Short Communication

First Occurrence of Cylindrospermopsin in Freshwater in France

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ABSTRACT: Eleven waterbodies in Western France dominated by cyanobacteria of the genera Aphanizomenon and Anabaena were analyzed in September 2006 for microcystins (MC) and cylindrospermopsin (CYN). CYN was detected for the first time in France in four of them in the presence of Aphanizomenon flos-aquae and in the presence of Anabaena planctonica in the other. The intracellular concentrations of CYN measured by LC-MS/MS ranged between 1.55 and 1.95 μg/L. The occurrence of CYN represents an additional health hazard to MC especially because Aphanizomenon flos-aquae is the third most common species in freshwaters in France.

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Keywords: cylindrospermopsin; Aphanizomenon flos-aquae; Anabaena sp.; drinking and recreational waters

INTRODUCTION

Massive proliferations of cyanobacteria are observed in many waterbodies on all continents as a consequence of increasing eutrophication (Whitton et al., 2000). Cyanobacteria are potent producers of different types of toxins such as neurotoxins, hepatotoxins, and dermatotoxins (Chorus and Bartram, 1999). Although the characterization of these toxins is well documented, only the group of microcystins (MC) is routinely analyzed in France during monitoring of recreational and drinking waters. In Brittany, the presence of toxic cyanobacteria in freshwater has been documented since 1995 (Vezie et al., 1997; Brient et al., 2008) with the presence of MC in nearly 65% of the sampled sites.

Recently, other cyanotoxins have also been detected in France: anatoxin-a and homoanatoxin-a from benthic cyanobacteria have been found in association with dog poison-
The two genera *Anabaena* in Germany (Preubel et al., 2005) and *Aphanizomenon* in Europe producing CYN, recent studies highlighted *Cylindrospermopsis raciborskii* (Stuken et al., 2006). Among the other species are also *Anabaena bergii* in Israel (Banker et al., 1997; Schaw et al., 1999); *Anabaena lapponica* in Finland (Spoof et al., 2006); *Raphidiopsis curvata* in China (Li et al., 2001); *Lyngbia wollei* in Australia (Schembri et al., 2001); *Anabaena curvispira* in Japan (Harada et al., 1994); and *Lyngbia wolfei* in Australia (Seifert et al., 2007).

Among these species, *Cylindrospermopsis raciborskii* has been detected in a reservoir in France (Briand et al., 2004) but did not produce CYN. It is important to bear in mind the small number of taxonomists in France who can identify this species taking into account that it has several synonyms in particular that of *Anabaena* or *Anabaenopsis raciborskii* (Stuken et al., 2006). Among the other species in Europe producing CYN, recent studies highlighted *Anabaena lapponica* in Finland (Spoof et al., 2006) and *Aphanizomenon flos-aquae* in Germany (Preubel et al., 2006); *Aphanizomenon ovalisporum* in Israel (Banker et al., 1997; Schaw et al., 1999); *Anabaena bergii* in Australia (Schembri et al., 2001); *Anabaena lapponica* in Finland (Spoof et al., 2006); *Raphidiopsis curvata* in China (Li et al., 2001); *Umezakia natans* in Japan (Harada et al., 1994); and *Lyngbia wolfei* in Australia (Seifert et al., 2007).

Since 2004, phytoplankton analyses, identification, and counting have been carried out weekly between May and October and thus allow to trace the presence of these two genera. Lakes Ribou (Cholet), La Dathée (Vire), and Etang au Duc (Plœrmel) are used for recreational activities and the production of drinking water. The other eight waterbodies are used for recreational activities only: Grand Lieu (Bouaye), Martigné Ferchaud, Marcillé Robert, Le Pertre, Apigné (Rennes), Vern sur Seiche, Boulet (Feins), and Chevreux. Their water volumes are all lower than 3 millions m$^3$ during the summer and their mean depth is less than 3 m.

The samples were carried out using a 1 L tube extending over the top meter of the water column in the swimming area.

