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ALGAL BLOOMS: EMERGING PROBLEM FOR HEALTH AND SUSTAINABLE USE OF SURFACE WATERS (B-BLOOMS)

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PART 2

GLOBAL CHANGE, ECOSYSTEMS AND BIODIVERSITY

- ATMOSPHERE AND CLIMATE
- MARINE ECOSYSTEMS AND BIODIVERSITY
- **TERRESTRIAL ECOSYSTEMS AND BIODIVERSITY**
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ALGAL BLOOMS: EMERGING PROBLEM FOR HEALTH AND SUSTAINABLE USE OF SURFACE WATERS (B-BLOOMS)

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Part 2:
Global change, Ecosystems and Biodiversity

FINAL REPORT

Algal blooms: emerging problem for health and sustainable use of surface waters (B-BLOOMS)

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ABSTRACT

The BBLOOMS project was a two-year program, which was primarily proposed to make a first assessment of the extent of present cyanobacterial blooms in Belgium and of the potential threat for the surface water resources. In this study, we addressed several aspects: (i) the extent and phenology of nuisance blooms in multiple-use Belgian surface waters, (ii) the taxonomic diversity of bloom-forming cyanobacteria, using traditional and genetic tools, (iii) the use of genetic markers to estimate whether the organisms are potentially toxic, (iv) the measurement of toxin concentration in field samples, and (v) the relationship between environmental variables and nuisance blooms in selected water bodies. Most field samples came from 4 reference lakes (Blaarmeersen lake in Flanders and three pre-dam lakes of Eau d'Heure in Wallonia) that were monitored intensively for 2 years. Twenty-three samples were taken for Blaarmeersen, in a continuous manner. Seventy-three samples were studied for the Eau d'Heure complex, but only when proliferations were observed. Additional samples were provided by summer samplings in a series of small lakes in Flanders carried out by the University of Gent. As this was insufficient to obtain a global view of the phenomenon, we have built BLOOMNET, a network of water managers and users, who received information about cyanobacterial blooms and how to collect them for subsequent analysis. The network contributed about forty samples from all regions.

This first study of algal blooms in Belgium has shown that the phenomenon was quite common when meteorological and environmental conditions were favourable.

The monitoring of the reference lakes provided the environmental and biological data to assess the feasibility of developing predictive models of cyanobacterial blooms. Environmental conditions favouring cyanobacteria were systematically high temperature, high pH and stable stratification, but potentially toxic taxa required high dissolved phosphorus, with relatively low inorganic nitrogen. Number of days without strong winds was also a factor favouring cyanobacteria blooms, as was the abundance of the larger filter-feeders (*Daphnia*, cyclopoid copepods) in the zooplankton. The prediction of total cyanobacteria biomass using Artificial Neural Networks (ANN) gave good agreement between predicted and observed values (R^2 = 0.95). The best predictive variables were total nitrogen and total phosphorus, along with the meteorological variables. This implies that key conditions for bloom prediction can be monitored relatively easily using weather stations and water sensors connected to data loggers.

In total, 93 BLOOMNET bloom samples from Flanders, Wallonia and Brussels were obtained, with 81 coming from Flanders (69 different waterbodies). The bloom samples were obtained from March till October. The toxigenicity tests by amplification

of *mcyB/E* genes appeared adequate to select samples that could be submitted to an analytical measurement of microcystins by HPLC. When 12 samples with one or none of the *mcy* genes were tested, none gave detectable concentrations of microcystins. In contrast, half of the samples selected with both *mcy* genes indeed contained measurable amounts of microcystins in the conditions used. In total, seventy-nine percent of the Flemish BLOOMNET samples tested by PCR harboured toxigenic taxa (double positives for the *mcyB/E* genes), and 90% for the Walloon BLOOMNET samples (11 samples tested). Due to technical problems, measurements of microcystins by a sub-contractor were restricted to a limited number of samples, and mainly carried out on lyophilized biomass. Of the 46 Flemish BLOOMNET samples positive for the two *mcy* genes, 54% actually contained total microcystins, in a range of concentration from 18 µg $[g\ DW]^{-1}$ to 2651 µg $[g\ DW]^{-1}$. In 13/21 Flemish lakes, the microcystin-LR concentrations are higher than the threshold of a French recommendation used to forbid the access to the recreation waters (25 µg/L microcystin-LR). *Microcystis* was the dominant cyanobacterial genus in all samples where microcystins were detected. However, *Planktothrix* blooms were also observed, and in 2 cases, coincided with microcystin detection. Among the reference lakes, lake Blaarmeersen does not seem to be prone to toxic blooms, whereas toxigenic proliferations were observed in the Eau d'Heure lakes but could not be confirmed by microcystin measurements due to technical problems. The neurotoxin, anatoxin-a, was also measured by HPLC but could not be detected by the subcontractor in any of the samples selected for analysis of this toxin, even in the presence of massive bird kills. Other cyanotoxins were not tested.

The protein phosphatase inhibition assays used for detection and analysis of cyanobacterial hepatotoxins did not provide satisfactory results in this study. It seems that it produced false positives, probably because the targeted enzyme system also responds to a wide variety of noncyanobacterial toxins and metabolites.

The molecular diversity of the cyanobacteria was also studied on the basis of 16S rRNA sequences. Two methods were used, Denaturating Gradient Gel Electrophoresis (DGGE) and construction of clone libraries.

