Relevance of Intra- and Extracellular Cyanotoxins for Drinking Water Treatment*

Bedeutung intra- und extrazellulärer Cyanotoxine für die Trinkwasseraufbereitung

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Summary: Bloom-forming cyanobacteria have been observed in eutrophic waterbodies including drinking water reservoirs all over the world. In this connection investigations about the relevance of intra- and extracellular cyanotoxins for drinking water treatment were carried out in laboratory- and pilot-scale experiments. An algae growth phase depended toxin release from cyanobacteria was obtained naturally caused from cultured cyanobacteria (*Microcystis aeruginosa*) and in an eutrophic reservoir containing *Planktothrix rubescence*. Results from laboratory-scale tests using cultivated cyanobacteria and pilot-scale experiments at an eutrophic reservoir underline the induced toxin release during conventional water treatment. Additional to the known toxin release using pre-oxidation, it was obtained the first time that the application of flocculation/filtration also effects in toxin release under the conditions investigated, possibly caused by turbulences in pipes and pressure gradients in filters.

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Zusammenfassung: Die Beeinträchtigung der Trinkwasserqualität infolge oftmals überraschender Algenmassenentwicklungen stellt die Wasserwerke in vielen Ländern der Welt vor erhebliche Probleme. In diesem Zusammenhang wurde das Verhalten intra- und extrazellulärer Cyanotoxine im Prozess der Wasseraufbereitung anhand von Laborversuchen und an einer kleintechnischen Versuchsanlage untersucht. Die natürliche Freisetzung der Cyanotoxine wurde, abhängig vom Wachstumsstadium der Cyanobakterien, sowohl an einer *Microcystis aeruginosa*-Kultur als auch an einer mit *Planktothrix rubescens* belasteten eutrophen Talsperre nachgewiesen. Im Ergebnis von Labor- und kleintechnischen Versuchen an kultivierten bzw. natürlich vorkommenden Cyanobakterien wurde festgestellt, dass im Prozess der konventionellen Aufbereitung eine induzierte Toxinfreisetzung stattfindet. Neben der bereits bekannten Freisetzung bei der Voroxidation konnte erstmals auch die Freisetzung von Cyanotoxinen im Prozess der Flockung/Filtration, wahrscheinlich hervorgerufen durch Turbulenzen im Leitungssystem und Druckgradienten in Filtern, nachgewiesen werden.

1 Introduction

Bloom-forming cyanobacteria have been observed in waterbodies including drinking water reservoirs all over the world. Several strains of these microorganisms have the ability to produce potent toxins as secondary metabolites, the so-called cyanotoxins. These have caused many animal deaths and have also been implicated in cases of human illness, e.g. in U.S.A., Australia, China, Brasil, and Great Britain [1-7]. According to their effects on health, cyanotoxins are divided into hepatotoxins (liver damaging, tumor promoting; microcystins, nodularin, cylindrospermopsins), neurotoxins (nerve damaging; anatoxin-a, saxitoxins), and dermatotoxins (dermatitis; aplysiatoxins, lyngbyatoxins) [8, 9]. Since cyanotoxins have been found in drinking waters, a Guide-line Value of $1.0 \mu g/L$ for microcystin-LR in drinking water is proposed by the World Health Organisation (WHO) [10].

In nature, cyanotoxins are characterised by the state of their occurrence, cell bound (intracellular) and dissolved (extracellular), respectively. Toxin release into water may be due to both natural toxin release, caused by naturally biological process of cell lysis, and by artificial induction through cell destruction in treatment processes (induced toxin release). The latter is of particular interest in drinking water production.

The state of the art in water treatment of cyanobacteria and their toxins is non-uniform because of the complexity of the objectives. Some work has been done on potentially useful technologies such as flocculation/filtration [11-13], biodegradation [14-17], oxidation [18-23], and adsorption [24-27], but these studies are often fundamental, have been focused solely on one treatment technology, or restricted to solving only local well-defined problems. All of the applied treatment processes have advantages and disadvantages depending e.g. on the local and seasonal situation, genera and state of growth of cyanobacteria, the presence of particular toxin types, as well as the technological equipment available and treatment conditions.

