Managing the Risk of Emerging Algae Toxins in Drinking Water: Development of a Targeted Analytical Approach


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Abstract: The objective of the study was to identify appropriate analytical scenarios to assess the release of emerging toxins in water resources and manage the risk for drinking water. A comprehensive toolbox was developed for the monitoring of cyanobacteria and cyanotoxins in ten water resources used for drinking water production and known for regular algae blooms. Six toxins - microcystins, cylindrospermopsin, anatoxins, nodularin, saxitoxin and BMAA - were targeted. High performance HPLC-MS/MS methods were implemented in parallel with rapid kits and a real-time PCR method was developed to identify specifically the microcystin producing species. Those analytical methods were found in good agreement and very sensitive and selective.

Low levels of eutrophication were obtained during those campaigns leading to limited occurrence of toxins. Results obtained on one site are detailed in this paper.

Finally, recommendations could be drawn to manage the risk related to various emerging algae toxins, based on the use of a phycocyanin probe on-site as early-warning system. When a threshold value is reached, treatment and monitoring strategies are implemented in parallel.

Keywords: cyanobacteria; cyanotoxins; PCR; drinking water; phycocyanin; monitoring freshwater

INTRODUCTION
Cyanobacteria are present in all ecosystems under various forms and sizes and tropical species of algae have started to appear in Europe (Brient 2008-1, Cadel-Six 2007). They present a potential health risk through the production of toxins that can be released into natural waters. Among the more than 2000 cyanobacterial species, many produce toxins including neurotoxins and hepatotoxins, which have often caused massive animal deaths and more rarely, human deaths.

As a consequence, water professionals have to manage the health risks associated with the occurrence of algae in waters intended for recreational use (bathing) or drinking water production (Baudin 2007, Chorus 1999, Carmichael 2001, Knappe 2004). Regulations concerning algae toxins are rapidly evolving worldwide. While MC-LR is the most commonly regulated for drinking water, Maximum Contaminant Levels (MCL) have appeared for other microcystins, anatoxin-a, cylindrospermopsin, nodularin or saxitoxin. Appropriate and complementary analytical tools are needed to provide a relevant characterization of the resources, which is a pre-requisite for operational risk management. Those include alert tools which can help operators to anticipate algae blooms, avoid operation failure and guarantee safe drinking water production, as well as high performance laboratory methods which provide precise levels of contamination.

METHODOLOGY
Monitoring and analytical strategy
Six toxins considered as a priority for operators were targeted: microcystins (7 variants), cylindrospermopsin, anatoxins, nodularin, saxitoxins and BMAA (Metcalf 2008). Monitoring campaigns were performed in ten drinking water treatment sites over 4 summer months, following the analytical strategy summarized in figure 1 and based on a large panel of analyses.

The raw water samples were usually collected at a treatment plant intake, and an algal biomass sample (“algae scum”) was collected with a plankton net in the surface water at a place where the bloom was most visible. ELISA tests for cylindrospermopsin, microcystins and saxitoxins were systematically carried out on scum samples. Whenever the number of
cyanobacteria at the raw water intake exceeded 200 cells/ml, quantitative PCR targeting Microcystis aeruginosa toxin producing genes was carried out. When the number of cyanobacteria in the raw water exceeded 2000 cells/ml, samples were selected for analysis of microcystins and nodularin, cylindrospermopsin and anatoxin A using two different HPLC-MS/MS methods and treated water was tested as well. Approximately ten samples were selected on the basis of the dominant algal species for analysis of BMAA (results not discussed in this paper) by HPLC-fluorescence.

**Figure 1:** Analytical strategy followed during the campaigns.

Rapid monitoring
In freshwaters, cyanobacteria are the only microorganisms to produce significant quantities of phycocyanin (PC) and its derivative allophycocyanin. This specific pigment can be monitored on-line using dedicated probes (Brient et al, 2008). The TriOS microFlu-blue sensor used in this study was a submersible and miniaturized fluorometer (diameter 48 mm and length 200 mm), equipped with a red diode provided with a filter, having an excitation wavelength of 620 nm, an emission wavelength of 655 nm and a band-width of 10 nm. More details on the...
functioning of the probe are available on the web site: www.trios.de/. The linearity of the probe signal was tested with a phycocyanin commercial extract over the range 0 to 200 µg/L.

