the treatment train (Table 19).

MICROCYSTINS

As shown in Table 19, the good efficiency for removing cyanobacterial cells from the raw water (>99%) does not automatically lead to an acceptable elimination of the produced toxins. MC elimination varies between the two spot checks. However, these differences are minor, if the standard deviations are taken into account. The standard deviations are the result of a triple determination of the MC concentrations by HPLC-DAD, ELISA and PPA. But, except for the MC detection after sand filtration (ASF) on 01/08/2001, the results of these three detection methods were in the same range. Nevertheless, these two spot checks show on average elimination rates of below 40% for flocculation and below 60% for combined flocculation and filtration. The proportion of extra cellular (free) toxin increases from 17.5 % in raw to 97.9 % after flocculation and filtration. The high concentration of free toxin in raw water from

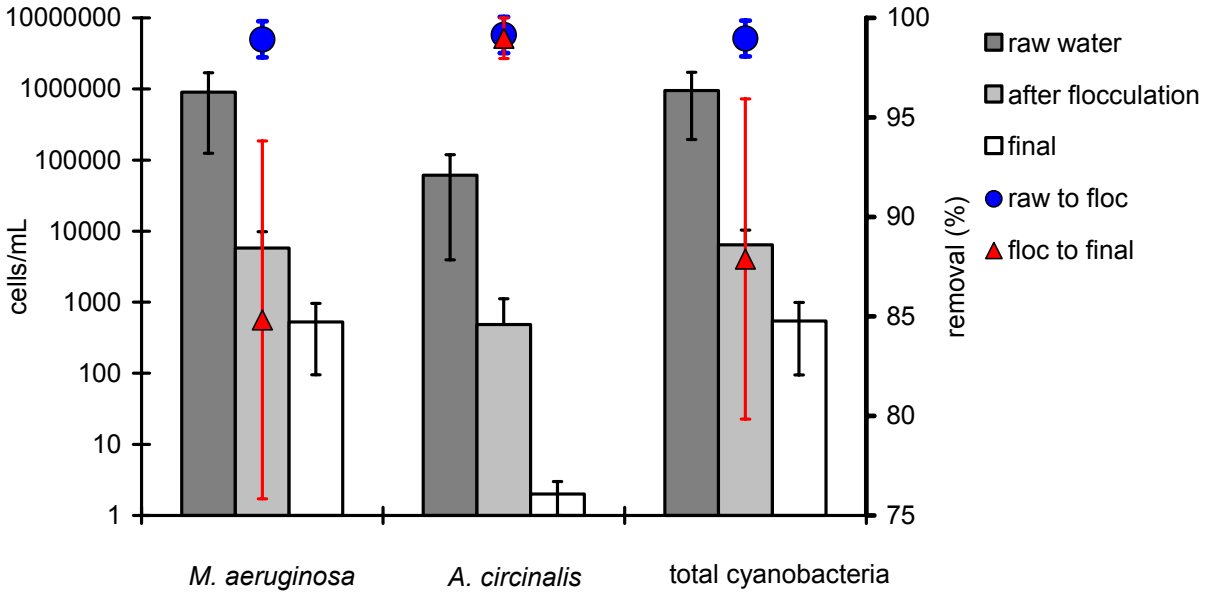


Figure 28. Efficiency of water treatment train at Gordonbrook Dam to remove cells between 05/06/2001 and 01/08/2001 (n=15 for raw water to flocculation, n=5 for raw water to final water and flocculation to final water).

22/08/2001 can be explained by a collapsing *M. aeruginosa* bloom at this time point and therefore an increasing lysis of cyanobacterial cells. The lower elimination of MCs by flocculation may be caused by this fact. Flocculation is effective in removing cells, but not in eliminating free microcystins (*372, 373*).

In addition, freeze-dried material of the surface bloom from 01/08/2001 was analysed and found to contain 67 µg MC/g DW, whereas in sludge after flocculation only 5 µg MC/g DW were detectable, obviously a result of cell lysis during and after flocculation. More than 93% of the toxin shows the same retention time as MC-LR. Another molecule (less than 7%) with the typical UV-spectrum of MCs was not further identified. Based on the estimate that only approximately 1/3 of the biomass of the material was *M. aeruginosa* (see Table 19) and the fact that *A. circinalis* has not been shown to produce MCs, *M. aeruginosa* contains about 200 µg MC/g DW in this case.

		01/08/2001			22/08/2001		
		RW	**AF**	**ASF**	**RW**	**AF**	**ASF**
cells/mL	***M. aeruginosa***	54,000	570	165	25,500	NI	220
	A. circinalis	151,000	2,300	2	55,900	NI	80
% removal	***M. aeruginosa***	-	98.9	99.7	-	-	99.1
	A. circinalis	-	98.5	99.9	-	-	99.9
toxin (ng/L)	**MCs**	820 ± 160	470 ± 150	310 ± 350	740 ± 170	640 ± 230	570 ± 70
	PSPs	68	40	33	79	45	30
% removal	**MCs**	-	42.5	61.9	-	13.1	22.6
	PSPs	-	41.0	51.0	-	42.9	62
% free toxin	**MCs**	17.5	97.9	97.9	43.5	99.5	100

Table 19. Removal of cyanobacterial cells and reduction of toxin concentration during the drinking water treatment at Gordonbrook Dam (RW: raw water, AF: after flocculation, ASF: after sand filtration (and flocculation), NI: not investigated).

PARALYTIC SHELLFISH POISONS

The detected concentrations of PSPs are below 100 ng/L in the investigated samples. The extraction procedure of the samples was optimised for MCs and not for PSPs, so

only a small part of the PSPs could be detected. Parallel investigations of Queensland Health Scientific Services (QHSS) with optimised sample preparation for PSPs show concentrations of 15 and 17 µg/L in raw water at 01/08 and 22/08/01, respectively (personal communication with QHSS). Nevertheless the results point to insufficient elimination of PSPs of ~40% after flocculation and ~60% after flocculation and filtration.

Toxin concentrations and composition at North Pine Dam

In North Pine Dam, *Cylindrospermopsis raciborskii* is the predominating species (Table 17).

EFFICIENCY OF WATER TREATMENT TRAIN IN WATER WORKS AT NORTH PINE DAM

The efficiency to eliminate cylindrospermopsin from raw water is shown in Figure 29. The concentration of cylindrospermopsin in raw water of 1.17 µg/L was reduced by flocculation to 0.63 µg/L (46%), and by filtration and chlorination to below the detection limit of 0.2 µg/L (100%). In raw water 20.5% of cylindrospermopsin is dissolved, whereas 38.1% of the toxins are released from cells after flocculation. No microcystin was detectable in these samples.
Additionally, a small pilot water treatment system was investigated for the capacity to remove cylindrospermopsin. The system employing different filtration materials is able to remove cylindrospermopsin to below detection level of 0.2 µg/L. In raw water, a concentration of 1.09 µg CYL/L was detected, with a free toxin quota of 22%.

DISCUSSION

Huge cyanobacterial densities make things difficult for the local authority at Kingaroy/Gordonbrook Dam

COMPETITION BETWEEN *MICROCYSTIS* AND *ANABAENA*

Both predominant species occurring in the raw water of this water works are known toxin-producers showing extremely high cell numbers perennially. While *M. aeruginosa* reached extremely high cell densities, toxin concentrations never exceeded 8.0 µg/L. Interestingly, the highest toxin levels were not found to coincide with the highest cell counts. In contrast, toxin levels increased at the beginning of the

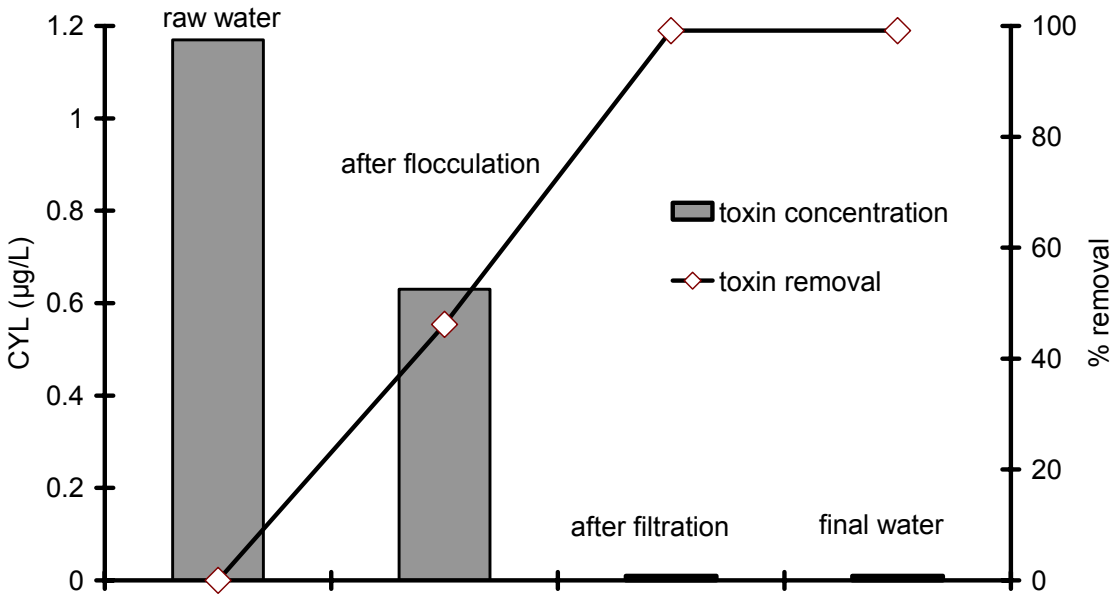


Figure 29. Efficiency of water treatment to remove cylindrospermopsin from raw water at North Pine Dam near Brisbane; expressed as µg CYL/L (left axis) and % removal (right axis).

exponential growth phase, when *M. aeruginosa* had to compete with *A. circinalis* for the dominant position in the lake. After suppression of the competitor, MCs concentrations decreased to a non-detectable level (Figure 22-24). This is in contrast to the situation with *A. circinalis* and the levels of PSPs. There is a strong correlation between PSP concentrations and cell densities (Figures 21-24 and calculated in Figure 25). The competition between these two cyanobacterial species becomes obvious in the Figures 22-24. In 10/98 *A. circinalis* starts growing until *M. aeruginosa* begins to grow in 11/98. In this phase *Microcystis* produces high toxin concentrations relative to their cell densities. This results in a breakdown of the existing *A. circinalis* bloom with up to 50,000 cell/mL. After this collapse, *Microcystis* reduces its toxin production to amounts under the detection limit, while cell densities reach 200,000 cells/mL. It seems that *A. circinalis* tries to compete again and starts producing PSPs (to make it better than 11/98), but *M. aeruginosa* is powerful enough and restarts microcystin production for a short period to make the proportions clear. In 10/99 *A. circinalis* starts PSP production simultaneously with the exponential growth phase (in contrast to 10/98, see above) and is successful in suppressing the growth of *M. aeruginosa*. Even a short attack of *M. aeruginosa* can be parried by *Anabaena* with high toxin production over three months. Different the

situation in February to April 2000: both species are growing, both are producing their toxin. However, *Microcystis* is successful and reaches more than 400,000 cells/mL. But again (compare to 12/98) the PSP concentration increases successfully followed by a short collapse of the *Microcystis* bloom in March 2000. But, the battle is lost for *Anabaena*, *Microcystis* dominated the reservoir for the next 10 months. Over this period, no microcystin was detectable. From March 2001 to September 2001 the situation was similar and the competition continues. This discussion assumes, that *A. circinalis* does not produce any microcystin. Although 2 out of 24 French strains were able to synthesise MCs (*557*), no MCs production can be found in Australian strains (*548*), but the possibility cannot be excluded.

In the case of *Microcystis aeruginosa* there are two possible explanations for this phenomenon of varying MC-concentrations :

- this species can switch toxin production on and off, depending on the situation given in the environment
- there are two or more strains of *M. aeruginosa*, "toxin producers" and "non producers". The "non producer" grows slowly as long as the competition between the "toxin producer" and *Anabaena* exists. After the environment has been more or less cleaned from interspecies competitors, the "non producer" has the advantage, in that it need not waste energy and nitrogen for toxin production. Thus, genetic heterogeneity is possible in cases of monospecific blooms as several studies have described toxic and non-toxic laboratory cultures of the same species, even when isolated from the same population (*49, 558, 559*). In another study the correlation factor between MC-LR and densities of *M. aeruginosa* only explained 38-48% of the variation in concentrations of MC-LR, whereas 70% of the samples that contained no detectable MC-LR contained *M. aeruginosa* (>1000 cells/mL) (*54*).

Such a competitive mechanism is probable because possible ABC transport genes have been shown to collocate to genes coding for toxin synthesis enzymes (*42*). Investigations concerning allelopathic effects of cyanobacterial secondary metabolites (*560*) to other cyanobacteria (*561*), eukaryotic algae (*60*), diatoms (*562*) and higher plants (*563*) have shown allelopathic effects. However, while such transporters and/or receptors for these secondary metabolites are unknown, the question concerning this phenomenon can not be answered satisfactorily.