In lake Blaarmeersen, no real cyanobacterial blooms were detected by DGGE. The most dominant cyanobacteria were affiliated to *Synechococcus, Limnothrix redekei* and *Anabaena/Aphanizomenon*. The cyanobacterial community in lake Blaarmeersen differed from season to season and from year to year. During the stratification period, there was also a difference between the community in the epilimnion and hypolimnion. A clone library on a July sample yielded a majority of sequences belonging to *Anabaena/Aphanizomenon* (OTU I), and quite a lot of *Synechococcus* ones (OTU IV, V, VI). A minor component corresponded to *Limnothrix redekei* (OTU IX). Four measurements of microcystins by HPLC in 3 samples over 2 years were negative.

Considering two lakes of Eau d'Heure (30 samples), in Falemprise, a bloom of *Anabaena/Aphanizomenon* and in Ri Jaune, a proliferation of *Synechococcus* were detected by DGGE. The cyanobacterial community differed significantly between the two lakes and between the two years. With clone libraries, a July sample of Falemprise confirmed the dominance of cyanobacteria affiliated to *Anabaena/Aphanizomenon* (OTU I) whereas in August in Féronval, it was a different lineage in this cluster (OTU II) that was dominant. In Ri Jaune, in August, a much larger diversity was present with sequences of *Woronichinia naegeliana* (OTU VII), *Synechococcus* (OTU IV, V) and *Microcystis* (OTU VIII). Due to technical problems, it is not known if any Eau d'Heure blooms was toxic.

For BLOOMNET samples, DGGE revealed also that the most dominant bloom formers were *Microcystis, Planktothrix* and *Anabaena/Aphanizomenon*. *Planktothrix* was found at significant lower temperatures and at significant higher concentrations of nitrate in comparison to *Anabaena* and *Aphanizomenon*. A clone library of Parc des Sources (Woluwé, Brussels), where microcystin was detected, was dominated by *Planktothrix* (OTU III) sequences and a few *Aphanizomenon* (OTU I*)*.

The geographic scope of this study has been biased towards recreational and fishing ponds in Flanders, as the majority of samples came from that region. When tested for toxigenicity, 79 % of these samples showed the presence of two genes involved in the synthesis of microcystins, and of the 61 samples tested by HPLC (including 46 with both *mcy* genes), 25 indeed contained measurable amounts of these cyanotoxins. Thirteen out of the twenty-one lakes with measurable microcystins should be closed for recreation, using the threshold value of a French directive. In Wallonia and Brussels, sporadic blooms also showed some toxicity, but not enough analyses could be carried out.

KEYWORDS: algal blooms, cyanotoxins, nutrients, meteorological conditions, cyanobacteria, diversity, detection, early-warning, *mcy* genes, 16S rRNA

INTRODUCTION

Algal blooms, mass developments of algae and cyanobacteria floating at the surface of waterbodies, have become a recurrent and increasingly important phenomenon in freshwaters worldwide over the last decades. These nuisance blooms, and in particular those dominated by cyanobacteria, represent major potential hazards for human and animal health, and interfere in various negative ways with the use of surface waters for e.g. drinking water supply, recreation, irrigation, fisheries. While in other European countries, initiatives have been taken to monitor algal blooms in surface waters, there was a striking lack of knowledge of incidence and nature of algal blooms in Belgian surface waters. The participants in BBLOOMS, as well as other research groups, had recently accumulated evidence suggesting that water blooms, particularly by toxic cyanobacteria, may be a common phenomenon in Belgian surface waters, with still uncertain consequences for public and ecosystem health.

The B-BLOOMS project associated Belgian research groups from three universities (Universities of Liège, Gent and Namur) having expertise in taxonomy and ecology of phytoplankton, as well as in molecular diversity of cyanobacteria. The first general objective of BBLOOMS was to document the extent, nature (genotypic diversity, identification of toxins), the phenology and ecology of nuisance blooms in Belgium. In other words, this two-year program was primarily proposed to make a first assessment of the extent of cyanobacterial blooms in Belgium and of the potential threat for the surface water resources. As cyanobacterial blooms are known to develop in specific environmental conditions (most blooms develop in stratified eutrophic "standing" water bodies), the project incorporated the development of predictive models: is it possible, from easily monitored environmental conditions, to predict where and when potentially toxic cyanobacteria blooms could occur, i.e. to set an early-warning system from environmental monitoring? Third, as the problem may well appear as being real, B-BLOOMS also puts an emphasis on the development of tools for detecting potentially harmful algal blooms in Belgian surface waters, which could be used later on to set up a country-wide monitoring network. Companies and agencies involved in the management and exploitation of freshwater resources were involved from the beginning, both in a user committee following the study and as members of a network (BLOOMNET) designed for sampling phytoplankton blooms in various freshwater bodies.

Notably, at the start of this project, there was no Belgian laboratory where routine measurements of microcystins by HPLC could be conducted. Therefore, during the course of the project, the laboratory of Namur has developed this methodology and carried out some analyses at the end of the project. However, Belgium is still missing facilities to analyse other cyanotoxins (neurotoxins) in water

samples and to identify cyanotoxins in tissues of animals, in case of massive bird and fish kills.