In general, flocculation/filtration is effective for cell removal but ineffective for extracellular toxin elimination. Previous studies have neglected the importance of extracellular toxin release caused by system turbulence and pressure gradients in filters during flocculation, as well as by application of pre-oxidants [11-13]. Since cyanotoxin biodegradation depends strongly on the local situation, e.g. cyanobacterial composition, climatic conditions, adapted microorganisms, and occurrence of oxygen, there are no comprehensive and comparable data available so far [14-17]. It is known that chemical oxidation and adsorption technologies are effective for extracellular toxin removal. However, chemical oxidation and adsorption seem to be questionable in relation to cell lysis, toxin release, and biological stability of the water, as well as competitive adsorption of DOC [18-21, 24-27]. Disinfection with chlorine-containing agents can reduce the toxin concentration insufficiently and unknown, possibly harmful by-products can be formed [22, 23, 28]. Finally, additional techniques such as liming, membranes, and flotation seem to be effective for cell removal but have not been well examined so far [29].

According to that, the objective of the present study was the characterisation of both forms of toxin release. The obtained results base on the examination of cultivated cyanobacteria under laboratory conditions and long-term pilot plant studies in relation to conventional treatment trains (flocculation/filtration in combination with pre-oxidation).

2 Experimental

2.1 Cyanobacterial culture

Microcystis aeruginosa strain B 14.85 (Sammlung von Algenkulturen, University of Göttingen, Germany) was grown in 5 L of ZEHNDER medium [30] at 21-23 °C under continuous stirring and an illumination of 12 hours/day (ca. 80 µmol s /m² photons, day-

night-change). This strain of *Microcystis aeruginosa* is known to produce microcystin-LR.

2.2 Laboratory-scale experiments

Laboratory- scale experiments were carried out using a cultivated *Microcystis aeruginosa* strain B 14.85 described above. Cultures were harvested at the late exponential phase of growth for experiment 1 and at the stationary growth phase for the second experiment (Figure 1). The cultivated algal suspension was dissolved with raw water from a cyanobacteria-containing reservoir (relation 1:3).

Figure 1

Flocculation was performed using a 2 L flocculation reactor and the flocculants ferric chloride sulphate β (Fe) = 6 mg/L) and aluminium sulphate β (Al) = 4 mg/L), respectively, as follows: dosage of the flocculants, rapid stirring (200 1/min, 1.0 min), dosage of flocculant aid ('Praestol'', β = 0.1 mg/L), rapid stirring (200 1/min, 1.0 min), slow agitation (40 1/min, 15 min), sedimentation for 20 min, and membrane filtration (Isopore, 0.4 µm).

Ozone was generated from absolutely pure oxygen (purity 3.5) by a laboratory ozone generator (Sorbios GSG, $\beta(O_3) = 50 \text{ mg/L}$ in the gas phase). Stock solutions of ozone ($\beta(O_3)$ ca. 0.03 mg/mL water) were prepared as published by Hoigné and Bader [31]. The exact amount of ozone in the stock solutions was determined by the Indigo reagent before each test [32]. All investigations were carried out in "reactor" flasks (1.0 L) as batch experiments. The reaction was started by adding a defined amount of the ozone stock solution yielding to $\beta(O_3) = 0.1 \text{ mg/L}$ and 0.2 mg/L, respectively. The flask was then closed, shaken, and the reaction was stopped by adding a solution of sodium sulfite (5 mL, $\beta = 5 \text{ mg/mL}$) after a reaction time of 5 minutes.

2.3 Pilot-scale experiments

Pilot-scale investigations were carried out using the pilot-scale system presented schematically in Figure 2.

Figure 2

The pilot-scale system consists basically of a flocculation reactor and a filtration unit. Additionally, the dosage of pre-oxidants (ozone, permanganate) as well as powdered activated carbon is possible. Technological parameters and dosages ranges are included in Figure 2. Pilot-scale experiments were carried out as long-term attempts over the filter run period controlled by continuous measurements of pH, turbidity, and filter pressure.

The investigations were performed using a raw water from an eutrophic German reservoir (DOC ca. 5 mg/L) containing cyanobacteria of the genera *Planktothrix rubescence* up to biovolumina of 10 mm³/L. The dominant cyanotoxin produced by this population of *Planktothrix rubescence* was identified by tandem-MS as a demethylated variant of microcystin-RR (m/z 1023) reported previously as [DAsp³]-MC-RR or [Dha⁷]-MC-RR [33].

2.3 Cyanobacteria and cyanotoxin analysis

Determination of cell number and biovolumina was performed by microscopy after sample preservation with Lugols iodine [34]. For *Planktothrix rubescence* the length of the filaments was measured and recalculated.

Sample preparation was carried out using careful filtration (cellulose nitrate filters, 0.45 µm) of water samples for separation of intra- and extracellular cyanotoxins.

Cyanotoxin analysis included both extracellular toxins determined by a combination of ion-pair supported solid-phase extraction (SPE) coupled with HPLC-MS-MS [35] as

well as cell bound intracellular toxins determined by a method described by Fastner et al. [36].