Park (*564*) reported high concentrations of microcystin during the exponential growth phase of the bloom and release of microcystin from cells during senescence and decomposition. In contrast, *Anabaena circinalis* produces toxin in correlation to cell density. The PSP concentrations are higher than the levels for microcystins synthesized by *Microcystis*. Oh *et al.* (*509*) found correlations of MC concentrations versus chlorophyll-a and cell density, respectively, in a Korean lake with predominant *Microcystis* and *Anabaena*. In contrast, but also in agreement with our data, no correlation was observed to *Microcystis* or *Anabaena* densities. Cronberg *et al.* (*216*) even reported the highest toxin production at very low cyanobacterial biomass.

The facts, that cyanobacteria produce different amounts of toxins under different conditions and in different seasons are mentioned in the literature. Henriksen (*535*) found a 9-fold increase of MCs/g DW of *M. aeruginosa* during two months and a remarkable doubling of the microcystin concentration of *M. viridis* collected within a two-day interval. Other studies have explained the variations of toxin contents with solar radiation, water temperature, pH, primary production and oxygen saturation in the water (*57*) or with differences in the toxin production in the different growth phases and with seasonal changes in the predominant toxic or non-toxic species of *Microcystis* (*564*). Toxic and non-toxic strains within one field sample are well documented. Twelve different strains of *Microcystis* were isolated from one *Microcystis* bloom by Henning *et al.* (*534*), eight of which were toxic, 4 of them not. Briand *et al.* (*533*) suppose in a case of a perennial population of *P. agardhii* in France, that non–toxic strains grow in winter, whereas toxic ones grow in spring and autumn.

OTHER SOURCES FOR MCS IN RAW WATER

As another source for MCs, picocyanobacteria with a typical diameter of 0.2-2µm appear in all environments including lakes, dams and drinking water reservoirs (*565, 566*) and exist in mixed communities with other planktic scum-forming cyanobacteria (*567, 568*). These small cyanobacteria are difficult to see and to analyse but are also able to produce microcystin or microcystin-like compounds (*569*) and may influence the concentration of MCs in water samples.

DELAYED NATURAL DEGRADATION OF MCS AND PSPS

Another reason for the poor correlation between cell densities and measured toxins could be the kinetics of natural degradation of these two toxin groups. The pattern of PSP concentration shown in Figure 23 and 24 could be interpreted by slow degradation. Degradation of PSPs seems to be a more chemically-mediated process, and these toxins are able to persist in the water body for more than 3 months (*570*). In contrast, the most important degradation pathways for microcystins are enzymatic mineralisation by bacterial degradation and degradation photosensitised by humic substances (*196*). Because of the initial lag-phase in microbial activity and only low rates of photochemical degradation resulting from low radiation energy reaching and penetrating the surface, MCs can persist for days or even weeks. However, the presented data do not give any evidence, that free MCs persist for longer periods in the investigated reservoirs.

CYANOBACTERIA DURING TREATMENT TRAIN AND HUMAN HEALTH

Apart from reflections about the deeper sense of toxin production by cyanobacteria it is unlikely, that the toxins are synthesized to harm human population by drinking water. However, the water suppliers must eliminate potential harmful substances from the raw water in order to provide healthy drinking water for the population. MCs and PSPs elimination of 20-60% and 10-60%, respectively, after flocculation/filtration as observed in this study is comparable to the literature (*571*). Cell-bound MCs decrease while extracellular toxin concentrations remain constant after flocculation and filtration. These findings are in accordance with results presented elsewhere: in these investigations the concentration of extra-cellular secondary metabolites also remained constant after flocculation with aluminium sulphate (*572, 573*) or ferric chloride (*574*). However, the concentrations of flocculants used during these experiments were clearly lower (up to 4.8 mg/L) compared to the situation in the water works investigated in this study (120 mg/L). But the stability of cells during flocculation is discussed controversially. No additional release could be observed in several studies (*572, 573*). However, Drikas *et al.* (*575*) tested the efficiency of a treatment train with flocculation (65 mg/L) and sand filtration to remove *M. aeruginosa* cells from raw water and found that these processes cause little damage to cells and release a minimal amount of toxin. In

another study, flocculation and filtration also resulted in an increase of extracellular toxin after experiments with *M. aeruginosa* and *P. rubescens* (*450*). The researchers suggest turbulences in pipes and pressure gradients in the filter as reasons for their results. Experiments with cyanobacterial extracts resulted in toxin reduction by 11-32% after Al-flocculation (36-79 mg/L), sand filtration and chlorination (*378*). Additional PAC (5 mg/L) improved the removal of MCs only to 13-34%.

One possible explanation for the increase in extracellular toxin could be the degradation of the sunken sludge. This post-flocculation sludge and the cells remaining in the sand filter could explain the higher extracellular toxin concentrations after treatment in the daily routine of a water works. Thus, thorough removal of the sludge and timely backwashing of the filters are essential. In addition, the DOC content of the raw water is crucial for the success (*576*). Thus, for the removal of cyanobacterial cells the percentage of removal can not be estimated by laboratory investigations. The raw water conditions in the individual water works are different.

For elimination of PSPs during the treatment process, activated carbon in granular or powdered form was shown to be very effective (*577*). Thus, in this water works if *A. circinalis* reaches or has reached high densities the treatment with PAC is recommended, in order to adsorb the dissolved PSPs.

Although toxin could not be detected in tap water during a one-year investigation, the high cell numbers of *M. aeruginosa* in tap water (up to 10^4 cells/mL) and detection of PSPs in final water give enough reason to improve the efficacy of the water treatment train. Because of the detection limits of 0.5 and 0.2 µg/L for PSPs and MCs, respectively, a contamination with lower concentrations can not be excluded. The spot check investigations of final water with ELISA and PPA and HPLC result in concentrations near the detection limit. The efficacy of chlorine (0.5 mg/L) to destroy MCs and PSPs is doubtful (*417*). Furthermore, intact cells could be destroyed by chlorine during storage and the toxin set free into tap water. Mechanical pressure due to pumping of the water is also a potential causes for lysis of cyanobacterial cells during the passage from water works to households. The results have also shown that even if cell density is low in raw water, high cell numbers can still occur in final water tanks. This phenomenon could be explained by persisting high cell numbers in the final water reservoirs due to preceding periods with high cell densities in raw water

(also reported in (*380*)) or by high cell numbers in the sand filter which may be washed out with the next water charge.

OTHER CRITERIA FOR TASTY AND HEALTHY DRINKING WATER

The water works at Gordonbrook Dam has to remove not only cyanobacterial cells and toxins such as microcystins and PSPs, unpleasant smelling compounds including geosmin (produced by *A. circinalis* (*578*)) and *β*–cyclocistral (produced by *M. aeruginosa* (*579*)) must also be eliminated (*479, 580*), as the odour threshold level for these compounds is ~10 ng/L for geosmin and 19.3 µg/L for *β*–cyclocistral (*581*). The need to remove such compounds from drinking water is well known in Australia (*582, 583*). Flocculation with up to 4.8 $Al_2(SO_4)_3$ mg/L was shown to cause no additional lysis of *A. circinalis* cells and thus no increase in geosmin concentration after treatment (*572*). Drinking water in the supplied regions of water works 2 smelled strongly on days of spot-check investigations. This is evidence for the insufficient elimination of geosmin produced probably by *A. circinalis* in this case.

Combination of lower cyanobacterial cell densities and better treatment facilities guarantee safe drinking water at North Pine Dam/Brisbane

As expected, MCs could not be detected in raw water from North Pine Dam. Although only <1.0 µg CYL/L was found, a weekly monitoring of the raw water source is recommended, as cell densities at the time point of the spot-check were low compared to reported bloom events of *C. raciborskii* in this reservoir. *C. raciborskii* is, similar to *P. rubescens* in another climatic region, difficult to recognize from the surface because the population is stratified in deeper layers of the dam. To avoid other phytoplankton intake, many water works have set their water intake at a deeper layer. The well-equipped water treatment system at this dam eliminates more than 99% of the toxin during flocculation, sand filtration and chlorination. PAC was not added on the sample day. However, the treatment efficiency could be completely different with a higher cell number over a longer period**.**

CONCLUSIONS

Because of the low toxicity of the *M. aeruginosa* strains during the investigation, toxin concentrations of more than the guideline value of 1.3 µg/L (*458*) in tap water

were not detected. The missing correlation of cell densities and toxin concentration is an important fact for the water works. Relatively low densities of highly toxic strains must not be underestimated. One third of the raw water samples were contaminated with partly high concentrations of cyanobacterial toxins, although the predominating *Microcystis* strain seemed to produce no or low amounts of toxin during the investigated period. The water works has to deal with high cyanobacterial densities and a variety of toxins. The risk for a break-through of toxin concentrations beyond the Australian guidelines is possible. However, variations in toxin production during the year show, that monitoring of phytoplankton and measuring of toxin concentrations during the treatment steps is highly necessary.

CHEMICAL AND TOXICOLOGICAL CHARACTERISATION OF MICROCYSTIN BY-PRODUCTS AFTER OZONATION

Cyanobacterial toxins and especially microcystin-LR are in the focus of research since the late eighties of the last century and many studies were carried out with respect to the toxicity of microcystins (MCs), one type of cyanobacterial toxins. The toxic effects after ingestion of microcystins are of concern to drinking water suppliers who gather their raw water from cyanobacterial contaminated surface waters. Besides the removal or elimination of such toxins, suppliers must also consider products resulting from natural or oxidative degradation of microcystins and the possible toxic effects of these by-products. In the study presented here, MC-LR was oxidised with ozone in molar ratios ranging between 0.38 and 3.05 (MC-LR/ozone). Ozone (O_3) was shown to react within seconds with MC-LR to yield characteristic products, while the further changes in the following minutes were negligible. These by-products were further characterised by LC-MS. MC-LR decreases indirectly proportional to the ratio MC-LR/O_3. Because the MC-LR concentrations detected by HPLC-DAD and indirect ELISA were in the same range, it was supposed that no by-products with other retention times than the native MC-LR are responsible for the binding to the Adda-antigen used for the ELISA. The PPA shows even lower toxin concentrations than HPLC and ELISA for the samples after ozonation. Thus by–products after ozonation of MC-LR do not result in additional protein-phosphatase toxicity or binding to Adda-antigen.

INTRODUCTION

The presence of cyanobacterial toxins (e.g. microcystins) in drinking water supplies poses a serious health risk to humans due to their potential liver tumour promoting capacity (*366*). Toxic cyanobacteria occur almost ubiquitously, only the dominant species vary due to the trophic status of the surface water and the season. It is thus important to monitor cyanobacterial densities and toxin levels in water reservoirs and, in the case of a bloom, to remove these toxins by adequate water treatment procedures. Conventional water treatment e.g. flocculation and sand filtration is ineffective in reducing cyanobacterial toxin levels to below toxic concentrations (*417*). Ozonation has however been shown to be a very effective method to eliminate microcystins from water (*445*).

Ozone can react with compounds or organisms in the water in two different ways (*452, 584*):

- Ozone itself reacts mainly with microbial constituents and has thus a disinfection function, but ozone is relatively unreactive towards many inorganic and organic compounds.
- A hydroxyl radical is formed by decomposition of ozone. As hydroxyl radicals are highly reactive and non specific, this radical is the main oxidant for ozone-resistant organic or inorganic molecules. Thus, microcystins may possibly also be decomposed by this radical.

Shawwa *et al.* (*419*) found that eight moles of ozone are necessary for a complete conversion of 1 mole MC-LR. This reaction of ozone with MC-LR has been shown to process very rapidly. On the basis of the stoichiometeric requirements, the overall rate constant was calculated and is shown to differ depending on pH and temperature (from 2.32 x 10^4 $M^{-1}s^{-1}$ at pH 7 and 10°C to 1.61 x 10^5 $M^{-1}s^{-1}$ at pH 2 and 30°C). It has been mentioned that the reason for this difference could be the decomposition of ozone to unreactive hydroxyl ions under more alkaline conditions, which is also obvious from the lower oxidation potential of ozone under alkaline conditions (1.24V) compared with that under acidic conditions (2.07V) (*419*). The very fast degradation of MC-LR has also been described by Rositano *et al.* (*401*). MC-LR at a concentration of 166 µg/L was completely oxidised with less than 0.2 mg ozone/L. In the case of low concentrations of MC-LR versus high ozone concentrations, the kinetics of the reaction can be modelled as pseudo first order with respect to ozone (*383, 419, 585*). At high MC-LR concentrations the result of reaction kinetics between MC-LR and ozone can be modelled as second order with respect to both reactants (*419, 585*).