Microcystins, nodularins, cylindrospermopsins and saxitoxins (intra and extra cellular) were analysed using the ELISA test kit from Abraxis Bioscience. This test is an immunoassay for the quantitative and sensitive congener-independent detection of these different cyanotoxins in raw water samples.

Microcystins were also analysed by strip tests from Abraxis and Agdia with different limits of quantification: 1 µg/l for tap water (LOD >0,5 µg/l) and three detection levels of 2, 5, and 10 µg/l for raw waters. With these strip tests a microcystins antibody binds to the microcystins, producing a color line different from a control line. Where all the MC antibody is bound to MC in a sample no line will form indicating a positive result.

**Specific chemical and biological analyses**

*Microcystins and Nodularin*: seven microcystins (LR, YR, RR, LA, LY, LW and LF) and nodularin were quantified in the dissolved phase using a protocol adapted from the ISO 20179:2005 standard protocol. 500 ml of filtered water were extracted on C18 cartridges (JT Baker, 6 ml, 500 mg). The HPLC column used for separation of the 8 toxins was a Waters Atlantis T3 (2.1*150 mm, 3 µm pore size). The mobile phase consisted of water+0,05% formic acid (A) and acetonitrile+0,05% formic acid (B). The gradient was started with 75% (A) held for 1 min and ended with 100% (B) after 15 min with final hold for 2 min. The instrument used was a triple stage quadrupole (TSQuantum AM from ThermoFinnigan) used in ESI positive mode.

Two transitions were monitored for each analyte. Limits of quantification over the whole procedure (LOQ) ranged from 4.5 ng/l (MC LY) to 7.8 ng/l (MC RR). Recoveries determined by spiking a river water ranged between 60 and 100% for all toxins. The analysis of particulate toxins retained by filtration involved freezing/thawing of the sample, contacting the filter with 1 ml of a mixture of water/methanol 20/80 (V/V%) in a 15 ml Falcon tube under sonication during 30 min. Filters were then left in contact with the solvent during 24 h at 4°C, followed by 10 min sonication. The supernatant was transferred into 2 ml Eppendorf tubes, centrifuged at 10,000 rpm for 15 min and the supernatant collected in a 2 ml vial before HPLC-MS/MS analysis.

*Cylindrospermopsin and anatoxin A*: these two toxins are too polar to be extracted by solid-phase extraction. Hence, a direct aqueous injection method in HPLC-MS/MS (positive electrospary mode) was used, as previously reported by Bogialli et al (2006) for anatoxin A. Separation was carried out using the same Atlantis T3 C18 column used for microcystins. The mobile phase consisted of HPLC water+0,1% formic acid (A) and methanol (B). The gradient started with 95% (A) and ended with 80% B after 10 min, held for 3 min. Cylindrospermopsin eluted after 8.4 min, followed by anatoxin A at 8.94 min. Two transitions were monitored for cylindrospermopsin (from 466 parent ion to daughter ions at 194 and 336 amu) while three (from 166 parent ion to daughter ions at 91, 131, 149 amu) were monitored for anatoxin A. The method proved linear between 0.1 and 20 µg/l and spiking experiments in real raw water samples did not induce any shift in retention time or any ion suppression.

*Detection of mcy genotypes in Microcystis population by duplex real-time PCR*

A known volume of water samples (from 100ml to 1 L) was filtered through 0,5µm polycarbonate filter and stored at −20°C until analysis. Before DNA extraction using QiaAmp DNA mini kit (Qiagen), the filter was sonicated during 10 minutes with a Branson 3510 bath (20 Hz). In the final step, DNA was eluted in 100 µl of molecular biology-grade water and stored at −20°C until amplification.