3 Results and Discussion

3.1 Natural Cyanotoxin Release

Investigations of naturally induced cyanotoxin release were carried out using a cultivated strain of *Microcystis aeruginosa* as well as field measurements on an eutrophic German reservoir containing *Planktothrix rubescence*.

In Figure 3 cell numbers and concentrations of intra- and extracellular microcystin-LR (MC-LR) are presented versus the growth of the cultivated *Microcystis aeruginosa*. Obviously, intracellular MC-LR is generated during the exponential growth phase (day 0 - 9). A further generation of intracellular toxin was obtained during the stationary phase of growth (day 9 - 16) with a maximum level of 25 µg/L at day 13. Simultaneously to the decrease of the cell bound toxin content after day 13, the concentration of extracellular MC-LR increases up to $10 \mu g/L$ at day 16 caused by toxin release from cells in the surrounded medium. A sharp rise of dissolved MC-LR up to 35 µg/L after day 16 characterised the decaying algae growth phase.

Figure 3

The obtained relation between intra-and extracellular cyanotoxins in a cultivated *Micro-cystis aeruginosa* was confirmed also by cultivated cyanobacteria of the genera *Anabaena flos-aquae* and *Planktothrix rubescence* [37]. This indicates that from the middle of the stationary growth phase (*Microcystis, Planktothrix*) and the end of the exponential growth phase (*Anabaena*), respectively, the massive cyanotoxin release obtained was caused by the physiology of the cyanobacteria cells, only.

For example of natural populations, the occurrence of a continuous bloom of the filamentous cyanobacteria *Planktothrix rubescence* in a German reservoir, applied for drinking water supply (Apr 2000/Apr 2001), and the content of intra- and extracellular cyanotoxins (MC-RR variant m/z 1023 as dominant toxin), determined in two measurement campaigns (Nov/Dec 2000, Feb/Apr 2001), is shown in Figure 4.

Figure 4

Biovolumina up to 10 mm³/L and intracellular toxin concentrations up to ca. 3.5 μ g/L underline the relevance of cyanobacteria and cyanotoxins in the waterbody of the reservoir (Fig. 4). Determinable extracellular toxin concentrations (max. 0.2 μ g/L, Fig. 4) indicate the level of natural toxin release. However, concentrations of dissolved toxins obtained in the waterbody were relatively low (max. 10 % of the intracellular content) caused e.g. by dissolution, microbial and photocatalytic degradation.

In conclusion, the experiences of these measurement campaigns in connection with some positive results after investigations for toxin content in the waterworks practise led to the hypothesis that the behaviour of intracellular toxins during treatment has to be of relevance for drinking water production.

3.2 Induced Cyanotoxin Release

The investigations of induced cyanotoxin release during drinking water treatment processes (pre-oxidation, flocculation/filtration) were performed in laboratory-scale and pilot-scale experiments using a cultivated strain of *Microcystis aeruginosa* and a reservoir water containing *Planktothrix rubescence*, respectively.

3.2.1 Laboratory-scale Experiments

Laboratory-scale experiments were performed as described in Chapter 2 (Experimental) at two different growth phases of *Microcystis* culture (Fig. 1). The influents for the

batch experiments differed strongly in both the total cyanotoxin content and the relation between intra- and extracellular cyanotoxins. Whereas the influent of the exponential growth phase was characterised by 95 % of intracellular and only 5 % of extracellular MC-LR (total MC-LR 7,4 μ g/L, Table 1), a cyanotoxin relation of 1:1 was determined for the influent at the stationary phase (total MC-LR 16 μ g/L, Table 1).

Table 1

The results of the flocculation experiments using iron and aluminium salts are also summarised in Table 1 concerning cell removal as well as toxin removal and release.

As expected, whereas algal cells were removed, extracellular MC-LR was not eliminated by flocculation. However, the following observations are remarkable:

- Cell removal was higher in the stationary growth phase, generally.
- Flocculation with aluminium sulfate was more efficient in comparison to the iron salt.
- Concentration of extracellular MC-LR was increased in the stationary growth phase where cells are unstable, after flocculation (Table 1). The latter is of particular importance due to hypothesis that there could be a toxin release during flocculation.

The behaviour of intra- and extracellular MC-LR during batch experiments of pre-oxidation with two ozone concentrations are summarised in Table 2.