However, putative by-products after incomplete oxidation have neither been identified nor characterised adequately with respect to their toxicity. The aim of this study was to investigate if by-products caused by oxidation of microcystin-LR still show properties similar to those of the parent molecule, when tested with phosphatase inhibition assay, Adda-ELISA and HPLC-DAD.

MATERIAL AND METHODS

OZONATION

O_3 was produced in an ozone generator (type LN 103 AT, kindly provided by Ozonia, Duebendorf, Switzerland) by regulation of voltage (25–50 mA) and gas flow (166–208 cm^3/min) with oxygen as substrate. The concentration of microcystin-LR (Alexis, Switzerland) was checked photometrically using the absorption coefficient of 39,500 L mol^{-1} cm^{-1} (*335*). After determination of the MC-LR concentration (in 100% methanol) the samples were dried under nitrogen and redissolved in MilliQ (MQ)-H_2O. MQ-H_2O was ozonated and the concentration measured by the indigo method (*423*). After reaching the desired ozone concentration, the MC-LR (also dissolved in MQ-H_2O) was added to the sample. At different time points the reaction was stopped by adding 10µl 0.1M sodium-thiosulfate (Riedel-de Haën, Seelze, Germany) or by bubbling the sample with nitrogen for at least 20 seconds. The MC-LR was calculated to reach a concentration of 2.5 or 10 µg/mL in the reaction vessel. The amount of ozone was checked in a control experiment with ozonated water to achieve ozone concentrations between 0.05 and 1.2 mg/L. The experiments were carried out at 15°C +/- 2°C and pH 7. The experimental design is shown in Figure 30. After ozonation the samples were divided in two fractions and dried by speed-vac (ALPHA RVC, Christ, Osterode, Germany). One part of the sample was dissolved in MQ-H_2O for toxicity tests, the other was dissolved in 50% MeOH for HPLC and LC-MS.

ADDA-ELISA

The ELISA method was performed as described in chapter IV. Each sample was analysed three times in duplicate using a different 96 well plate for each assay. The mean values of each plate yielded the three values for calculation of the standard deviation (n=3).

RADIOACTIVE PROTEIN PHOSPHATASE ASSAY (rPPA)

PPA was performed as described in chapter IV with ^{32}P-ATP as substrate for phosphorylase (Amersham, Freiburg, Germany) and with the catalytical subunit of protein phosphatase 1 as inhibited enzyme (Calbiochem, Darmstadt, Germany). yielded the three values for calculation of the standard deviation (n=3).

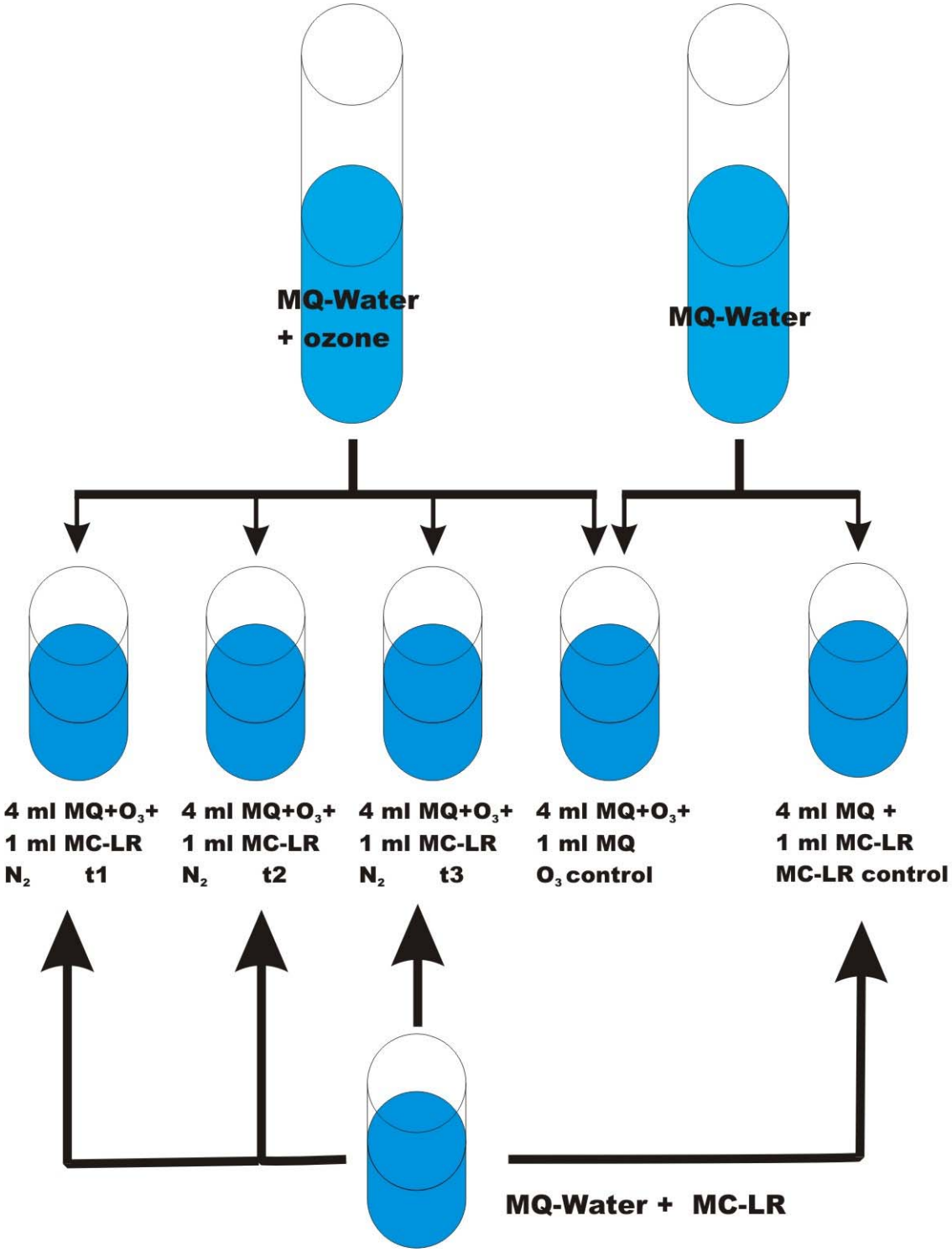


Figure 30. Experimental design for ozonation experiments. t1, t2 and t3 represent the times of reaction-termination which varied between the individual experiments.

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

Samples (in 50% methanol) before and after ozonation were analysed according to the method described by Lawton *et al.* (*526*) by injection into the following HPLC-system: Diode Array Detector SPD-M10A, Auto Injector SIL-10AD, Column Oven CTO-10AC, System Controller SCL-10A, Liquid Chromatograph LC-10AT, Degasser DGU-14 A, Shimadzu; Column: Grom-Sil ODS-HE, 250 × 4,6 mm, 5 µm.

MASS SPECTRA ANALYSIS

Mass spectra were obtained on a LCQ Duo (Finnigan, USA) equipped with an IonSpray (pneumatically-assisted electrospray) source.

SPECTROPHOTOMETRY

For measurement of the UV-spectra the samples (in MQ-H_2O) were scanned from 200 to 300 nm with a Beckman DU 640 spectrometer before and after ozonation.

RESULTS

Ozonation

Microcystin-LR concentrations ranging between 2.5 and 10 µg/mL (2.51 and 10.04 µM) were oxidised with 0.06 to 1.2 mg O_3/L (1.25 to 25 µM). The decrease of ozone in MQ-water was measured in a control experiment with MQ-H_2O instead of MQ + MC-LR. The decrease of ozone was found to be a typical first order reaction as shown in Figures 31 and 32. The slight differences in ozone decrease between the individual experiments were a consequence of performing the experiment in open vessels: for every ozone measurement 0.9 ml of sample were necessary. Thus after each sampling the ratio of volume to water-surface area decreased consequently followed by an increased loss of ozone into the surrounding gas-phase. The experiments were carried out with volumes from 5 to 20 ml ozonated MQ-H_2O. Thus, the changes of volume to water-surface ratio during the experiment were different between the individual experiments. However, the outcome of the experiments is not influenced by the ozone degradation kinetic, because the reaction of the ozone with microcystin is very fast. If MC-LR is the only substrate, ozone or the hydroxyl radical react completely with the MC-LR until one of the reaction partners is exhausted.

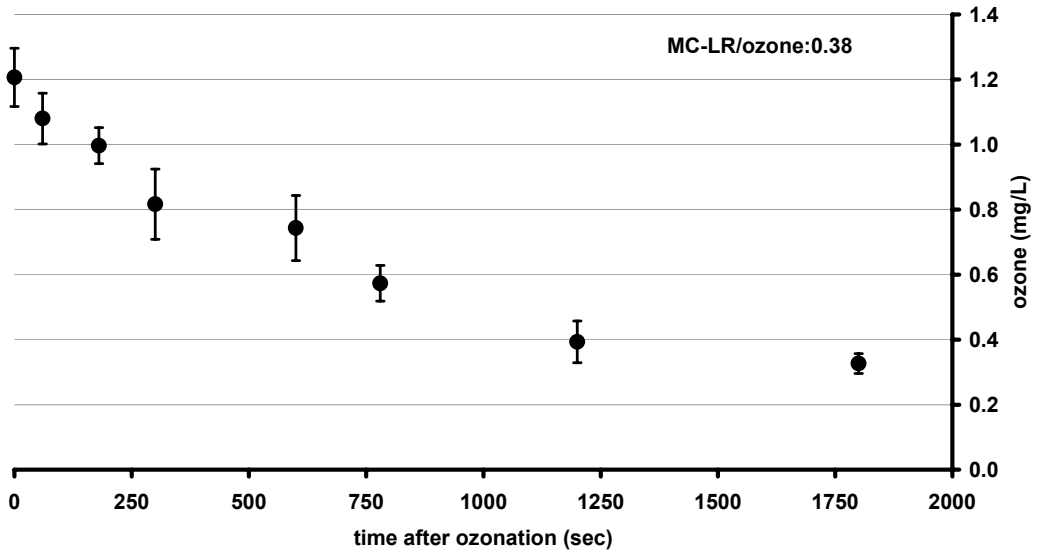


Figure 31. Control ozonation using a MC-LR/ozone ratio of 0.38 with an initial ozone concentration of 1.2 mg/L.

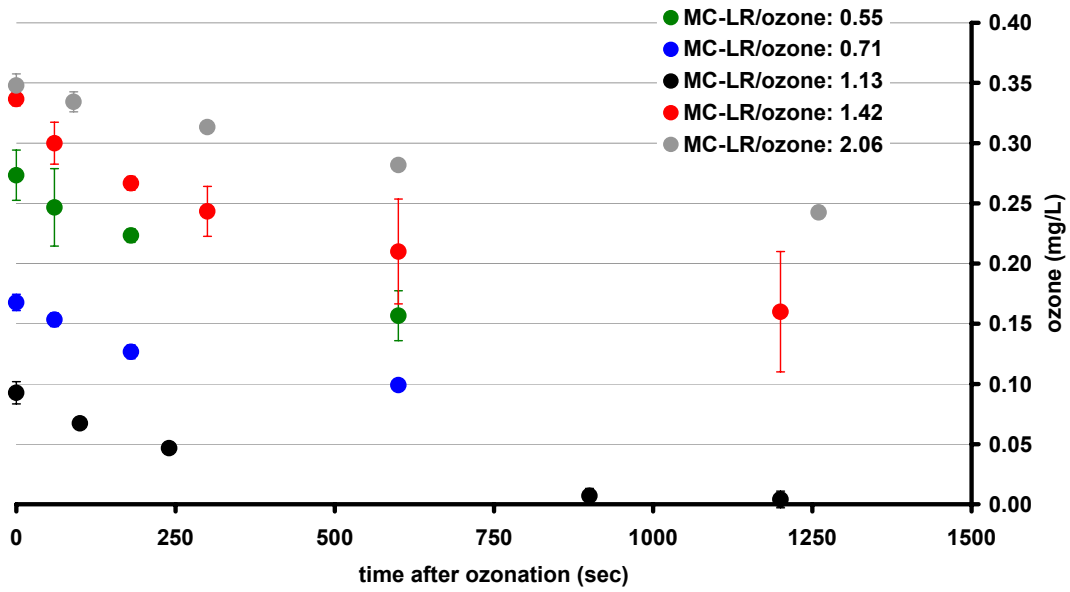


Figure 32. Control ozonation using a MC-LR/ozone ratio of 0.55 to 2.06 with an initial ozone concentration of 0.09, 0.17, 0.27, 0.34 and 0.35 mg/L.

Correlation between MC-LR concentration after ozonation and the MC-LR/ozone ratio.