### Analytical Methods

The fresh phytoplankton material was analyzed within 48 h of sampling. The counting of the cells was carried out with a Nageotte cell whose volume is 50 μL after concentration on a polycarbonate filter of 1 μm. Less than 200 mL of sample is filtered. The filter is washed with 1 mL of the original solution and aspirated with a pipette and injected in the Nageotte cell. This cell is divided into 40 bands and the number of bands observed is defined when at least 40 colony or filaments are counted (Brient et al., 2008).

MC concentrations were determined with a HPLC with diode array detection (HPLC-DAD) and a variable-wavelength UV detector operating at 238 nm. Samples were harvested by filtration and filters were suspended in 1 mL of 85% methanol in water and centrifuged at 7000 g for 7 min. The separation was performed on a Microspher C18 reverse-phase column (3 μm) under isocratic conditions at a flow rate of 1 mL/min in a mobile phase of 10 mm ammonium acetate and acetonitrile (7.4:2.6) for 20 min. As MC-LR was the standard used, concentration was expressed as μg MC-LR/L and μg Eq MC-LR/L for other MC.

For CYN analysis, a subsample of 200 mL was filtered on 1 μm glass fiber filter and dried to 40°C. The determination of CYN was performed by LC-MS/MS as described in details previously (Fastner et al., 2007). This analysis corresponded to intracellular CYN only in this preliminary investigation.

### RESULTS

CYN was found in six of the 11 waterbodies sampled. (Table 1) Characteristic ion chromatograms from standard CYN and of a sample from Boulet are shown in Figure 1. Among the genera potentially producing CYN, the species identified in the corresponding toxic samples were the...
following: *Aphanizomenon flos-aquae, Aphanizomenon gracile, Aphanizomenon issatchenki*, (called now *Cuspido-thrix issatchenki*), *Anabaena planctonica*, and *Anabaena spiroides*.

*Aphanizomenon flos-aquae* was present in four of them as the dominant species in Ribou with a biomass of 85,600 cells/mL and La Datheé with a biomass of 82,000 cells/mL.

For two other sites *Aphanizomenon flos-aquae* was present but nondominant, with 5000 cells/mL in Vern/Seiche and 16,000 cells/mL in Marcillé Robert. The pond at Le Pertre indicated traces of CYN in the presence of *Aphanizomenon gracile*, whereas there was neither *Aphanizomenon flos-aquae* in the September sample nor in any of the samples covering the period May to October. The site of Boulet

<table>
<thead>
<tr>
<th>Sites</th>
<th>Ribou</th>
<th>La Datheé</th>
<th>Vern/Seiche</th>
<th>Boulet</th>
<th>Marcillé Robert</th>
<th>Le Pertre</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYN (µg/L)</td>
<td>1.65</td>
<td>1.55</td>
<td>1.88</td>
<td>1.95</td>
<td>Traces&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Traces&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Dominant species</td>
<td><em>Planktothrix agardhii</em></td>
<td><em>Woronichinia</em></td>
<td><em>Anabaena planctonica</em></td>
<td><em>Anabaena planctonica</em></td>
<td><em>Planktothrix agardhii</em></td>
<td><em>Anabaena spiroides</em></td>
</tr>
<tr>
<td>Second most dominant species</td>
<td><em>Aphanizomenon flos-aquae</em></td>
<td><em>Microcystis aeruginosa</em></td>
<td><em>Aphanocapsa sp.</em></td>
<td><em>Anabaena spiroides</em></td>
<td><em>Oscillatoria sp.</em></td>
<td><em>Aphanizomenon flos-aquae</em></td>
</tr>
<tr>
<td>Third most dominant species</td>
<td><em>Aphanizomenon Issatchenki</em></td>
<td><em>Aphanizomenon flos-aquae</em></td>
<td><em>Anabaena planctonica</em></td>
<td><em>Oscillatoria sp.</em></td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Total cyanobacteria (cell/mL)</td>
<td>404,000</td>
<td>561,000</td>
<td>48,000</td>
<td>84,000</td>
<td>593,000</td>
<td>160,800</td>
</tr>
<tr>
<td>Dominant species (cell/mL)</td>
<td>266,600</td>
<td>367,000</td>
<td>17,000</td>
<td>38,500</td>
<td>373,000</td>
<td>100,800</td>
</tr>
<tr>
<td>Second most dominant species (cell/mL)</td>
<td>85,600</td>
<td>100,000</td>
<td>16,000</td>
<td>21,100</td>
<td>192,000</td>
<td>60,000</td>
</tr>
<tr>
<td>Third most dominant species (cell/mL)</td>
<td>22,400</td>
<td>82,000</td>
<td>5200</td>
<td>12,000</td>
<td>16,000</td>
<td>–</td>
</tr>
</tbody>
</table>