Table 2

Generally, the total MC-LR and intracellular MC-LR level decreased in both growth phases and the toxin degradation by oxidation with ozone is remarkable. However, ozone dosages applied for pre-oxidation are not sufficient for the complete elimination of intracellular cyanotoxins (Table 2). In contrast, an increasing extracellular concentration of MC-LR could be obtained at the exponential growth phase, definitely caused by toxin release from the algal cells (Table 2). Toxin release can also be assumed for the

stationary growth phase. However, due to the overlapping of toxin degradation and release, the dissolved content of MC-LR released from the algal cells could not be exactly quantified.

Summarising the laboratory-scale experiments with a *Microcystis* culture it can be concluded that:

- The relation between intra- and extracellular cyanotoxins determined by the algae growth phase is important for a comprehensive risk assessment of cyanobacteria-containing waters for drinking water supply.
- Cyanotoxin release induced during drinking water treatment seems to be of interest, especially for treatment processes with raw waters from lakes or reservoirs.

However, laboratory-scale experiments using biological materials can only be the base for several hypothesis. Morphological differences between algal cultures and natural algal populations as well as problems of transferability to waterworks practise (turbulences, pressure gradients) demonstrate the limits of laboratory-scale experiments for the assessment of toxin removal under conditions of practice.

Long-term pilot-scale investigations using cyanobacteria-containing raw waters are needed for confirmation of the results of laboratory-scaling.

3.2.2 Pilot-Scale Experiments

Long-term pilot-scale experiments were carried out at various points of algae growth in a German reservoir between October 2000 and April 2001 (Fig. 4). Due to overlapping growth phases in the natural *Planktothrix rubescence*-population under investigation no single growth phases could be defined for the experiments. The results obtained in three various flocculation experiments (2.6 mg/L aluminium as aluminium sulfate, 0.1 mg/L Praestol as flocculant aid) with *Planktothrix*-biovolumina between 4 and 8 mm³/L are summarised in Figure 5.

Figure 5

As expected, algal cells were pre-concentrated and cell-bound cyanotoxin was removed in the filter bed, respectively. In contrast, the content of extracellular toxin increased directly after flocculation (Dec. 2000) and in the filter bed (Nov./Dec. 2000, March 2001) up to concentrations between 0.20 and 0.70 μ g/L in the filter effluent (Fig. 5). Depending on the growth phase, connected with the various stability of algal cells, the significant toxin release during flocculation/filtration can be explained by destabilising of the algal cells caused by hydraulic stress (turbulences) as well as pressure gradients in pipes and filters.

Generally, it is remarkable that the mechanical pressure is recognised to be a common problem in water technology treating algae-containing waters. So, it is not amazing that, in contrast to former studies [11-13], the release of cyanotoxins during flocculation/filtration could be reported for the first time.

In conclusion, the results obtained in pilot-scale tests confirm and underline the hypothesis of the possibility of toxin release during conventional water treatment, as assumed from laboratory-scale experiments.

A second complex of investigations illustrates the influence of pre-oxidation, commonly used for inactivation of the motility of algal cells, with permanganate and ozone, respectively, to the behaviour of intra- and extracellular cyanotoxins during treatment. In Figure 6 the application of permanganate and ozone, respectively, in combination with flocculation/filtration is compared with treatment without pre-oxidation.

Figure 6

Obviously, the application of permanganate effected in a sharp increase of dissolved cyanotoxins (= toxin release) up to 0.85 μ g/L. The reason should be a reduction of cell stability by the oxidant. No oxidative degradation of cyanotoxins could be obtained using permanganate. The decreased level of intracellular toxin was reached by flocculation/filtration, only.

In contrast to permanganate, the application of ozone yielded to both the degradation of intracellular cyanotoxins and the interim toxin release in the filter bed as overlapping effects. Although a decrease of extracellular toxins was observed in the filter effluent, the ozone dosage commonly applied for pre-oxidation was not sufficient for complete degradation of dissolved toxins. Besides the direct oxidation of toxins after ozonation the main reduction is located on the filter bed. This result indicates the complexity of the process and shows that the dose of ozone, the contact time, and the situation in the filter bed play an important role for toxin degradation.

Summarising the pilot-scale experiments it can be concluded that the hypothesis of toxin release during several conventional treatment processes deduced from laboratory-scale could be confirmed. Both flocculation/filtration and pre-oxidation give effects in the release of cyanotoxins from intra- to extracellular. Even with the application of ozone a complete degradation of cyanotoxins could not be reached.

5 Conclusions

An algae growth phase depended toxin release from cyanobacteria was obtained naturally caused from cultured cyanobacteria (*Microcystis aeruginosa*) and in an eutrophic reservoir containing *Planktothrix rubescence*.