The ratio of MC-LR concentration to ozone is important for the expected residual concentration in the sample. As presented in Figure 33 the remaining percentage of MC-LR after ozonation increases with the quotient MC-LR/ozone. Experiments with six different MC-LR/ozone ratios were carried out. Toxin concentration ranged from below 1% to more than 70% of the initially added amount. The results of HPLC and Adda-ELISA in the experiments with MC-LR/ozone ratios of 0.71 and 2.06 with 3 and 70% of the initially added amount, respectively, were without interpretable differences. However in the majority of the cases the Adda-ELISA yielded slightly higher toxin concentrations. Different percentages of degradation could be determined in the MC-LR/ozone 1.42-experiments. While the HPLC and Adda-ELISA results are comparable, the rPPA gives three to four fold lower values. The three-fold lower results of HPLC compared to Adda-ELISA could be seen in the experiment with a MC-LR/ozone ratio of 1.13 (Figure 33). However, high standard deviations of the ELISA results lower the significance of this difference. Figure 34 gives a more detailed view of the more strongly ozonated samples with lower residuals of MC-LR. Because the sensitivity of the applied HPLC-method was not sufficient to analyse MC-LR concentrations below 300 nM, no toxin could be detected in experiments with a higher ozone concentration (0.38, 0.55). A ratio of 0.38 for MC-LR/ozone results in a reduction of toxicity by 99.1 and 99.9 % as detected by Adda-ELISA and rPPA, respectively. Because a ratio of 0.38 was sufficient for an almost complete degradation it can be assumed that approximately three moles of ozone are necessary to mineralise one mole MC-LR under the condition applied within the experiments (pH 7, 15°C).

If the ratio MC-LR/ozone is plotted versus the percentage of remaining MC-LR, the correlation between the two parameters can be described either as an exponential (Figure 35) or as a linear function (Table 20). Both result in correlation factors (R^2) of greater than 0.9 (Table 20).

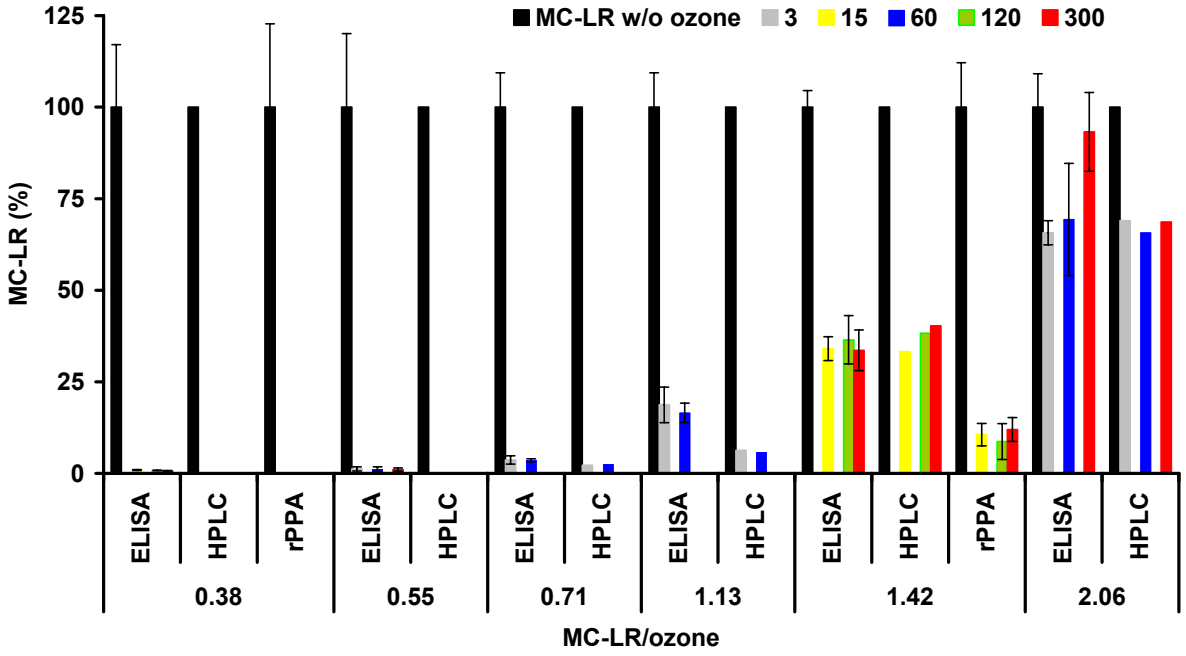
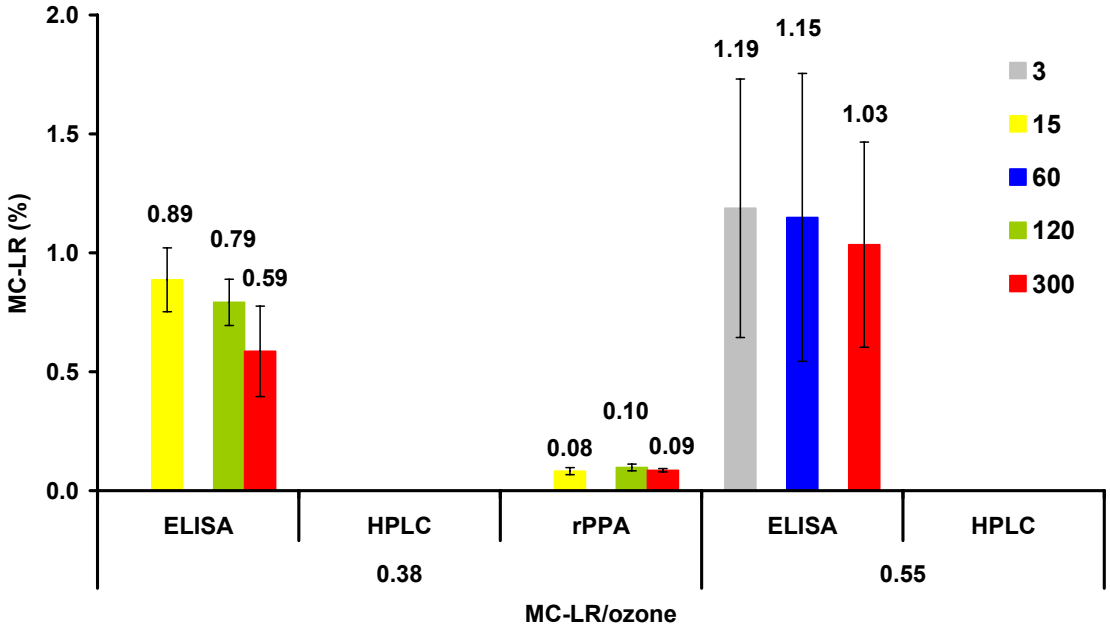


Figure 33 (above) and 34 (below). Remaining MC-LR after ozonation. Shown are the percentages of MC-LR, which were still detectable after increasing duration (3, 15, 60, 120, 300 seconds) of ozonation (100% = MC-LR sample without ozonation but the same sample preparation, see Figure 30 **Figure 34** gives a more detailed view of the more strongly ozonated sample.



Adda-ELISA	HPLC
$y = 42.092 x - 22.712$	$y = 52.148 x - 41.032$
$R^2 = 0.9517$	$R^2 = 0.9293$
$y = 10.328 x^{2.83}$	$y = 6.623 x^{3.911}$
$R^2 = 0.9764$	$R^2 = 0.923$

Table 20. Comparison of exponential and linear regression of the plots in **Figure 35**.

The results of mass spectrometry analysis help in assessing the coherence between the MC-LR/O_3 ratios and the toxicity of samples after ozonation. Although an exact quantification is not possible with the applied MS-method, the tendency is clearly recognisable. As shown in Table 21 the relative absorbance of the MC-LR peak at 995 (m/z) decreases with a decreasing ratio of MC-LR/ozone.

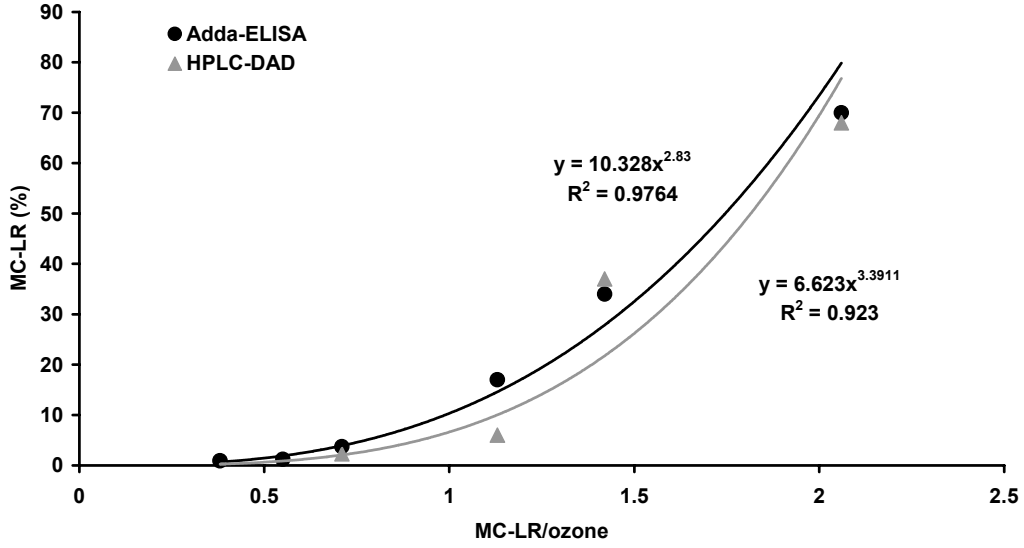


Figure 35. Percentage of remaining MC-LR detected by HPLC and Adda-ELISA plotted versus the applied MC-LR/ozone doses.

By-products after ozonation of MC-LR

In Europe and Northern America ozonation is a widespread drinking water treatment technique (*432, 586-590*). Although many of these water works must eliminate cyanobacteria from drinking water, little is known about the possible by-products after ozonation.

In this study relatively high concentrations of microcystin (2.5 to 10 µg MC-LR/mL) were ozonated to investigate possible pathways of degradation during the ozonation process. Mass spectra analysis yielded mainly masses between 794 and 836 (Table 21), which can be interpreted as shown in the postulated degradation scheme in Figure 36. The Adda side-chain was attacked at different positions, whereby the cleavage directly at the ring structure occurred most frequently (MC1, Figure 36). Microcystin-LR/ozone ratios between 0.55 and 1.42 showed almost the same by-product pattern. At a three-fold higher ozone availability (MC-LR/ozone: 0.38), the most frequently detected mass was 815, which could be a lineralised microcystin as shown in Figure 36 (MC5,).

The absorption maxima of the ozonated samples shift from 238 nm (maximum of MC-LR) to a peak at 215 nm. In addition the range between 200 and 240 shows a conspicuously increased absorption. While no difference existed between 15 min and 300 min reaction time after ozonation, the absorption-slope of the sample with more ozone per MC-LR is slightly lower (MC-LR/ozone: 0.38 with absorption lower than 1.42, Figure 37 a-e).

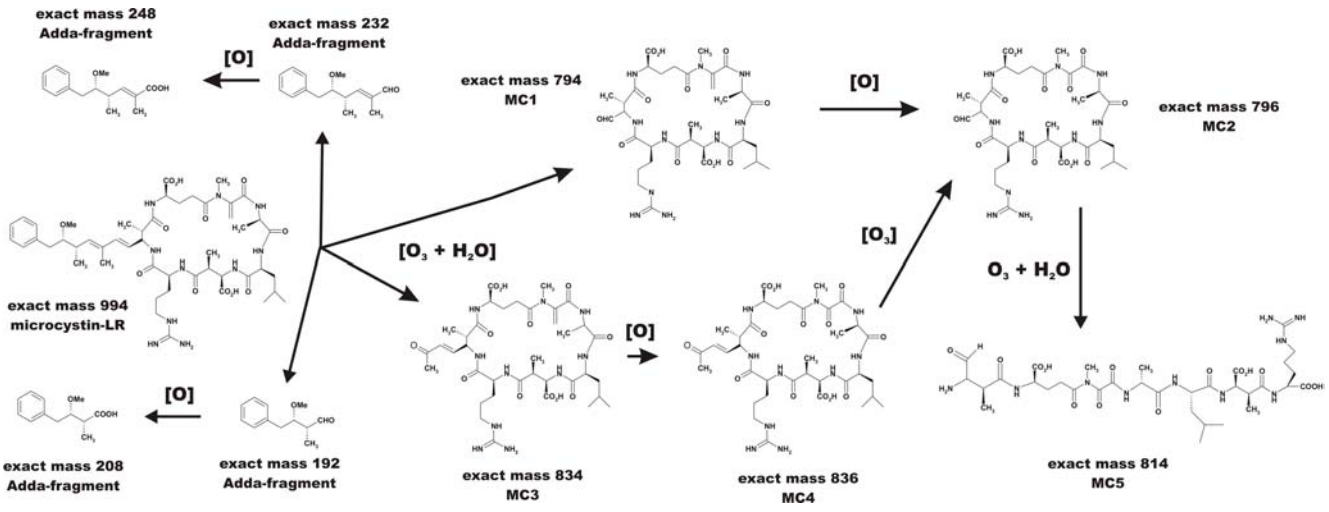


Figure 36. Possible degradation pathways after ozonation of MC-LR, developed according to the detected ions shown in **Table 21**.