<sup>a</sup>Traces: < limit of quantification (0.01 µg/L).

Fig. 1. Reconstructed ion chromatograms of a cylindrospermopsin standard (retention time 4.50 min) and a sample from Lake Boulet (Feins) with the MRM transitions of m/z 416 > 194 and m/z 416 > 176 characteristic for cylindrospermopsin. Chromatographic conditions were as in Materials and Methods.
indicated the existence of CYN in the presence of *Anabaena planctonica* (38,000 cells/mL), although there were no other species of *Aphanizomenon* or *Anabaena*.

The three sampling sites at Apigné and Chevreux were characterized by the absence of *Aphanizomenon flos-aquae* and *Anabaena planctonica* but by the presence of the species *Aphanizomenon aphanizomenoides*, *Aphanizomenon issatchenki*, and *Aphanizomenon gracile* without detection of CYN. The remaining two sites at Martigné-Ferchaud and Etang au Duc indicated the presence of *Aphanizomenon flos-aquae* without CYN.

The 11 waterbodies studied for intracellular CYN show the presence of this cyanotoxin with concentrations varying from traces to 1.95 μg/L for biomasses of potential producers from 16,000 cells/mL to 85,000 cells/mL.

It is also interesting to note that the two hepatotoxic types of cyanotoxins, CYN and MC, were present at the same time in four waterbodies: Le Pertre, Marcillé Robert, Ribou, and La Dathée (Table II).

Three other waterbodies Grand Lieu, Apigné, and Martigné-Ferchaud contained cyanobacteria producing MC but not CYN.

**DISCUSSION**

In this preliminary study, we highlighted the possible role of *Aphanizomenon flos-aquae* and *Anabaena planctonica*, as CYN producers on the waterbodies Ribou and Dathée, Vern/Seiche, and Boulet. This hypothesis is in agreement with recent publications in Europe where the two species were reported in the presence of CYN (Preubel et al., 2006; Spoof et al., 2006). The toxin concentrations found in this study are also similar to those reported for *Cylindrospermopsis raciborskii*, namely 1 μg/L of CYN with 20,000 cells/mL (McGregor and Fabbro, 2000). The presence of traces of CYN in Le Pertre may be explained by the presence of *Aphanizomenon gracile* as this species is a suspected CYN producer in Germany (Rücker et al., 2007).

This investigation highlights that in France waterbodies used for human activities should be monitored for cylindrospermopsins, as well as for the routinely monitored MC, in the presence of species of *Aphanizomenon* and *Anabaena* genera. This is of special importance as *Aphanizomenon flos-aquae* is the third most dominant species after *Planktothrix agardhii* and *Microcystis* sp. in France (AFSSA, 2006).

The maximum concentration of CYN allowed in France for drinking water is 0.3 μg/L (AFSSA, 2006). Threshold values relate to the intracellular fraction of CYN but should also considered extracellular dissolved fraction. Indeed, several recent studies have demonstrated the predominance of dissolved CYN of around 80% of total CYN in the presence of *Cylindrospermopsis raciborskii*, *Aphanizomenon ovalisporum*, and *Aphanizomenon* sp. (Schaw et al., 1999; Rücker et al., 2007). This study has exclusively considered intracellular CYN and thus most probably underestimated the concentration of total CYN in these shallow lakes. It is worth noting that the treatment of drinking water does not satisfactorily remove CYN (Falconer and Humphage, 2006).