In the duplex real-time PCR, two target gene regions were amplified: the intergenic spacer region within the phycocyanin (PC) operon and the mcyB region, which carries out one step in
microcystin biosynthesis (Tillett et al 2000). The used primers and probes sequences as well the amplification reaction conditions are those previously described by Kurmayer and Kutzenberger (2003) and Briand et al (2009). DNA extracted from raw water samples was systemically ten-fold diluted and 5 µl from this dilution were amplified in a final PCR volume of 50 µl. For each sample, the potential PCR inhibition was controlled by adding, in a supplementary PCR tube, a known amount of DNA extracted from pure culture of Microcystis aeruginosa PCC 7806 (final amount 5 ng per reaction mix) to the DNA sample. No PCR inhibition was observed if the difference between the Ct measured in the inhibition control of the respective sample and the Ct measured with M. aeruginosa DNA alone was less than 0.5 cycle. In case of evidence for PCR inhibition, real-time PCR assay was redone in presence of T4 gene 32 protein (Roche) as described by Kreader (1996).

RESULTS AND DISCUSSION:

Validation of Phycocyanin probe (all sites)
The two graphs below (Figure 2) plot the concentration of phycocyanin as a function of algal biomass (left) and biovolume (right) measured at all sites during all monitoring campaigns.

Figure 2: Phycocyanin concentration as a function of algal biomass (left) and biovolume (right).

With $R^2$ coefficients equal to 0.83, these graphics show that phycocyanin concentration is well correlated with the number of cyanobacterial cells and the algal biovolume, and hence indicate the relevance of using the phycocyanin pigment as a marker for the monitoring of cyanobacterial biomass. Therefore this probe can be used as an early warning tool upstream of drinking water plants; exceeding a predefined phycocyanin level can trigger complementary analytical methods (counting/identification/detection/quantification of toxins). This probe allows to anticipate the onset of algal blooms in the resource and take appropriate actions at the treatment plant level.

Validation of the PCR method
The linearity of the duplex real-time PCR signals was first checked by spiking known amounts of Microcystis aeruginosa cells PCC 7806 (as enumerated by microscopy) in 1 L of ultrapure sterile water (from $10^3$ to $10^6$ cells/L), as shown in figure 3. For pure water, no dilution of the DNA extract was used, whereas dilution of at least 10 fold factor was needed for surface water samples. As only 100 ml of raw water samples were usually filtered on polycarbonate membrane, the detection limit for this method validated on surface water was of $10^5$ cells/L (100 cells/ml) considering the ten-fold dilution on the DNA extract. In the case study presented in this paper (hereafter), 5 samples over the eight samples analyzed by PCR required the addition of T4 gene 32 protein in order to remove all PCR inhibition and keep the same sensitivity.
Figure 3: Linearity of the relationship between the real-time Ct for PC and mcy genes and the initial concentration of *Microcystis aeruginosa* PCC 7806 in water samples.

**Case study at a specific site**

This study site located near the Loire valley in France is a reservoir with high level of eutrophication (DOC around 8 mg/l) and yearly algal blooms lasting for several months. This reservoir water is used for the production of drinking water by clarification followed by first stage GAC filtration, ozonation and chlorine disinfection.

Figure 4: Evolution of algal biomass, chlorophyll a and phycocyanin at the study site

Figure 4 shows the evolution of algal biomass (cyanobacteria and other algae), of chlorophyll a and phycocyanin (as measured by the probe) at this site. The number of algae, primarily blue-green, peaked at 300,000 cells/ml during the month of June. This was followed by a drastic decrease in July (although the number of algae always remained above 2000 cell/ml), during which period the fluoroprobe was set up. The probe signal started to increase in early August at the start of a new bloom period. The probe signal (µg/l of...
phycocyanin) peaked during mid-september when the number of cyanobacteria reached a new maximum near 250,000 cells/ml. Overall the probe signal correctly detected the onset, maximum, then subsequent decrease of the cyanobacterial bloom while the level of chlorophyll a (measured with a second probe) remained approximately constant as the bloom was entirely dominated by blue-green algae. The distribution of the various species of cyanobacteria also shows significant variations over time (see figure 5), with Aphanizomenon, Planktothrix, Woronichinia, and Oscillatoria as the dominant species. Even though the dominant species were not microcystin producers in a few samples, microcystin was always present, showing that even a low occurrence of producing species can lead to detectable levels of MC.