In this report, we have addressed the following specific questions in a multi-disciplinary manner:

1) What is the extent and phenology of nuisance blooms in multiple-use Belgian surface waters?
2) What is the taxonomic diversity of bloom-forming algae, particularly cyanobacteria, on the basis of traditional and genetic tools? Which markers, based on classical (microscopy, pigments) and molecular methods (genomic sequences) can be used to easily and quickly estimate whether the organisms are potentially toxic?
3) What is the toxin concentration in the samples? Are these values higher than guideline values proposed in other countries and by WHO?
4) Which are the environmental conditions related to triggering of nuisance blooms in selected model systems? How can these parameters be used as a basis for the development of models for the prediction of future blooms?

State-of-the art on cyanobacteria blooms

Algal nuisance blooms : what are they and what is their ecology?

Algal BLOOMS are made by proliferations of algae that form visible masses and scum covering the water surface. In the marine environments, eukaryotic microalgae like dinoflagellates, raphidophytes, haptophytes and diatoms cause important health, environmental and socio-economic problems. In freshwaters, the bloom-formers are generally cyanobacteria (often named 'blue-green algae') though cases of toxic blooms of dinoflagellates in freshwater have been observed (Rengefors 2001). It is estimated that 40 to 70% of cyanobacterial blooms release toxins in the water. In addition to this hazard, negative effects include also the aesthetic problems (discolouration, production of scum), the release of malodorous compounds like geosmin, the clogging of filters used for drinking water, and the anoxia due to intense oxygen consumption. Toxic cyanobacteria have been reported in surface waters used for recreational activities and drinking water supply in almost all European countries. At the international level, cyanotoxins were found at least in 57 countries on 5 continents (Chorus & Bartram 1999, Codd et al. 1999). Though the list of cyanobacterial genera including toxin-producing members is expected to increase, the most frequent producers in our regions belong to the planktonic genera *Microcystis, Nostoc, Anabaena, Nodularia, Aphanizomenon, Cylindrospermopsis, Gloeotrichia and Planktothrix*. However, different strains of the same species, or different samples of one bloom may vary in toxin production (Codd et al. 1999). Cyanobacterial toxins, or **cyanotoxins**, have caused animal poisonings (e.g.

livestock, waterfowls, pets) and problems for recreation and production of safe drinking water.

According to their effects on health, cyanotoxins are divided into **hepatotoxins** (liver damaging, tumor promoting: microcystins, nodularins, cylindrospermopsins), **neurotoxins** (nerve damaging: anatoxin-a, anatoxin-a(S), saxitoxins), and **dermatotoxins** (dermatitis: aplysiatoxins, lyngbyatoxins). Microcystins are the most common cyanotoxins worldwide. They are specific inhibitors of the eukaryotic protein phosphatases 1 and 2A.

Main exposure routes are the drinking water, the inhalation and swallowing of water during sports (e.g. swimming, kayak) and showering, and irrigation of crops (Codd et al. 1999). Recognizing the importance of these compounds, the World Health Organisation (WHO 1997) has proposed a Guideline Value of 1.0 µg/L for microcystin-LR in drinking water (Chorus & Bartram 1999). An exceptional case of direct exposure happened when haemodialysis using water from a reservoir containing blooming cyanobacteria was fatal to 60 Brazilian patients (Jochimsen et al. 1998). More frequent, though unrecognized, are probably health problems related to bathing and sporting in waters where cyanobacteria are blooming. Recently, the death of an american teenager was hypothesized, after autopsy, to be due to cyanobacteria producing anatoxins (Stewart et al. 2006a, http://www.jsonline.com/story/index.aspx?id=167645). In Belgium, this might be the most relevant risk for the population, especially children and teenagers who might swallow water when swimming and playing. The WHO guidelines include three levels of alert for recreational waters: 1) low probability of adverse health effects from waters with 20,000 cyanobacterial cells/mL or 10 µg chlorophyll *a*/L (if cyanobacteria are dominant); 2) moderate probability of adverse effects from waters with 100,000 cells/mL or 50 µg chlorophyll-*a*/L; and 3) high probability of adverse effects from contact with and/or ingestion/aspiration of cyanobacteria at scum-forming densities (WHO 2003). To address the problem in more direct manner, a French recommendation of the circular DGS/SD7a-N°2003-270 of 4 June 2003 gives a value of 25 µg/L microcystin-LR to forbid the access for bathing in recreation waters. Epidemiological studies are unfortunately still rare. A recent cohort study in Florida and Australia showed that subjects exposed to high levels of cyanobacteria in recreational waters were more likely to report symptoms after such exposure than subjects exposed to low levels of cyanobacteria. Respiratory symptoms were most evident, but of low severity. However, no cyanotoxins could be measured in the majority of the studied water bodies (Stewart et al. 2006b). Thus, more studies are needed to better assess the risks linked to exposure to cyanobacterial blooms and toxins.

The chemical structures of harmful compounds range from cyclic peptides, alkaloids to lipopolysaccharides. The cyclic peptides are the most stable toxins and

are quite resistant to heat, hydrolysis and oxidation. In general, treatments to eliminate cyanobacteria and toxins are expensive and must be thoroughly tested to avoid creating even more toxic compounds (EPA 2001).

Cyanobacterial bloom formation in fresh water bodies usually require a combination of environmental conditions, among which high nutrient loading, low N:P ratio, high pH (low CO_2), water column stability and long water residence time are key factors (Maberly et al. 1994, Reynolds 1998).