m/z	possible molecules	MC-LR/O_3					
		0.38	0.55	0.71	1.13	1.42	2.06
135	$[PhCH_2CH(OCH_3)]^+$	NI	+	+	+	NI	+
213	$[Glu - Mdha + H]^+$	-+	---	---	---	-+	---
375	$[C_{11}H_{14}O$ (Adda-fragment) - Glu - Mdha$]^+$	---	++	---	---	---	+
431	$[M + 2H - 134]^{2+}$	++	+	+	+	+	+
509	$[M + Na + H]^{2+}$	---	---	---	---	---	+
520	$[M + 2Na]^{2+}$	---	---	---	---	---	+
795	$[M1 + H]^+$	+	+++	+++	+++	+++	+
797	$[M2 + H]^+$	+	+	+	+	++	+
815	$[M1 + Na]^+$ or $[M5 + H]^+$	+++	++	---	---	---	---
817	$[M2 + Na]^+$	---	---	++	++	+	+
835	$[M3 + H]^+$	---	++	++	++	---	+
837	$[M4 + H]^+$	+	---	+	+	---	---
861	$[M + 2H - 135]^+$	+	---	+	---	---	++
981	$[M + H - CH_2]^+$	---	---	---	---	---	+
995	$[M + H]^+$	---	---	--	--	--	-+

Table 21. MC-LR $(M+H)^+$ and the potential by-products after oxidation with different ozone concentrations specified as ratios MC-LR/ozone of six individual experiments. The relative abundance of the ions are compared to the control experiment (MQ-H_2O and MC-LR). The results are graded from --- for not detected to +++ for a strong increase in abundance compared to the MC-LR control sample. Adda (2S,3S,8S,9S)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid; Glu glutamic acid; Mdha methyldehydroalanine, NI not investigated.

Toxicity of ozonation by-products

Three test systems were employed for the detection of MC-LR and the toxicity of possible by-products. For the interpretation of the toxicity results it is important to have a closer look at the different methods. The protein phosphatase assay tests the capacity of the microcystins and the potential by-products to inhibit protein phosphatases. Because the by-products have not been separated from the educt, the inhibitory capacity is the sum of the MC-LR residual and the by-products. The ELISA detects the Adda-side chain of the MC/nodularin-family and is therefore not specifically designed to bind only entire MC/nodularins, but is sensitive to every molecule with an intact Adda side-chain (*525*). In HPLC, only molecules with the same chemical properties before and after ozonation can be assumed to be identical

to microcystin-LR. The loss of single amino acids and even the variation or loss of single groups change the chromatographic properties. The UV-spectrum of MC-LR with its maximum at 238 nm depends on the conjugated double bonds in the aromatic portion of the Adda-group and is amplified by double bonds of the methyldehydroalanine. Thus, changes within the Adda side-chain and the methyldehydroalanine result in a loss or decrease in absorbance at 238 nm.

HPLC-DAD analysis of MC-LR-samples after ozonation showed new peaks at a wavelength of 238 nm. The samples treated with three-times more ozone than MC-LR (MC-LR/ozone: 0.38) were separated into two new peaks (P4 and P5) after ozonation (Figure 38). The samples with a ratio of 1.42 MC-LR/ozone were divided into three peaks. One peak showed the same retention time as MC-LR. LC-MS results of this sample confirmed the assumption, that the peak corresponds to MC-LR, because a peak at the mass of M/Z 995 was present. A second peak with the identical retention time as P5 of the 0.38 sample and a third peak P2, which eluted earlier than the native MC-LR, occurred in the chromatograms. No change was observable between the different time points (t3-t300 after ozonation) at which the reaction was stopped by bubbling with nitrogen or by adding sodium-thiosulfate. The samples with reaction times of 3, 15, 60, 180 and 300 seconds differed neither when analysed via HPLC (Figures 33, 34, 38) nor with Adda-ELISA (Figures 33, 34) or rPPA (Figures 33, 34). The concentrations detected by HPLC and by ELISA were in the same range. No toxin was detectable by HPLC in the samples with 0.38 and 0.55 (MC-LR/ozone). As mentioned before, the detection limit for MC-LR using HPLC was around 300 nM (300 µg/L). Consequently, no peak could be expected in these samples, because the ELISA showed clearly lower concentrations. A remarkable difference is however recognisable in the samples with MC-LR/O_3 ratio of 1.13 where 3% and 1% of the MC-LR were detectable using Adda-Elisa and HPLC, respectively. Only two test series were analysed with the radioactive PPA. Although the concentrations for the MC-LR control were in the same ranges as (or even slightly higher than) the results from ELISA and HPLC, the concentrations of the ozonated samples were three (experiment 1.42) and seven times (experiment 0.38) lower than the result with Adda-ELISA (Figure 33).

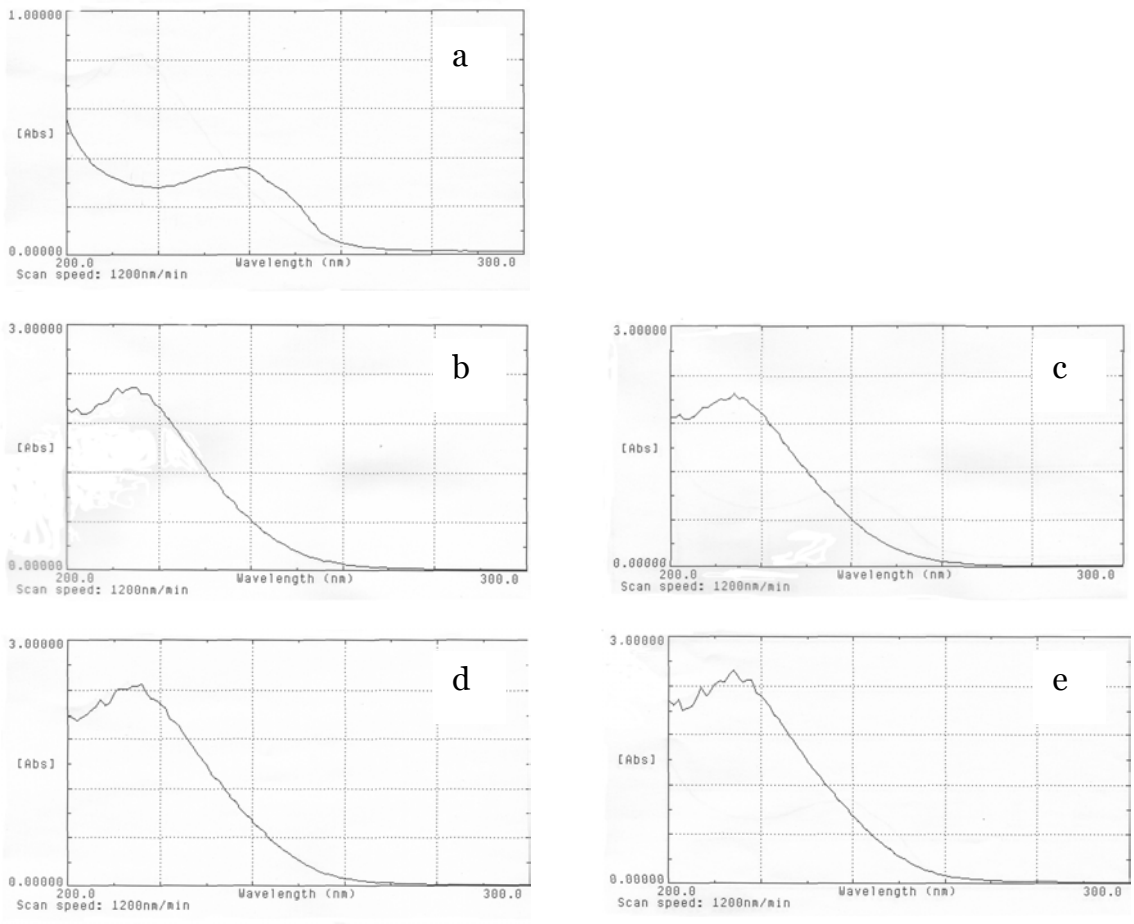


Figure 37. UV-scans of samples from experiments with MC-LR/ozone ratios of 0.38 and 1.42. **a:** MC-LR (10µM) before ozonation; **b:** after ozonation with 26 µM ozone (0.38), t=15; **c:** after ozonation with 26 µM ozone (0.38), t=300; **d:** after ozonation with 7 µM ozone (1.42), t=15; **e:** after ozonation with 7 µM ozone (1.42), t=300.

DISCUSSION

By products after ozonation of MC-LR

The masses 861 (431, m/z) and 135 as well as 213 and 375 are known from LC-MS analysis of MC-LR (*591, 592*). In contrast, the identified MC-LR-by-products in the range between 794 and 836 are not typical for MC-LR by-products during LC-MS measurements. Thus, it is likely that the presented pathway (Figure 36) is typical for the degradation of MC-LR during ozonolysis. In a comparable study, MC-LR ozonolysis resulted in masses of 852, 758, 569, 383 and 309 (m/z) after 2 seconds incubation time (*372*). However, neither the toxin nor the ozone concentration were specified by the researchers, thus no further discussion is warranted.

In the same study, HPLC analysis (238 nm) of an ozonated sample resulted in three new peaks. These new peaks, as well as the pattern of the peaks (one peak before and two after the educts) are similar to the results after HPLC- analysis in this study (Figure 38). However, neither in the presented nor in any other study have these peaks been further characterised.

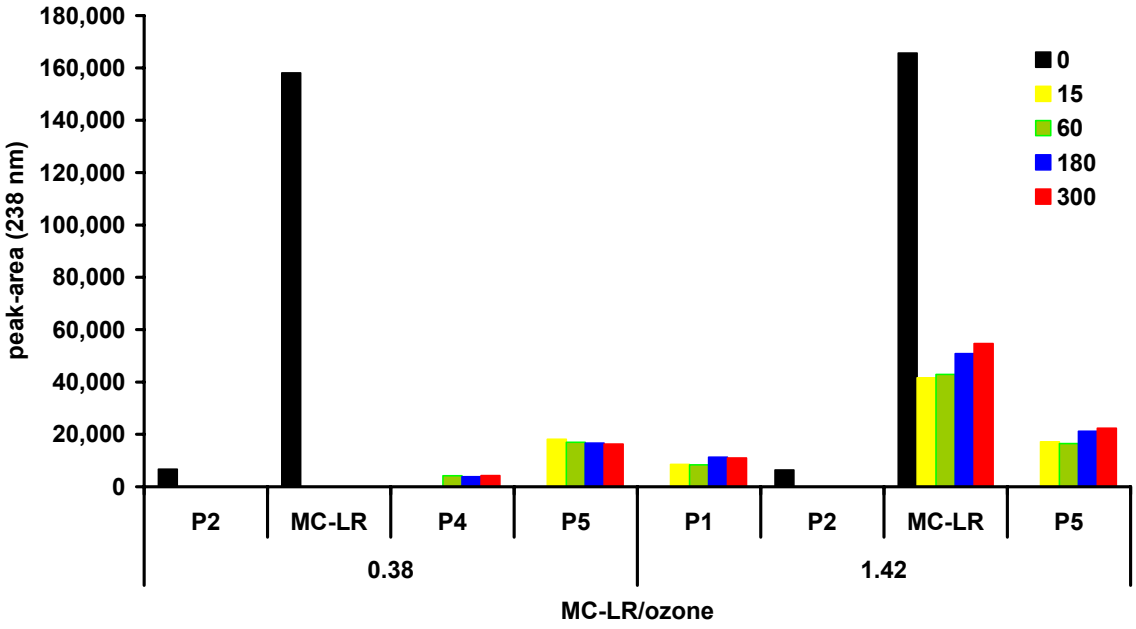


Figure 38. HPLC analysis shows at each case two new peaks (P) for MC-LR oxidised with a ratio of MC-LR/ozone of 1.42 and 0.38 (peak area at 238 nm). No difference exists with respect to the peak area between the reaction times t=15 seconds to t=300 seconds (see also **Figure 33**).

The different MC-LR/ozone ratios resulted in almost the same by-product pattern except for the lowest and highest ozonation. In the latter case more ozone was available per MC-LR molecule, so that the 794 (m/z) by-product could react further to yield the 815 (m/z) by-product by accomplishing a ring opening. Once the ring was cleaved, the molecule was destabilised and further mineralised, which led to many low molecular products. Masses below 400 (m/z) were difficult to identify with the applied MS-method, because the noise in this range was high. Only three masses (375, 213, 135) could be assigned to possible by-products. It is furthermore possible that some of the smaller molecules were lost during the sample preparation procedure, particularly during the sample preparation process. For example, the Adda fragments shown in Figure 36 could not be demonstrated by LC-MS.