Results from laboratory-scale tests using a cultivated *Microcystis aeruginosa* and pilotscale experiments at an eutrophic reservoir containing *Planktothrix rubescence* underline the induced toxin release during conventional water treatment. Additional to the known toxin release using pre-oxidation, it has to be pointed out that the application of flocculation/filtration also effects in toxin release under the conditions investigated, possibly caused by turbulences in pipes and pressure gradients in filters.

Following from these results it is essential to minimise toxin release during water treatment, especially, for the large number of waterworks dealing with massive cyanobacterial blooms using flocculation/filtration and disinfection.

Further systematic investigations should contribute in relation to additional treatment techniques which can guarantee the minimisation of cell destruction (e.g. membranes) and an effective removal of extracellular toxins (e.g. powdered activated carbon).

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Table 1:Induced cyanotoxin release during laboratory-scale flocculation
experiments in different growth phases of a cultivated Microcystis
aeruginosa

Induzierte Cyanotoxinfreisetzung in Laborversuchen zur Flockung in verschiedenen Wachstumsphasen einer kultivierten *Microcystis aeruginosa*

	Exponential phase			Stationary phase		
	Cells/m	MC-LR _{intra} in µg/L	MC-LR _{extra} in µg/L	Cells/m	MC-LR _{intra} in µg/L	MC-LR _{extra} in µg/L
	L			L		
Influent	268000	7.1	0.31	483000	8.1	7.9
β (Fe) = 6 mg/L	64000	n.d.	0.34	207000	n.d.	8.4
$\beta(Al) = 4 \text{ mg/L}$	15400	n.d.	0.33	80000	n.d.	8.9

MC-LR: microcystin-LR intra: intracellular extra: extracellular n.d.: not determined Table 2:Induced cyanotoxin release during laboratory-scale ozonationexperiments in different growth phases of a cultivated Microcystisaeruginosa

Induzierte Cyanotoxinfreisetzung in Laborversuchen zur Oxidation mit Ozon in verschiedenen Wachstumsphasen einer kultivierten *Microcystis aeruginosa*

	Exponential phase			Stationary phase		
	MC-LR _{total} in µg/L	MC-LR _{intra} in µg/L	MC-LR _{extra} in µg/L	MC-LR _{total} in µg/L	MC-LR _{intra} in µg/L	MC-LR _{extra} in µg/L
Influent	7.4	7.1	0.32	16	8.1	7.9
$\beta(O_3) = 0.1 \text{ mg/L}$	5.7	4.3	1.4	7.4	4.6	2.8
$\beta(O_3) = 0.2 \text{ mg/L}$	3.8	2.3	1.5	7.8	4.4	3.4

MC-LR: microcystin-LR intra: intracellular extra: extracellular

Figure captions

Fig. 1: Definition of the growth phases of a cultivated Microcystis aeruginosa selected for laboratory-scale experiments
 Darstellung der für die Laborversuche ausgewählten Wachstumsphasen der kultivierten Microcystis aeruginosa

Fig. 2:Scheme of the pilot-scale system (PAC: powdered activated carbon)Aufbau der kleintechnischen Versuchsanlage

- Fig. 3:Cyanotoxin release of a cultivated Microcystis aeruginosa (MC-LR:
microcystin-LR, intra: intracellular, extra: extracellular)Cyanotoxinfreisetzung aus einer kultivierten Microcystis aeruginosa
- Fig. 4:Cyanotoxin release of a natural bloom of *Planktothrix rubescence* in a
German reservoir (MC-RR: microcystin-RR)

Cyanotoxinfreisetzung aus einer natürlichen Blüte von *Planktothrix rubescence* in einer deutschen Talsperre

- Fig. 5: Behaviour of intra- and extracellular cyanotoxins during flocculation and filtration in pilot-scale experiments using a *Planktothrix rubescence*-containing raw water (intra: intracellular, extra: extracellular)
 Verhalten intra- und extrazellulärer Cyanotoxine bei der Flockung und Filtration in kleintechnischen Versuchen unter Verwendung eines mit *Planktothrix rubescence* belasteten Rohwassers
- Fig. 6: Behaviour of intra- and extracellular cyanotoxins during pre-oxidation with permanganate and ozone in pilot-scale experiments using a *Planktothrix rubescence*-containing raw water (intra: intracellular, extra: extracellular)

Verhalten intra- und extrazellulärer Cyanotoxine bei der Voroxidation mit Kaliumpermanganat und Ozon in kleintechnischen Versuchen unter Verwendung eines mit *Planktothrix rubescence* belasteten Rohwassers

(Figure 1)



(Figure 2)



(Figure 3)



(Figure 4)



(Figure 5)



(Figure 6)