In addition to the formation of proliferations, the question of the variability of toxin production by the cells is of key importance to determine the concentration of the toxin finally available in the environment. Field studies have shown that the same factors that influence cyanobacterial growth (light and nutrients) also influence the microcystin dynamics (in function of cyanobacterial biomass). Laboratory cultures with isolated strains have shown that the microcystin contents of cyanobacteria could vary by a factor 2-3 in response to environmental conditions, but there was a large variability between different strains of *Microcystis* (Kardinaal & Visser 2005). Toxic and non-toxic *Microcystis* strains grown with variable nitrogen and phosphorus concentrations showed different growth rates and toxin productions and it was concluded that high levels of nitrogen and phosphorus in freshwaters may favour the growth of toxic strains over non-toxic ones (Vézie et al. 2002).

Identification of bloom-forming cyanobacteria: classic taxonomy and genetics

The identification is a first hint that a toxin-producing taxon is present, but it is only the first step in the assessment of toxicity. Indeed, from the same bloom, toxic and non-toxic strains can be isolated but there is little information about factors that regulate such ratio. One strain may produce one or more cyanotoxins, and their productions are influenced in various proportions by environmental factors such as phosphate, nitrate, light, pH, T° (Utkilen & Gjolme 1992, Vézie et al. 2002). However, Kurmayer et al. (2002) noted that some morphotypes were less likely to be toxigenic. Indeed, morphotypes identified as *Microcystis wesenbergi*i did not contain the *mcyB* gene, and only 17% of *M. ichthyoblable* did. So, there are probably cases where a careful determination of the morphology can be useful to predict the microcystin production, but this possibility must be still tested on a large scale. Moreover, the size (and thus the age) of the colonies seem to be important. Indeed, it was also demonstrated that the proportion of microcystin-producing colonies depended on colony size, with larger colonies producing more often microcystins (Via-Ordorika et al. 2004).

Molecular tools have been introduced to alleviate the shortcomings of morphological identification of cyanobacteria. Indeed, the latter may be confused by the morphological plasticity of the organisms and disagreements between taxonomic schemes and authors. Moreover, the morphological and genotypic diversity generally

do not coincide (Lyra et al. 2001, Wilmotte1994, unpublished). For toxin-producing cyanobacteria, the molecular taxonomic markers generally used included the 16S rRNA, the spacer between the genes encoding the 16S and 23S rRNA, *cpcBA*, *rpoC1, rbcLX* and *nifH* (Otsuka et al. 1999, Beltran & Neilan 2000, Baker et al. 2001, Dyble et al. 2002, Gugger et al. 2002, Rantala et al. 2004). They generated useful information and novel insights on the phylogeny and geographic distribution of the studied taxa. This is why this project has combined morphological and molecular approaches. With this combination of tools, the information obtained on the identification and quantification of cyanotoxins can be put in the right context, and relations between the samples, their physico-chemical features, the taxa, and the toxins can be examined.

Analysis and toxicity assessment of cyanotoxins

In the early studies, the mouse bioassay was used to determine the toxicity of cyanobacterial bloom samples. Several alternative bioassays and methods are now available to screen the toxicity of samples and quantify toxins. For example, the protein phosphatase inhibition assay (PPIA) is a sensitive and specific test to detect cyanobacterial hepatotoxins (microcystins and nodularins) (Ward et al. 1997). This method is well suited to discriminate the toxic samples but as it gives a measure of the total hepatotoxin concentration, it has to be complemented by an analytical method, such as HPLC analysis with diode array detector. More sophisticated and costly methods include GC-MS, MALDI-TOF, NMR and ELISA (Blaha & Marsàlek 2000, Fastner et al. 2001).

Genetic markers of toxins production

Microcystins, the cyclic heptapeptide hepatotoxins are the most common cyanobacterial toxins worldwide and are mainly produced by the freshwater cyanobacteria *Anabaena, Microcystis, Planktothrix* and *Nostoc*. There are more than 70 isoforms known at present, with various toxicities (Codd et al. 2005a). These cyclic peptide toxins are products of non-ribosomal peptide/polyketide synthesis. The microcystin synthetase gene clusters have been characterized from *Microcystis, Anabaena* and *Planktothrix* (Dittmann & Börner 2005). Microcystin synthetase genes (total size 55 kb including the modification genes) code for peptide synthetases, polyketide synthetases, and modification enzymes which assemble microcystins. The *mcy* gene cluster assembly consists of 10 bidirectionally arranged genes that reside in two operons (*mcyA-C* and *mcyD-J*) in *Microcystis aeruginosa*. In *Anabaena* 90, the genes are also transcribed from a bidirectional promoter region, whereas in *Planktothrix*, all genes except *mcyT* seem to be transcribed unidirectionnally. PCR primers have been designed, that enable the specific and sensitive detection of several peptide synthetase genes (*mcyA, mcyB, mcyC*) (Baker et al. 2001, Tillett et

al. 2001, Nonemann & Zimba 2002) in *Microcystis* strains and water samples. More recently, Rantala et al. (2004) have determined the sequences of *mcyE, mcyD* and *mcyA* of *Nostoc*, *Anabaena, Planktothrix* and *Microcystis*. These authors also suggested that the genes encoding the **nodularin** synthetase (found in *Nodularia spumigena*) are derived from those encoding the microcystin synthetase. In addition, putative genes have been found for **cylindrospermopsin**, a potent hepatotoxin produced by *Cylindrospermopsis raciborskii*, and species of *Anabaena* and *Aphanizomenon.* On this basis, specific PCR primers have been designed (Schembri et al. 2001).