In the case of the low ozonation experiment (MC-LR/ozone 2.06) only small amounts of by-products could be identified, the signals between 794 and 836 being very weak. Seventy percent of the toxin were still in the original state at 995 (m/z). The observed ions at 861 (m/z) and 135 (m/z) are typical for LC-MS analyses (*591*) and can not be attributed only to ozonolysis, although the signal in the ozonated samples was slightly higher compared to the control MC-LR.

The maximum of the UV-spectra in samples after ozonation shifted to the lower wavelength of 215 nm. This wavelength is typical for peptide bonds, however, less peptide bonds would be expected to exist in the ozonated samples than in the intact molecule. Thus this possibility seems unlikely. But as well as the wavelengths from 210-220 nm, the absorption between 220 and 240 nm is also increased several fold (Figure 37 b-e). A huge variety of compounds absorb in these wavelengths. The Woodward rules for conjugated carbonyl compounds or the Woodward-Fieser rules for dienes lead to a complex system of very different possible by-products such as aldehydes, carboxylates and hydroxylamines. All of them are possible by-products and could be the reason for the observed increase of absorption between 200 and 240 nm. Thus, to identify this variety of compounds detailed chemical investigations are necessary, which were beyond the scope of this study.

The UV-spectra after 15 and 300 seconds reaction time are without apparent differences. Together with the analytical data from HPLC, Adda-ELISA and rPPA this represents strong evidence for a very fast reaction of the ozone molecule with the MC-LR. Slight differences are recognisable with respect to the absorption intensity of the peak at 215 nm between the experiments with the ratios 0.38 and 1.42 (MC-LR/ozone). The decrease could be a result of a further degradation or mineralisation of MC-LR to compounds which show lower absorption.

Possible toxicity of by-products after ozonation of MC-LR

More than 99% of the MC-LR have been destroyed after oxidation with three-times as much ozone as toxin. Thus, in the presented study, approximately three moles of ozone were necessary to destroy one mole of toxins. In contrast, eight moles of ozone were required for complete oxidation of each mole of MC-LR in experiments carried out by Shawwa *et al.* (*419*). Data presented by Rositano *et al.* (*401*) tend to the same range for approximately 6-8 moles ozone per mole MC-LR. Given that the conditions of ozonation were different in each of these three experiments and that it is known

that the reaction of ozone is very temperature-sensitive (*593*), the congruence of the data is satisfactory. In addition, the number of experiments in the critical range was low in each of the three studies, thus the stoichiometric requirements could only be estimated. In the study carried out by Rositano *et al.* (*401*) and in the study presented here (Figure 34) residuals of MC-LR could be detected in the experiments used to calculate the amounts of ozone necessary to degrade one mole MC-LR. The potential toxicity of the by-products could not be measured by Rositano *et al.* (*401*) and Shawwa *et al.* (*419*), because only HPLC was used for the detection of the residuals of the educt (MC-LR). In the study presented here, the by-products detected by LC-MS lost part of their Adda-side chain, which is responsible for binding to the antibody in the Adda-ELISA used. In the more strongly ozonated samples the concentrations detected by ELISA were generally higher than the values detected by HPLC. Although high concentrations of Adda-fragments could not be detected by LC-MS, it is possible that even relatively low concentrations of these fragments were detected by Adda-ELISA. The noise in the applied LC-MS method and the great number of possible MC-LR by-products have made a detailed analysis very difficult. Because the Adda-side chain is very important for the inactivation of the protein phosphatases 1 and 2a (*110, 113*), by-products without this side chain are less potent in the inhibition of enzymes. The Adda-fragment lacks the correct three-dimensional structure of an MC-LR molecule for an optimal binding to the active centre of the catalytic subunit of the protein phosphatases. This could be one explanation for the three- to seven-times lower toxicity in the rPPA. However, also compared to HPLC analysis, MC-LR concentrations were three- to four-times lower (experiment with MC-LR/ ozone 1.42). Thus, although uncertainties within the applied rPPA method cannot be excluded, no additional protein phosphatase inhibiting capacity was recognisable.

The cleavage of the Adda-side chain and the further degradation of the MC-LR by ozonation is an appropriate tool for the detoxification of microcystins during drinking water treatment. However, to ensure the safety of the drinking water with respect to by-products after ozonation of MC-LR additional toxicity tests with purified and chemically characterised by-products should be carried out. In addition, in the daily routine of drinking water treatment plants the raw water includes a mixture of natural organic matter, which influences the efficacy of the microcystin detoxification (*419*). Thus, the purified and toxicologically characterised by-products can be compared to potential products, which occur during the drinking water treatment

process. This knowledge may be very helpful in optimising the treatment methods for cyanobacterial loaded raw water in order to avoid toxic substances in drinking water consumed by humans .

GENERAL DICUSSION

RISK ASSESSMENT FOR POSSIBLE NEGATIVE HEALTH EFFECTS CAUSED BY INGESTION OF CYANOBACTERIA BY HUMANS

Cyanobacteria are responsible for oxygenic life on earth through their photosynthetic activity and have had more than 3.5 billion years to develop the production of a broad variety of molecules. Polypeptides such as anabaenopeptins, aeruginosins, microcystins, nodularins, microginins or microviridins and alkaloids such as anatoxin-a, saxitoxins and cylindrospermopsins are not necessary for the primary metabolism. The function of these molecules either within or outside the cell is presently unknown, only some hypotheses exist as explained in detail in chapter I. Overall, hundreds of molecules from these families have been structurally identified and many more new compounds will probably be discovered within the next years. Dozens of cyanobacterial species and families produce these secondary metabolites. Every surface water can act as habitat for cyanobacteria. Parameters such as climate, trophic status and morphology of the water body decide on the composition of the cyanobacterial community in the respective water body. As well as the advantages of these organisms as nitrogen fixers, as succession pioneers, as first link in the food chain or as potential sources for pharmaceutically relevant compounds (*77*), these molecules can also have disadvantages for humans. The scope of the investigations presented here are comprised of the possible occurrence of cyanobacterial toxins in drinking water, the best treatment methods to remove or degrade these toxins and the assessment of possible risks for human health with special focus on the role of microcystins. However, because drinking water is only one of the possible exposure routes for cyanobacterial toxins, the other routes must be considered to develop a reasonable risk assessment. The main routes and possibilities for human contact with cyanobacterial toxins are:

(I) Accumulation of cyanobacterial toxins in the food chain, e.g. contaminated food after irrigation with toxin-rich water, cyanobacterial blooms in rice fields as well as microcystins accumulated in fish, crayfish and shellfish.

(II) Dermal, nasal or oral (accidental ingestion) contact during recreational use of water.

(III) Blue-green algae food supplements.

(IV) Drinking water and intoxication during hemodialysis.

The order of importance of the individual routes varies between countries and depends on many factors such as climatic condition, eating habits of the local population, source of drinking water and the drinking water treatment in the respective region. Possible ways of ingestion are discussed below. The potential exposure routes are sorted by perceived increasing importance (set by the author):

(I) Microcystins are known to be taken up by cultivated plants such as lettuce (*Lactuca sativa*) (*296*) and the common bean *(Phaseolus vulgaris) (295)*, if the toxins are present in the irrigation water or the growing media. As some cyanobacteria can fix nitrogen from the atmosphere and hence provide a valuable nitrogen source for the growing rice plants after lysis of the cyanobacterial cells, cyanobacteria are welcome in rice fields (*594*). However, little is known about uptake mechanisms into the plants and the concentration of toxic cyanobacterial compounds in rice fields. Thus, it is impossible to assess the risk for human health arising from these sources before detailed investigations are carried out into these topics. Microcystins (and other cyanobacterial toxins) can also accumulate in fish, crayfish and shellfish. While the toxin concentrations in the edible parts of fish are relatively low (however, the liver is to be avoided (*271*)), shellfish and also crayfish are a potential source of high concentrations of microcystins and nodularins. Vasconcelos (*305*) has calculated the weights of edible parts of fish (7 g), crayfish (0.7 g) and shellfish (0.1 g), the ingestion of which are sufficient to attain the provisional WHO guideline values for MC-LR in drinking water (see calculation in chapter I and Table 22). The calculations from Vasconcelos are based on the maximum amounts of MCs (*305*), which have been found in the edible parts of fish (0.3 µg MC-LR/g), crayfish (2.7 µg MC-LR/g) and mussels (16 µg MC-LR/g). Shellfish is also known as a source of saxitoxins (paralytic “shellfish” poisons), however, these saxitoxins are mostly produced by marine dinoflagellates and are thus not in the scope of this risk assessment. Only people with a traditionally high proportion of sea (or freshwater) food within their daily diet may be exposed acutely to high concentrations or chronically to low concentrations. No data exist concerning accumulation of cyanobacterial toxins in livestock, although in Australia and alpine regions of Switzerland (*415*) these animals depend on cyanobacterial contaminated drinking

water. In an Australian study, no carry-over of microcystins into milk could be observed in cattle (*306*).

(II) Many of the toxic representatives of cyanobacteria show mass development in the summer, during the time many people use water bodies for recreation. The harmful concentrations for an acute intoxication with the most abundant cyanobacterial toxin in freshwater is calculated as follows: On the basis of mouse studies (*142, 145, 291, 409*) it can be estimated, that a single intake of 25 µg MC has no adverse effects on young children (10 kg), which are the group with the highest risk (*424*). In 2% of 128 samples from recreation sites near Berlin, Germany, more than 100 µg MC/L water could be detected. If children consume 250 ml of this highly contaminated water, the safe level of 25 µg/l would be exceeded (*424*). Infants playing in shallow water with a high density of perhaps decomposing cyanobacterial bloom could potentially be highly endangered with respect to illnesses resulting from cyanobacterial toxins. According to correspondence with colleagues from all over the world, even heavy cyanobacterial blooms do not prevent water bodies from being used for recreational purposes by the local population. Thus, an acute health risk through recreation can not be excluded. Therefore in several countries such as Australia (*595*), Germany (*596*), Canada and Scotland, warning signs have been installed at water bodies used for recreation to inform the people about the potential danger. Dense and therefore visible concentrations of cyanobacteria prevent most people from swimming in such "contaminated" water bodies. The possible ingestion of cyanobacterial toxins via this route is limited to the summer season, however a subchronic exposure with cyanobacterial toxins can occur. In tropical regions an all-year round exposure of swimming and playing children is possible.

(III) Nowadays cyanobacterial (blue-green algal) supplements (BGAS) represents an important economic branch (*286*), while being sold mainly in the industrialised countries. In addition, several regions worldwide such as Mexico, northern Africa or China have a history of the use of algae as a food source (*286*). The scientific knowledge about possible toxins produced by the cyanobacterial species consumed in these countries is poor. Although the provider of BGAS state that they screen out MC-levels of more than 1 µg/g DW (*286*) in their products, independent investigations of BGAS have demonstrated toxin concentration of up to 35 µg MC-LR equiv./g DW (*149, 283*). The instruction leaflets of these supplements suggest, that adults and children should ingest 1.5-2 g BGAS for several weeks or months. Thus, in the worst

case scenario children could ingest up to a 30-fold amount of MC equivalents as provisionally considered safe by the WHO for MC-LR in drinking water based on a human being of 60 kg. Although samples with a toxin contamination of more than 10 µg MC-LR equivalents/g DW are the exception, seven of eight blue-green algae products tested recently have shown more than 1.0 µg MC-LR equiv./g DW in Adda-ELISA and cPPA (unpublished results, S.J.H.). Own investigations and the study from Lawrence *et al.* (*283*) have shown differences in detectable toxin amounts when employing ELISA, PPA and HPLC-DAD, LC-MS/MS. Thus the values obtained can only be taken as rough estimation of the microcystin content in BGAS. Nevertheless, and despite the possible health promoting constituents as declared by the providers, these supplements contribute to the daily intake of cyanotoxins. Gilroy *et al.* (*149*) suggested a safe level for microcystins in BGAS of 1.0 µg/g DW. The basis for this value was the study of Fawell *et al.* (*409*) with an NOAEL of 40 µg/kg-day divided by 10 for intraspecies, 10 for interspecies and 10 for subchronic to chronic extrapolation, evidence for tumour promotion and database inadequacies. This resulted in 0.04 µg/kg-day x 60 kg divided by 2 (2 g consumption rate) = 1.2 µg/g. This safe level was rounded to 1.0 µg/g and adopted by the Oregon Health division as a provisional regulatory standard for BGAS products on 23/10/1997.