![Figure 5: Distribution of the various species of cyanobacteria at the study site.](image)

**Results from toxin detection/quantification methods**

Globally, concentrations of toxins were rather low, despite significant algae contents in a few samples. The highest concentration of MC was observed on the first sampling day and reached 150 ng/l at this site and a few hundreds ng/L at other sites (results not shown). A semi-quantitative comparison of the various analytical methods for microcystin (MC) is shown in table 1. PCR and HPLC-MS/MS methods appear as the most sensitive, as expected given their detection limits: microcystins are always detected, whereas the other methods showed negative results in some samples. The ELISA kit was able to detect the positive event of June when MC LR reached 79 ng/l. On the other hand it did not detect the September 14 event when MC LR was found at 30 ng/l by HPLC-MS/MS, in excess of its theoretical limit of detection (10 ng/l), indicating a possible antagonistic effect. Results from HPLC-UV always remained below the 0.2 µg/l limit of detection of this method for MC, making it of little use as an early warning tool.

**Table 1: Semi-quantitative comparison of the various analytical methods for microcystin**

<table>
<thead>
<tr>
<th>Microscopy</th>
<th>M. aeruginosa</th>
<th>PCR mcy</th>
<th>Strip kit</th>
<th>ELISA kit</th>
<th>HPLC-MS MS intracellular MC</th>
<th>HPLC-MS MS extracellular MC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microscopy</td>
<td>LQ: 100 cell/mL</td>
<td>1/7</td>
<td>1/7</td>
<td>3/6</td>
<td>3/7</td>
<td>7/7</td>
</tr>
<tr>
<td>PCR</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Strip kit</td>
<td>Microcystin +</td>
<td>LQ: 0.5 µg/L</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>ELISA kit</td>
<td>Microcystin +</td>
<td>LQ: 0.01 µg (equ.LR)/L</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>HPLC-MS MS</td>
<td>intracellular MC</td>
<td>0.005 - 0.01 µg/L</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>extracellular MC</td>
<td>dep. variant</td>
<td>-</td>
<td>-</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

NA: not available

HPLC-MS/MS method enabled to show that microcystin LR was the major variant; only one sample displayed the presence of other ones (see table 2).
Table 2: Concentrations of the individual variants of microcystins, measured by HPLC-MS/MS (no analysis on 6/7).

<table>
<thead>
<tr>
<th>Concentrations (ng/L)</th>
<th>22/6</th>
<th>6/7</th>
<th>20/7</th>
<th>3/8</th>
<th>17/8</th>
<th>31/8</th>
<th>14/9</th>
<th>28/9</th>
</tr>
</thead>
<tbody>
<tr>
<td>microcystin RR</td>
<td>55</td>
<td>&lt;LD</td>
<td>&lt;7,8</td>
<td>&lt;7,8</td>
<td>&lt;7,8</td>
<td>&lt;7,8</td>
<td>&lt;7,8</td>
<td>&lt;7,8</td>
</tr>
<tr>
<td>nodularin</td>
<td>&lt;LD</td>
<td>&lt;LD</td>
<td>&lt;LD</td>
<td>&lt;LD</td>
<td>&lt;LD</td>
<td>&lt;LD</td>
<td>&lt;LD</td>
<td>&lt;LD</td>
</tr>
<tr>
<td>microcystin YR</td>
<td>17</td>
<td>&lt;LD</td>
<td>&lt;6,4</td>
<td>&lt;LD</td>
<td>&lt;LD</td>
<td>&lt;6,4</td>
<td>&lt;6,4</td>
<td>&lt;6,4</td>
</tr>
<tr>
<td>microcystin LR</td>
<td>79</td>
<td>8</td>
<td>13</td>
<td>14</td>
<td>6</td>
<td>31</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>microcystin LA</td>
<td>&lt;LD</td>
<td>&lt;LD</td>
<td>&lt;LD</td>
<td>&lt;LD</td>
<td>&lt;LD</td>
<td>&lt;LD</td>
<td>&lt;LD</td>
<td>&lt;LD</td>
</tr>
<tr>
<td>microcystin LY</td>
<td>&lt;LD</td>
<td>&lt;LD</td>
<td>&lt;LD</td>
<td>&lt;LD</td>
<td>&lt;LD</td>
<td>&lt;LD</td>
<td>&lt;LD</td>
<td>&lt;LD</td>
</tr>
<tr>
<td>microcystin LW</td>
<td>&lt;LD</td>
<td>&lt;LD</td>
<td>&lt;LD</td>
<td>&lt;LD</td>
<td>&lt;LD</td>
<td>&lt;LD</td>
<td>&lt;LD</td>
<td>&lt;LD</td>
</tr>
<tr>
<td>microcystin LF</td>
<td>&lt;LD</td>
<td>&lt;LD</td>
<td>&lt;LD</td>
<td>&lt;LD</td>
<td>&lt;LD</td>
<td>&lt;LD</td>
<td>&lt;LD</td>
<td>&lt;LD</td>
</tr>
<tr>
<td>Total MC</td>
<td>151</td>
<td>8</td>
<td>13</td>
<td>14</td>
<td>6</td>
<td>31</td>
<td>9</td>
<td></td>
</tr>
</tbody>
</table>