Use of models to predict harmful algal blooms

Simulation and prediction of cyanobacteria blooms has been approached with several mathematical models (Patterson et al. 1994, Reynolds & Irish. 1997, Wallace & Hamilton 2000). The Artificial Neural Network (ANN) models can be applied successfully for predicting the composition of algal assemblages and the occurrence of blooms. For instance, a ANN model developed for the lake Kasumigaura (Japan) was able to predict the blooms of three algal and cyanobacterial taxa, but failed for *Oscillatoria* (Wei et al. 2001). Recknagel et al. (1997) succeeded in predicting cell abundance of three major cyanobacteria genera in three lakes, located in Japan and in Finland. Therefore the development of predictive tools for potentially and actually toxic blooms seems possible, provided a data base containing data from adequate monitoring of the relevant factors is constructed.

The situation in Belgium at the start of B-BLOOMS

In 2003, there was recently accumulated evidence from Belgian surface waters that recurrent nuisance blooms were probably widespread. In Flanders, toxic *Microcystis* samples had been investigated by van Hoof et al. (1994). In Wallonia, the first microcystin-producing bloom was found in a pond near Liège (Wirsing et al. 1998) but several eutrophic reservoirs used for recreation and drinking water supply were potential sites for the development of toxic cyanobacteria. For instance, a study started in 2002 has detected a "blue-green" bloom (*Aphanizomenon flos-aquae*) in the lake of Falemprise (Hainaut), where swimming is allowed (Descy et al. unpublished). These scattered data suggested that nuisance algal blooms might be widespread in Belgian freshwaters, but the risks had never been assessed and the ponds and reservoir management simply ignored this risk.

During the B-BLOOMS project, in 2005, Willame et al. warned that in 64 blooms from Southern Belgium, Luxembourg and Northern France, 53% of the samples contained measurable amounts of microcystins.

Given the worldwide and European level initiatives to monitor blooms, it is crucial that this expertise is also developed for Belgian surface waters. The

standards used in EC directives (e.g. CD 98/83/EC) are generally based on the WHO's guidelines (e.g. 1 µg/L microcystin-LR drinking water). So, in the future, it will be mandatory to dispose of the adequate methodologies to measure the cyanotoxin concentrations in drinking water and to determine the efficiency of water treatment processes. Not only drinking water but also recreational waters should be monitored. In 2003, there was no Belgian laboratory which had yet developed routine analytical methods for cyanotoxin analysis.

Components of the B-BLOOMS project

The project was based on environmental monitoring, screening and analysis of algal samples for pheno- and genotypic identification of nuisance algae, detection and identification of cyanobacterial toxins, and development of a model for prediction of nuisance blooms.

Two types of samples were studied. The first one came from 4 reference lakes (Blaarmeersen lake in Flanders and three pre-dam lakes of Eau d'Heure in Wallonia) that were monitored intensively for 2 years. The second set of samples was sporadic and consisted of bloom samples brought by scientists and members of BLOOMNET. BLOOMNET was a first attempt to build a network of persons able to inform us about the development of blooms and, ideally, able to take samples for us and process them for our analyses. Therefore, we have produced a bilingual video about sampling and further handling of samples for B-BLOOMS' analyses. The persons who contributed to BLOOMNET were mainly employees of environmental administrations and ministries (Vlaamse Milieu Maatschappij [VMM], Institut Bruxellois de Gestion de l'Environnement [IBGE], Région Wallonne [RW], Police de l'environnement...) and members of associations linked to water activities (fishermen, bird watchers, etc). This network also has contributed to raise the awareness of water managers, to better estimate the situation and the global incidence of blooms. The purpose of the network was to store all information in a database (BLOOMBASE), so that it could be used to construct a predictive ANN model (BLOOMODEL).

Molecular markers for taxonomic identifications were used to determine the genotypes in local samples in order to test and improve the specificity and sensitivity of molecular tools for bloom characterization and monitoring. The toxigenicity (presence of *mcy* genes, revealed by PCR) was used to screen the samples and select those that would be subjected to more costly and time-consuming analyses (PPIA assay and HPLC) for the toxins themselves. These two analyses were carried out by a sub-contractor (CRITT-BioIndustries, France) and at the end of the project, by the laboratory of Namur.

MATERIAL AND METHODS

1. Monitoring of reference lakes in Wallonia and Flanders

1.1. The reference lakes: the 3 pre-dam lakes of Eau d'Heure and Blaarmeersen

In Wallonia, the 3 eutrophic pre-dam lakes of the Eau d'Heure complex have been sampled in 2003 and 2004 as reference lakes for the B-BLOOMS project, while the Blaarmeersen lake was chosen in Flanders and sampled during the same period (figure 1).

In addition to the opportunity of exploiting data from a relatively dense monitoring program, the 3 pre-reservoirs of the Eau d'Heure complex were selected because they are eutrophic lakes prone to stratification where algal blooms were likely to occur. They are also important recreation sites in Wallonia, where few water bodies are accessible to the public for recreational activities (swimming, boating, fishing). One of these lakes (Falemprise) is an official swimming area, submitted to microbiological control according to the existing EC directive. However, despite the potential risks for the human population linked to the possibility of toxic cyanobacteria development, phytoplankton in these 3 lakes was not monitored by the Walloon authorities.