(IV) In a worldwide view the daily drinking water is the main source for the incorporation of cyanobacterial toxins for humans. As listed in Tables 1 and 15, the published cases of cyanobacterial toxins in raw water, during drinking water treatment and even in finished water are numerous and problematic worldwide. Microcystins have been reported in final water in Argentina, Australia (this study), Bangladesh, Canada, Czech Republic, China, Finland, France, Germany (this study), Latvia, Thailand, Switzerland (this study) and USA (for individual references see Table 15 in chapter VI). Up to 9000 cells/ml (occurred in Australia, see chapter VII) were detectable even after treatment in Argentina, Australia, Finland, Germany, Israel and Italy (Table 15). It must be assumed that this is only the tip of the iceberg, because water works are not interested in publishing reports regarding toxins or cyanobacterial cells in their raw or final water. In many cases worldwide the drinking water is simply not screened for cyanotoxins. In addition, more than one billion people have no access to “treated” drinking water, in many cases the drinking water is simply boiled, which does not destroy most of the known cyanobacterial toxins. In contrast, most of the people affected by gastro-enteritis at Itaparica dam/Brasilia (see

Table 6) boiled their drinking water before use. It is likely that the boiling caused lysis of the cyanobacterial cells resulting in toxin release. People who drank water without a previous boiling probably excreted the majority of the ingested cyanobacterial cells and were therefore less affected.

The mentioned facts in this paragraph and the discussions in the individual chapters of these thesis confirm the necessity to set guidelines for microcystin concentration in drinking water. The calculation of the TDI and the resulting provisional WHO guideline value of 1.0 MC-LR/L is described in detail in chapter III. Two other examples are described below to underline how many possibilities exists to be affected by microcystins or other cyanotoxins.

In Scania, Sweden, the drinking water distribution system was erroneously coupled to untreated river water at a sugar refinery (*276*). In this river high densities of *P. agardhii* occurred at this date and the water contained approximately 1.0 µg MC-LR equiv./L. In the following days 121 persons, who consumed the contaminated water, developed symptoms including diarrhoea, headache, vomiting, fever and muscular and abdominal pain. Pathogenic bacteria or viruses could be excluded as the cause for the illnesses. As another interesting fact, 100% of the tea-drinkers of the sugar refinery were sick in the days following the accident, while none of the coffee-drinkers was affected. It is probable that the toxins were filtered out by the coffee grinds. Thus, it can be assumed that cyanotoxins at least participated in the observed symptoms.

A well investigated accident occurred in a hemodialysis centre in Caruaru, Brazil (*265*). The water used for dialysis was insufficiently treated and contained different cyanotoxins. The consequence was the death of 76 patients, while in the respective liver samples from 39 victims in average 223 ng MC/g liver weight (*597*) could be detected (MC-YR, MC-LR, MC-AR). Reports from Portugal (*598*) and USA (*246*) also report illness of hemodialysis patients probably caused by cyanobacterial metabolites.

To develop an overall risk assessment, several assumptions must be set. The tolerable daily intake for microcystins must be assumed to be around 40 µg/kg-day, as shown by results from studies with mice (*409*) and underlined by a study with pigs (*355*). Uncertainty factors (UF) are set as following: 10 for intraspecies, 10 for interspecies and 10 for subchronic to chronic extrapolation (UF 1000). Some authors (*599*) add another factor of 3 for the tumour promotion capacity (TPC) of MCs (UF 3000). The result is multiplied by the body weight, which differs extremely between infants and

corpulent adults and is set at 5 kg for an infant, 10 kg for a child and 60 kg for an adult as estimated by Duy *et al.* (*599*). Also the daily consumption of drinking water is difficult to estimate. It differs between individuals, depends on the age of the person and is here assessed to be 0.75 L for infants, 1 L for children and 2 L for adults. The sum of these assumptions results in estimated safety levels if drinking water is the exclusive source of MCs (Table 22). The safety levels presented by Giloy *et al.* (*149*) concerning the BGAS and from Vasconcelos (*513*) for seafood/freshwater food (fish, crayfish and shellfish) are also calculated only for one source for microcystin intake. In the provisional guideline for drinking water the WHO set a multiplication factor (MP) of 0.8, that means 80% of the assumed daily MC-intake comes through drinking water. However, also this value varies from person to person and is probably age dependant. This age dependant assessment of the importance of the different routes for ingesting microcystins is outlined in Table 23 and expressed as multiplication factors (MP).

		Safety levels for total microcystins (µg MC total/day)	Assumed consumption of drinking water per day (L)	Safety levels for total microcystins (µg MC/L drinking water)
Infants	**- UF for TPC**	0.2	0.75	0.27
	+ UF for TPC	0.068		0.09
Children	**- UF for TPC**	0.4	1	0.4
	+ UF for TPC	0.133		0.133
Adults	**- UF for TPC**	2.4	2	1.2
	+ UF for TPC	0.8		0.4

Table 22. Safety levels for total microcystins for different stages of life, based on the risk assessment set by the WHO (*1*) and resulting provisional guideline values. UF uncertainty factor; TPC tumour promoting capacity

For example, the safety level for adults without the uncertainty factor for tumour promoting capacity is 2.4 µg MC-LR equiv./d (Table 22). This value is multiplied by factors (MP) in Table 23, which represents the importance of the individual routes of ingestion for the different stages of age. The main differences between adults and infants/children is probable, that children and infants drink more water during playing in the water or at the contaminated shore of a water body. In addition, it is

assumed that infants do not come into contact with BGAS, although even this can not be excluded. Therefore, the calculated safety levels are burdened with many uncertainties. However, the calculated safety values in Table 23 are distinctly lower than the concentration measured regularly in drinking water, BGAS, recreational waters and "freshwater food" (fish, crayfish and shellfish), especially if the TPC of MC is included. Therefore, cyanobacterial toxins and especially microcystins are a danger for human health and must be observed with more attention in the future.

Table 23. Safety levels for the individual ingestion pathways calculated individually. The values from Table 22 (total MC) are multiplied by the assumed factors (MF) of importance of the individual pathways for several age stages.

		Drinking water	BGAS	Recreational purpose	Food chain
Infants µg MC/d	**MF**	**0.85**	**0.05**	**0.1**	**0**
	- UF for TPC	0.17	0.01	0.02	0
	+ UF for TPC	0.058	0.003	0.007	0
Children µg MC/d	**MF**	**0.6**	**0.25**	**0.1**	**0.05**
	- UF for TPC	0.24	0.1	0.04	0.02
	+ UF for TPC	0.08	0.033	0.013	0.007
Adults µg MC/d	**MF**	**0.6**	**0.25**	**0.05**	**0.1**
	- UF for TPC	1.44	0.6	0.12	0.24
	+ UF for TPC	0.48	0.2	0.04	0.08

CYANOBACTERIA DURING DRINKING WATER TREATMENT: WHICH AIMS SHOULD BE REACHED?

In the case of surface water treatment many factors play a role with respect to the treatment efficacy. The drinking water treatment is optimised to eliminate colour, taste and odour as well as pathogens. Data from the literature and the results presented in this study have shown, that in addition to the treatment train the natural organic matter, the temperature and the pH of the water affect the efficacy of different treatment methods with respect to the elimination of cyanobacterial toxins.

The known toxin variants show different chemical properties and therefore require different treatment steps. Another important parameter is the dosage during drinking water treatment. How much ozone is necessary to eliminate pathogens from water? Which ozone/cyanobacterial cell/natural organic matter ratios lead to lysis of cyanobacterial cells? How much ozone is necessary to lyse the cells followed by an oxidation of the extracellular toxins? After which time periods is backwashing of the filter necessary? How effective is the biofilm of the activated carbon filter in degrading cyanotoxins? These are only a few question which cannot be answered adequately from laboratory-scale experiments. Laboratory experiments can only investigate very specific questions and give a rough estimation of the situation in water works. Because nearly every water works has its own kind of water treatment system and a specific composition of raw water during the year, routine toxin measurements followed by individual consulting would be necessary for every single water works.

Also, other questions arise from the ozonation of cyanobacterial toxins, e.g. microcystins. If microcystin-containing water is oxidised, the risk of potential toxicity of the products exists. Chapter VIII pointed out, that the degradation followed distinct routes. The data from Adda-ELISA and rPPA have shown no additional signals through these by products, however, other binding partners besides the protein phosphatases and therefore negative impacts on humans health are possible. However, it can be assumed, that in the case of a adequate treatment train, the by products as well as the native molecules are degraded by the subsequent filtration steps.

The detected levels of cyanotoxins within the food chain, in water bodies used for recreational purposes, the relatively high levels of microcystins in BGAS and in drinking water all over the world, together with the uncertainties with respect to toxicological and analytical data must be a warning for the responsible authorities to note the health risks for humans caused by cyanobacteria.

SUMMARY

No life without water. Indeed, the human being is not content with this simple maxim any more. Drinking water must fulfil distinct criteria of appearance, smell and taste. The exponential population growth and the subsequent problems with environmental pollution endanger the supply of inoffensive and healthy drinking water. For a long time pathogens (e.g. cholera, typhus) in drinking water caused epidemics. Analytical improvements have enabled the detection of other potential harmful organisms and molecules in drinking water: Cyanobacteria (blue-green algae) and metabolites synthesised by these organisms were proven to cause illnesses in humans. Different organ systems can be affected by these secondary metabolites. The best investigated cyanobacterial toxins are alkaloids (saxitoxins, anatoxin-a, cylindrospermopsin) and polypeptides (microcystins, nodularins). To avoid risks to human health, an appropriate drinking water treatment is necessary.

In one part of this study a laboratory-scale drinking water treatment system was built to mimic the processes in water works, which ought to remove cyanobacteria and their toxins from drinking water. Therefore, an ozonation and a variety of filtration steps were installed and their respective efficacies regarding the elimination of pure Microcystin-LR, extracts from *P. rubescens* and cells of *M. aeruginosa* and filaments of *P. rubescens* from raw water were investigated. The experiments have shown, that the detoxification efficacy of the installed treatment system is affected negatively by the concentration of total organic carbon in the sample. Furthermore, the ozone concentration regularly used in water works can lead to lysis and release of toxins followed by an increased amount of extracellular toxin after ozonation. However, the residual toxicity after ozonation could be successfully removed by the following filtration systems (quartzsand/pumice, activated carbon/quartzsand, slow sand). An additional risk is posed by the repeated use of filter material. Previously adsorbed microcystins can be redissolved out of the filter material by subsequent charges of water and break through to final water.

In addition to the investigations on a laboratory scale, samples from drinking water works in Australia (flocculation/filtration), Germany (flocculation/filtration) and Switzerland (ozonation/filtration) were screened with respect to cyanobacterial

toxins. The samples were taken both from raw water and after the different filtration steps. In raw water concentrations of more than 5.0 µg microcystin/L were detectable. However, after the respective treatment steps, the concentration was distinctly below the provisional WHO guideline value of 1.0 µg MC-LR/L in every case. As well as microcystins, saxitoxins and cylindrospermopsin could be detected in raw water of drinking water works in Queensland/Australia. These were also detectable in trace amounts in final water. In one of these water works the efficacy of flocculation/filtration to remove cyanobacterial cells was also investigated. In spite of a removal efficacy of ~ 99%, the initially extremely high cell numbers in raw waters resulted in a cell density in final water, which was repeatedly above 5000 cells/mL.

A further method to "produce" drinking water is to employ "natural" water cleaning by bank filtration. To prove the efficacy of this filtration to degrade/remove cyanobacterial toxins, samples were taken from Lake Hallwil/Switzerland and the respective drinking water well near the shore. Lake Hallwil is known for regular blooms of toxic *Planktothrix rubescens*. More than 99% of the microcystin concentration detected in the lake water could be filtered (and degraded) via bank filtration. Even though traces of microcystin could be measured in the wells near the shore of the lake, the concentration in final drinking water wells were distinctly below 1.0 µg/L.

The results show, that a regular monitoring of raw and final water is necessary to enable the water supply authorities to adapt the water treatment to the respective cyanobacterial density and to be in a position to exclude loading of the final water with cyanobacterial cells or toxins.

As well as the drinking water relevant data, information could be gathered concerning the ecological aspect of cyanobacteria and the function of their toxins. In Lake Hallwil the comparison of cyanobacterial density and toxin amount can be interpreted as evidence for a change between the dominance of subpopulations of *Planktothrix rubescens*, which cannot be differentiated morphologically. In Gordonbrook Dam/Queensland/Australia the predominating species changed several times during a two-year period. A temporal coherence of interspecies competition for predominance in the water body and the respective toxin concentration was recognizable. Once a species had reached predominance, the toxin amount per litre decreased in spite of increasing cell density. An allelopathic function of these secondary metabolites can be derived from these observations. A further

interpretation of this phenomenon could be a change in predominance from a toxin producer to a non toxin-producing subpopulation. This change in dominance could be formed after the predominance of one species has been established and interspecies competition no longer takes place, similar to the situation at Lake Hallwil as described above.

A further part of this thesis was the identification of the degradation route of microcystin-LR after ozonation and the possible toxicity of the resulting by-products. It could be shown that ozonation cleaves the Adda side-chain at different positions. This is independent of the ozone concentration and is followed by a break-up of the ring-structure and further mineralisation steps, which could not be identified in detail. Structural (Adda - ELISA) and functional (protein phosphatase – inhibitory assay; PPA) test systems resulted in no additional signals. This gives strong evidence that Microcystin-LR ozonation by-products do not have a detectable Adda-side chain (ELISA) and are no longer capable of protein phosphatases inhibition (PPA).