Except for cylindrospermopsin, which was detected only once in the algae scum (but never in the plain raw water samples), no other toxin was ever found. Although the results for total microcystins always remained well below the 1 µg/L WHO guideline, the constant presence of MC LR indicates that this particular water could have the potential, under favorable ecological conditions, to produce problematic levels of microcysts. This water hence deserves permanent monitoring with sensitive and rapid methods. Because of its high sensitivity and good rapidity, PCR could be used in combination with an ELISA kit, provided complementary methods are developed to detect the toxin-producing genes from Planktothrix agardhii and Aphanizomenon that appear as the major MC producing species at this site. HPLC-MS/MS would remain the method of choice for the confirmation of toxins identity and concentration and could be triggered above a given detection level by ELISA (possibly 0.3 µg/l). On the other hand, the interest of the strip test as an early warning tool for microcystins, seems dubious.

CONCLUSION

The data showed rather low levels of cyanobacteria in most raw waters, the occurrence of microcysts above 0.2 µg/L in only 4 of the 10 sites investigated and that of cylindrospermopsin in 2 sites. It was interesting to observe that microcystins could be found even with low levels of cyanobacteria (lower than 5000 cells/mL). No saxitoxin, nodularin, BMAA or anatoxin A were ever found.

The results shown in this paper for one selected study site allow to draw preliminary recommendations for monitoring strategies. A complementary set of analytical tools rather than a single method is necessary to take adequate actions during algal bloom situations. The phycocyanin parameter enables to anticipate the risks incurred during contamination of water bodies by cyanobacteria by detecting the onset of cyanobacterial blooms. The ELISA test kit provides a second alarm level that can be used to trigger quantitative analysis of toxins by HPLC. HPLC-MS/MS appears as a much more powerful tool than the standard HPLC-UV method, owing to its superior sensitivity and identification capability. The strip test kits are not sensitive enough to serve as early-warning tools. The PCR method implemented that was developed to specifically detect the presence of toxin-producing genes from Microcystis aeruginosa proved very sensitive and very well correlated with HPLC-MS/MS results. It is interesting that this method showed positive results even though Microcystis aeruginosa was not detected by classical microscopic methods and that microcystins were always detected even when known producers were not found at significant levels (see sample 20/7). To be fully operational, this approach should be completed by other PCR tests capable of identifying the main producers known to occur at a given site.

Finally, no toxins were ever detected in the treated water from the investigated site indicating the adequacy of the treatment line comprising both ozonation and GAC filtration.
REFERENCES


Carmichael WW et al 2001 Assessment of blue-green algal toxins in raw and finished drinking water. AWWARF Ed. 90815, 208 pp.