Similarly, Lake Blaarmeersen was chosen as reference site for Flanders because of its high socio-economical value (recreation) to the local community and because it was found susceptible to the development of surface blooms due to eutrophication. Surface blooms of cyanobacteria (*Anabaena* sp.) had already been observed in summer (June 1998).

1.1.1. Description of the Eau d'Heure lakes

The building of the Eau d'Heure dams started in 1971 and ended up in 1979. They are situated in the Eau d'Heure valley, between Cerfontaine and Silenrieux, about 20 km south of Charleroi (figure 1). The complex comprises 5 water bodies: the two main ones are related to the two main dams of Plate Taille and Eau d'Heure; the three others are pre-dams reservoirs of Falemprise, Ri Jaune and Féronval, built at the origin to limit the daily level fluctuations. They are also now devoted to several recreational and touristic activities. The whole complex represents 72 km of banks and a surface area of 600 ha. The pre-dam lakes are fed by rivers draining agricultural areas and receiving treated domestic sewage (see Verniers & Sarmento 2004 for details).

Nutrient inputs come mainly from agriculture and livestock, reach the lakes through surface and groundwater, and result in substantial eutrophication of the lakes. The water and ecosystem quality is particularly degraded in Falemprise and

Féronval, classified as poly-eutrophic lakes, while Ri Jaune is considered as meso-eutrophic. In the last 20 years, eutrophication has increased in the 3 lakes (Dehavay 1981, Verniers & Sarmento 2004). Cyanobacterial blooms have been observed in several occasions in the three pre-dam lakes (Verniers & Sarmento 2004).

1.1.2. Description of lake Blaarmeersen

Lake Blaarmeersen is a temperate, monomictic lake situated at the outskirts of the city of Gent in between the river Leie, the Watersportbaan, a canal, created in 1955, to improve water sports and the Ringvaart, another canal (figure 1). It is composed of two basins. It has a maximal length and width of 800 and 250 m, respectively, and a maximal depth of 15 m. The lake is fed only with rain and groundwater but connections with the Leie and the Ringvaart exists to remove some water in excess. It was created between 1974-1976. The sand was used to create a small hill (38 m), which served as an outdoor ski station. From 1982 onwards, the lake is used for recreation purposes (swimming, sailing, fishing and diving).

The biological and chemical parameters from the early history of this lake are well documented in a study of Geysen & Coppejans (1985). At that time the lake

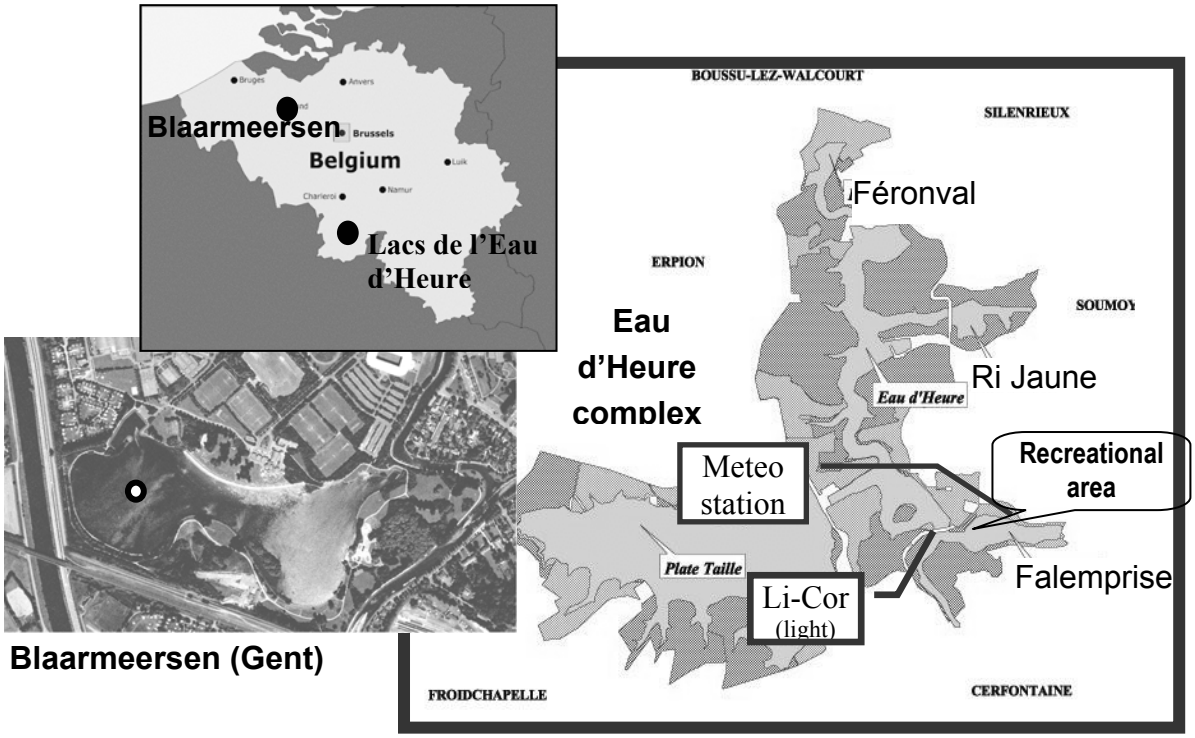


Figure 1: *Reference lakes : in Flanders, lake Blaarmeersen (Gent), and in Wallonia, lakes of Féronval, Ri Jaune and Falemprise, in the Eau d'Heure complex*

contained alkaline, oligotrophic water with low densities and diversity of phytoplankton and a well developed vegetation of macrophytes. In summer 2003, an extensive vegetation of *Nitellopsis obtusa*, a very rare macrophyte, which was believed to be extinct in Belgium, was rediscovered in this lake.