The knowledge regarding the efficacy of different drinking water treatment steps to eliminate and/or remove cyanobacteria and cyanobacterial toxins is summarised in this report. In addition, the importance of different routes of ingestion and the possible negative influences of cyanobacterial toxins on human health are assessed.

ZUSAMMENFASSUNG

Ohne Wasser kein Leben. Der Mensch begnügt sich allerdings nicht mehr mit diesem einfachen Grundsatz. Das Trinkwasser muss bestimmten Kriterien entsprechen, zumindest schmackhaft aussehen und angenehm riechen. Das exponentielle Bevölkerungswachstum mit dem damit verbundenen Problem der Umweltverschmutzung gefährdet die Versorgung mit gesundheitlich unbedenklichem Trinkwasser. Lange Zeit waren Pathogene im Trinkwasser Grund zur Besorgnis und Ursache großflächiger Epidemien (Cholera, Typhus etc.). Fortschritten in der Analytik machten die Detektion anderer potentiell gesundheitsschädlicher Moleküle im Trinkwasser möglich. Cyanobakterien (Blaulagen) bzw. von diesen Organismen produzierte Metaboliten erwiesen sich in den letzten Jahren als mögliche Verursacher gesundheitlicher Probleme des Menschen. Das Verbreitungsgebiet der Cyanobakterien erstreckt sich über den gesamten Erdball. Fossilien belegen ein erstes

Vorkommen von Cyanobakterien vor 3,5 Milliarden Jahren. Sie haben in der proterozoischen Zeit (“Zeitalter der Cyanobakterien”) vor ca. 2,5 Milliarden Jahren für den Sauerstoffanstieg in der Biosphäre gesorgt und damit die Voraussetzungen für aerobes Leben auf der Erde geschaffen. Cyanobakterien beherrschen den marinen, den limnischen und den terrestrischen Lebensraum.

Probleme im Trinkwasser verursachen Cyanobakterien nicht nur aufgrund ihres meist unangenehmen Geruchs und der unerwünschten Färbung des Wassers. Von diesen Organismen produzierte toxische Sekundärmetaboliten stellen ein Risiko für die Gesundheit der mit dem entsprechend belasteten Trinkwasser versorgten Menschen dar. Die chemische Struktur und die damit verbundenen Eigenschaften dieser verschiedenen Toxine ist unterschiedlich und umfaßt u. a. Alkaloide (Saxitoxine, Anatoxin-a, Cylindrospermopsin) und Polypeptide (Microcystine, Nodularine), die negativen Einfluß auf verschiedene Organsysteme des Menschen haben können. Um das Risiko für die Menschen zu begrenzen, ist eine geeignete Aufbereitung des Trinkwassers nötig.

In einem Teilprojekt der vorliegenden Arbeit wurde ein Trinkwasser-Aufbereitungssystem im Labormaßstab aufgebaut, um die Vorgänge während der Aufbereitung cyanobakterieller Wässer imitieren zu können. Hierfür wurde eine Ozonungsstufe und verschieden Filtrationsmaterialien installiert und deren Effektivität bezüglich der Eliminierung von reinem Microcystin-LR, von Extrakten aus *Planktothrix rubescens* und von intakten Zellen von *Microcystis aeruginosa* bzw. Trichomen von *P. rubescens* untersucht. Diese Versuche habe gezeigt, daß die Effektivität der Detoxifizierung cyanobakterieller Proben von einem hohen Gesamtkohlenstoffgehalt (TOC) des aufzubereitenden Wassers negativ beeinflußt wird. Außerdem kann Ozon schon bei Konzentrationen, die üblicherweise in Wasserwerken eingesetzt werden, zu einer Lyse der Zellen und Trichome und damit zu einer Freisetzung der Toxins führen. Die Resttoxizität nach Ozonung konnte jedoch durch drei verschiedene Filtrationssysteme, bestehend aus Quartzsand/ Bimsstein, Aktivkohle/Quartzsand und feinem Kies (Langsamsand) erfolgreich entfernt werden. Die Ergebnisse machten auch die Gefahr deutlich, daß vorher adsorbierte Toxine durch nachfolgende Microcystin - freie Chargen aus den Filtermaterialien gelöst werden und in das Reinstwasser durchbrechen können.

Ergänzend zu den Untersuchungen im Labormaßstab wurden Proben aus Trinkwasserwerken in Australien (Flockung/Filtration), Deutschland

(Flockung/Filtration) und der Schweiz (Ozon/Filtration) auf cyanobakterielle Toxine hin untersucht. Diese Proben wurden aus dem Rohwasser und nach den jeweiligen Filtrationsstufen entnommen. Belastungen des Rohwassers mit mehr als 5 µg Microcystin/L waren detektierbar, wobei die jeweils angewandte Aufbereitungsmethodik die Toxinkonzentrationen deutlich unter den von der WHO angegebenen Richtwert von 1,0 µg MC-LR/L reduzieren konnte. Im Rohwasser von Trinkwasseraufbereitungswerken in Queensland/Australien wurden neben Microcystin noch Saxitoxine und Cylindrospermopsin detektiert, die in Spuren auch noch im Reinstwasser nachweisbar waren. In einem dieser Wasserwerke wurde außerdem die Effektivität einer Kombination aus Flockung und Filtration bezüglich der Entfernung hoher cyanobakterieller Zelldichten untersucht. Im Reinstwasser dieses Wasserwerks waren nach Aufbereitung regelmäßig mehr als 5000 Zellen/mL zu messen. Bedingt durch extrem hohe Zellzahlen im Rohwasser führte auch die Entfernung von durchschnittlich 99% der Zellen nicht zu einer zufriedenenstellenden Qualität des Leitungswassers.

Eine weitere Methode der Gewinnung von Trinkwasser ist die sogenannte Uferfiltration. Probennahmen aus einem entsprechend genutzten Oberflächengewässer in der Schweiz und dem filtrierten Wasser in ufernahen Brunnen haben gezeigt, daß zwar mehr als 99 % der Microcystine gefiltert werden, jedoch noch Spuren von Microcystin in den Trinkwasserbrunnen vorhanden sind.

Die Resultate zeigen, daß regelmäßige Untersuchungen des Roh- und Reinstwasser notwendig sind, um die Aufbereitung den jeweils auftretenden Cyanobakteriendichten anpassen und eine Belastung der Reinstwassers mit cyanobakteriellen Toxinen ausschließen zu können.

Neben den trinkwasser-relevanten Daten konnten auch Informationen über ökologische Aspekte des Auftretens von Cyanobakterien und der möglichen Funktion der Toxine gesammelt werden. Im Hallwiler See/Schweiz weist der Vergleich von cyanobakterieller Zellzahl zu Toxingehalt auf einen Wechsel von Subpopulationen der Spezies *P. rubescens* hin, wobei diese Subspezies morphologisch nicht zu unterscheiden sind. Im Gordonbrook Dam/Australien konnte im Laufe zweier Jahre mehrmals ein Wechsel der dominierenden Cyanobakterien - Spezies beobachtet werden. Eine zeitliche Kohärenz zwischen interartlichen Konkurrenzsituationen um die Vorherrschaft im Gewässer und der jeweiligen Toxinkonzentrationen war zu erkennen. Nachdem eine Cyanobakterienart die Dominanz im Gewässer

übernommen hatte, ging die Toxinkonzentration trotz steigender Zellzahl zurück. Aus diesen Beobachtungen kann eine allelopathische Funktion dieser Sekundärmetabolite abgeleitet werden. Eine weitere Erklärung wäre ein Dominanzwechsel von einer toxischen zu einer nicht-toxischen Subpopulation der jeweiligen Spezies. Dieser Dominanzwechsel könnte sich zu dem Zeitpunkt ausbilden, zu dem die Dominanz der jeweiligen Spezies feststeht und keine interartliche Konkurrenz mehr zu befürchten ist, ähnlich zu dem bereits erwähnten Wechsel der Subpopulationen im Hallwiler See/Schweiz.

Ein weiterer Bestandteil der Arbeit war die Aufklärung des Abbauweges von Microcystinen während der Ozonung und die mögliche Resttoxizität. Unabhängig von der Konzentrationen an Ozon wurde die Adda-Seitenkette der Microcystine an verschiedenen Positionen abgespalten, gefolgt von einem Aufbruch der Ringstruktur des Restmoleküls und weiteren Mineralisierungsschritten, die nicht im Detail aufgeklärt werden konnten. Strukturelle (Adda - ELISA) und funktionelle (Proteinphosphatasen-Inhibitionsassay; PPA) Testsysteme ergaben keine zusätzlichen Signale. Diese Ergebnisse lassen den Schluß zu, daß Microcystin-LR Produkte nach oxidativem Abbau durch Ozon keine detektierbare Adda-Seitenkette mehr besitzen (ELISA) und diese Produkte nicht mehr imstande sind, Proteinphosphatasen zu inhibieren (PPA).

In dieser Arbeit ist der Wissensstand auf dem Gebiet der Effektivität verschiedener Trinkwasseraufbereitungsmethoden bezüglich der Eliminierung cyanobakterieller Toxine zusammengefaßt. Zusätzlich enthalten ist eine Beurteilung der möglichen Aufnahmewege und negativen Einflüsse dieser Toxine auf die menschliche Gesundheit.

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ACKNOWLEDGEMENTS

Unfortunately my patience to come up with intelligent sentences is exhausted at this point in time. Thus I ask everyone to forgive me, if I have forgotten to mention someone or if someone believes his/her contribution would be worthy of being honoured in a more detailed way. During the past 45 months a lot people were involved in the work presented here.

Special thank to my supervisor Dr. Daniel Dietrich for accepting me as a "revolutionist" in his lab. Beside the professional things I have learned, I have a lot of more stories to tell my grandchildren.

Thank to Dr. Eric von Elert for evaluating my thesis, for being a member of the examination committee, for helpful discussions concerning analytic of microcystis and for making HPLC-DAD available for parts of my work.

Thanks to Dr. Alexander Bürkle for being a member of the examination committee.

Not to forget Dr. Bettina Hitzfeld, who accompanied the main part of my thesis. Thanks for discussion, support in ticklish situations and also for many adventures besides the lab stuff.

Thanks to Dr. Glen Shaw from the National Research Centre for Environmental Toxicology (NRCET) for putting up with this tall German guy in his lab for 3 ½ months and for making the project about Australian water works possible.

Another important person involved with this work was Dr. Werner Fischer, who brought me into contact with cyanobacteria and their "nasty" but fascinating secondary metabolites. I will never forget our adventures in the swamps and plantations of New Orleans.

Thanks to Bernhard Ernst (special thanks for many hours of discussion about cyanobacteria and all the world and his wife), Alex Heußner (she brought organisation to my scientific environment), Anne Jungblut and Jörg Frommlet (the order of mentioning is random, both for numerous hours of information exchange, discussion and fun at this and the other end of the world), Dr. Evelyn O' Brien (for inestimable help with my spoken and written English and for teaching me the most important Gaelic expression: Sláinte), Anke Prietz (special thanks for the cactus), Karin Rieder (for helping me with so much administration stuff), Dr. Frank "Judas" Schwöbel (another member of the "New Orleans faction", for telling the story of the fearful chicken and the fox and for pointing out that Christina Lampert is the chicken and not me), and, last but not least, Birgit Wasserrab (for a lot of discussions about the hard life as a scientist and numerous pleasant days in Konstanz, Rotorua and other places all over the world and special thanks for asking me every day "When will it finally be Christmas?").

Thanks to Corinne Garnett. She was the main reason, why my time in Brisbane was as "tremendous" as it was. To work and to live with her was great. Thanks also to Dr. Wasa Wickramasinghe, who was my contact person at NRCET and is now a good friend. In addition, thanks to Vanessa Burgess, Dr. Jochen Müller (who brought German soccer techniques to Brisbane), Nick Osborn (I have still welts on my hands), Ian Steward and everyone else at NRCET.

Thanks to Dr. Billy Day (Department of Pharmaceutical Sciences, University of Pittsburgh, USA) for analysing some of my microcystin samples and for very helpful discussions about mass spectrometry and chemical details.

Thanks to Judith Blom (Institute of Plant Biology, University of Zurich, Switzerland) for providing the A7 strain and for help with HPLC-MS analysis. Thanks also for showing me Zurich at night and many talks about scientific life and the meaning of life per se.
Thanks to Murray Smith and Dr. Mike van den Heuvel (both Forrest Research, Rotorua, New Zealand) for making LC-MS analysis in Rotorua possible.
Special thank to the German Federal Environment Foundation and the Universitätsgesellschaft Konstanz e.V. for funding my work in Konstanz and Brisbane and above all thanks to my parents and grandparents for funding and supporting my scientific career.
And thanks to my son Jonas for being as he is.