In the absence of a global toxicity method which would identify each type of toxins, cyanobacteria monitoring in France should include testing for MC and for CYN. However, to reduce costs, testing for CYN could be preferentially conducted in the presence of *Anabaena* and *Aphanizomenon* genera. Indeed, the determination of the species themselves of these genera is not easy (Komarek and Kovacic, 1989). *Aphanizomenon flos-aquae* is relatively simple to identify by the shape of its colony but this is not the case for the *Anabaena* genus and other species of *Aphanizomenon* by the shape of their solitary filaments (Hindák, 2000; Stüken et al., 2006). Moreover, the distinction between *Anabaena* and *Aphanizomenon* is also criticized because the equivalent phenotypes for certain species justify the existence of only one genus.

CYN is an alkaloid which is difficult to detect when it is enzymatically-bound within the cells of animals or plants (Duy et al., 2000). Very few studies exist on the impacts of CYN on primary producers and their transfer through the foodweb (White et al., 2005) and as a protein synthesis-inhibitor on plants (Metcalf et al., 2004; Kinear et al., 2007). The inhibiting effects of CYN on the metabolism of *Sinapis* mustard seedlings have been demonstrated with 50% reduction in growth (Vasas et al., 2002) and of bioaccumulation in an aquatic macrophyte having inhibiting effects (White et al., 2005). The relative impact of CYN on different aquatic organisms is not known although for MC the ingestion of toxic cyanobacterial cells was found more toxic than when they were exposed to the soluble form (Lance et al., 2006). CYN and MC are two hepatotoxins with different synthesis pathways whose combined effect are unknown (Falconer, 2005). Moreover unlike MC which kill mice relatively quickly within 1–2 h after a single dose, CYN toxic effect on hepatocytes is delayed and progressive, causing death usually within 24–120 h in mice (Hawinks et al., 1985; Runnegar et al., 1994).

The presence of CYN in waterbodies reinforce the need to ban copper sulfate in reservoirs used for drinking water as the inhibition of degradation of soluble CYN has been reported (Smith et al., 2008). Considering that CYN is abundant in the extracellular fraction and that it can persist in the water for weeks without degradation in some settings.

**TABLE II. Distribution of CYN and MC from waterbodies**

<table>
<thead>
<tr>
<th>Water Bodies</th>
<th>Le Pertre</th>
<th>Marcillé Robert</th>
<th>Ribou</th>
<th>La Dathée</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYN (μg/L)</td>
<td>Traces</td>
<td>&lt;0.2</td>
<td>0.72</td>
<td>0.43</td>
</tr>
<tr>
<td>MC LR (μg/L)</td>
<td>0.72</td>
<td>&lt;0.2</td>
<td>1.65</td>
<td>1.78</td>
</tr>
<tr>
<td>Total equi MC-LR (μg/L)</td>
<td>0.72</td>
<td>0.43</td>
<td>1.78</td>
<td>1.67</td>
</tr>
</tbody>
</table>

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(Wormer et al., 2008), the monitoring of CYN must be regarded as being highly recommended.

The presence of two hepatotoxins MC and CYN at the same time in waterbodies (Table II) confirms the need for the management of water used for drinking or recreational purposes with monitoring measures base on cells numbers (Griffiths et al., 2003) and MC concentrations, not exclusively.

In conclusion, it is necessary to better understand the mechanisms of CYN excretion and the possible influences of environmental conditions for its production. The results of this investigation also show that CYN and MC can be present together in waterbodies demonstrating the need for carrying out studies on the combined effects of these hepatotoxins at the ecological level and on human health.

REFERENCES


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