1.2. Sites, sampling occasions and measurements

In Wallonia, the 3 pre-dam lakes of the Eau d'Heure complex have been sampled weekly from February/March to mid-September and then every second week till mid-October/November, at the maximal depth position in each lake (figure 1). The B-BLOOMS project also benefited from the sampling and results obtained in the framework of the "Eau d'Heure Project" (Verniers & Sarmento, 2004).

Phytoplankton samples of Lake Blaarmeersen were taken fortnightly (2003, n = 17) or monthly (2004, n = 11) between February 2003 and December 2004 on two depths (1 m and 8 m), with a vertical point water sampler, at a fixed position in the lake where the water depth is maximal.

In Flanders as in Wallonia, on each sampling occasion, measurements for obtaining vertical profiles of temperature, oxygen, pH and conductivity were carried out. Water transparency was measured with a Secchi disk, and light penetration was also measured using underwater and surface PAR sensors.

At Eau d'Heure, a meteo station was installed at the "Relais de Falemprise" (Eau d'Heure complex) (figure 1) for continuous recording of air temperature, rain, and wind during the sampling period. A Li-Cor system was also installed for recording surface and underwater light, with a 15 min. logging time.

Water samples were taken at different depths in all lakes studied by means of a Van Dorn bottle for nutrient and biotic (phytoplankton, molecular, toxins) analyses, in the epilimnion. Sampling was designed in each lake taking into account the conditions of the water column, which was usually stratified from mid-spring to mid-autumn. Zooplankton samples were taken with a Schindler trap equipped with a 63 µm mesh size plankton net, in the epilimnion. In Blaarmeersen, in summer, the hypolimnion was also sampled. In Eau d'Heure lakes, zooplankton was sampled every second sampling day (once a month) in 2003, and on each sampling occasion in 2004. In Blaarmeersen, zooplankton was sampled fortnightly in 2003 (but monthly in winter) and monthly in 2004.

2. Screening of Belgian lakes for algal blooms by the BLOOMNET network

The contacts we had for implementing BLOOMNET (workshop and personalized contacts) resulted in different agreements for collaboration and collection of samples.

In 2004, an agreement of monitoring of recreational waters in Wallonia was taken with the Division Générale des Ressources Naturelles et de l'Environnement (DGRNE), Division of water – "Direction des Eaux de Surface" . Monitoring and conditioning of BLOOMNET samples was carried out by the Société Wallonne Des Eaux (SWDE). In addition, other contacts were made occasionally, whenever blooms

were noticed, at the initiative of the B-BLOOMS teams or by diverse people from universities, institutes, administration and natural reserves. In Flanders, most of the samples were taken by the UGent team and some by BLOOMNET (Vlaamse Vereniging van Hengelsportverbonden [VVHV], Provinciale Visserij Comissie [PVC], Vlaamse Milieu Maatschappij [VMM], Instituut voor Natuur- en Bosonderzoek [INBO] and the BELSPO MANSCAPE project) and came from a wide variety of surface waters, most of them relatively small (fishing and urban ponds)

3. Pigment analysis

In Wallonia, High Performance Liquid Chromatography (HPLC) analyses have been carried out on algae samples, both for reference lake monitoring and BLOOMNET samples. Water samples were processed as in Descy et al. (2005), allowing estimating chlorophyll *a* (Chl *a*) biomass of Chlorophytes, Chrysophytes, diatoms, Cryptophytes, dinoflagellates and cyanobacteria T1 and T2 expressed in Chlorophyll *a* equivalents. Although the distinction of the two types of cyanobacteria was based solely on pigment composition, cyanobacteria T1 corresponded usually to coccoid forms (unicells or small colonies) while cyanobacteria T2 corresponded mostly to larges colonies or filaments. In Flanders, only total algal biomass (Chlorophyll *a*) was determined by HPLC analysis of acetone extracts obtained as described above.

4. Microscopic identifications

For Blaarmeersen, phytoplankton counts were made under microscope at 400 X, generally at the generic level for non-cyanobacteria and at the species level for cyanobacteria, and converted into biomass. For Eau d'Heure lakes, relative abundance of cyanobacterial species compared to total cyanobacterial biomass (from HPLC) were determined, while global phytoplankton composition was coded in 5 abundance classes.. For BLOOMNET samples, at least specific counts of cyanobacteria were carried out and pictures of taxa were taken.

5. Zooplankton countings and filtration rates

For Eau d'Heure lakes, individuals have been counted under inverted microscope at 100 X (till reaching at least 100 individuals of the dominant taxa). Adult crustaceans and rotifers were identified at the species level, copepods were classified in cyclopoids and calanoids while nauplii were simply counted. Filtration rates were estimated from taxa abundances and mean body length (30 measurements per cladoceran and copepod species, 10 per rotifer species, once a month), using the general formulas of Knoechel & Holtby (1986). For Blaarmeersen, adult cladocerans and rotiferans were counted at the species or genus level and copepods were classified in cyclopoids and calanoids, nauplii and copepodites.

6. Studies of the genotypic diversity of bloom-forming cyanobacteria

6.1. Sampling and DNA extraction

A portion (generally 100 to 400 ml) of the sample was filtered through 0.2 µm Supor filters (Pall Life Science, USA) for molecular analysis, except for lake Blaarmeersen where GSWP-filters were used, and immediately frozen. The Supor filters were conserved in 2 ml lysis buffer (40 mM EDTA, 400 mM NaCl, 0.75 M sucrose, 50 mM Tris (HCl; pH 8.3). The filters were kept frozen at -20°C before analyses.

DNA was extracted from the filters as described by Zwart et al (1998) for Blaarmeersen lake and as described by Boutte et al. (2006) for Eau d'Heure lakes and the additional bloom samples. The DNAs were exchanged between the laboratories of Gent and Liège to enable further analyses based on the same DNA.

6.2. Denaturating Gradient Gel Electrophoresis (DGGE)

16S rRNA gene sequences were amplified using the nested PCR protocol of Zwart et al. (2005). DGGE was essentially performed as described by Muyzer et al. (1993). Equal amounts of PCR products were loaded onto 8% (w/v) polyacrylamide gels (1 mm thick, in 1× TAE [20 mM Tris acetate (pH 7.4), 10 mM acetate, 0.5 mM disodium EDTA]). The denaturing gradient contained 35-60% denaturant [100% denaturant corresponded to 7 M urea and 40% (v/v) formamide]. Electrophoresis was performed for 16 h at 75 V and the temperature was set at 60°C. Finally, the gels were stained with ethidium bromide and photographed on a UV transillumination table with a CCD camera. The most important bands were cut out, purified by DGGE and sequenced. A BLAST search was done to find the most related organisms. Furthermore, the profiles were aligned using the program Bionumerics 3.0 (Applied Maths BVBA, Kortrijk, Belgium). The software performs a density profile through each lane, detects the bands and gives a value to the band intensity. A matrix was compiled based upon the relative contribution of individual bands to the total band signal in each lane. The statistical programs Primer 5 (Clarke & Gorley 2001) and Statistica 6.0 (StatSoft, Inc., 1995) were used to analyse the cyanobacterial community structure.

6.3. Construction of clone libraries

Five samples were used for the construction of clone libraries: Falemprise (01/07/03), Féronval (19/08/03), Blaarmeersen (18/07/03, 1 m depth), Ri Jaune (31/08/04), and one additional BLOOMNET pond (Parc des Sources, Woluwé, Brussels on 22/07/04) DNA of the samples was amplified and cloned as described by Boutte et al. (2005). However, for the first three samples, the forward primer was 16S27F (Taton et al. 2003). Each positive clone was sequenced using primer 1092R

(Houzrek et al. 2005). Sequencing reactions were carried out using an ABI Prism system 377 (PE Applied Biosystems, USA) by the company Genome Express (France). Sequences were manually corrected and the chimera were identified with CheckChimera (Cole et al., 2007) and removed.

An alignment was constructed with ClustalW (Thomson et al. 1994) and edited manually with BioEdit (Hall 1999). When duplicate sequences were present, only one was chosen as representative and the number of copies was indicated between brackets. The most similar sequence was found by the option 'Sequence Match' of the Ribosomal Database Project (Cole et al. 2007) and added to the alignment, as well as the outgroup *Escherichia coli*. A phylogenetic analysis was carried out with the construction of a Neighbor-joining tree on a distance matrix corrected with the formula of Jukes and Cantor, using the software TREECON (Van de Peer & De Wachter 1994). Trees were constructed for each clone library as well as for the global dataset. The latter tree was also used for the delineation of OTUs (groups of sequences sharing more than 97% similarity) by DOTUR (Schloss & Handelsmann 2005).

7. Molecular studies of diversity of cyanotoxin genes and design of tools for monitoring toxins

The samples that were sent to the sub-contractor CRITT-BioIndustries for measurements of microcystins were selected on the basis of the detection of *mcyB* and *mcyE* genes.

7.1. *mcyE/B* detection

DNA of samples was used for PCR reactions with primers *mcy*F1/*mcy*R1 and *mcy*F1/*mcy*R2 as described by Nonneman & Zimba (2002) which amplify a fragment of 320 bp inside the *mcyB* gene of *Microcystis,* and with primers *mcy*E-F2/*mcyE*-R4 as described by Rantala et al. (2004) which amplify a fragment of 810 – 812 bp inside the *mcyE* genes of several genera (*Microcystis, Planktothrix, Anabaena, Nostoc*).

7.2. RFLP of *mcyE*: fast assessment of the identity of the toxin-producer

The *mcyE* amplicon was submitted to a digestion with the restriction enzyme *AluI*. The enzyme was chosen with the tool 'Restriction analysis of DNA' on the website http://insilico.ehu.es/restriction/ as it generated different banding patterns for *Planktothrix, Anabaena* and *Microcystis*. For the latter, two profiles are possible. The fragments were separated by electrophoresis is a 2% agarose gel (HR agarose, Biotools, Spain) in TAE for 55 mins at 110 V. The gel pictures were digitalized and the size of bands was determined with the software GelCompar 2 (Applied Maths, Belgium).

8. Determination and measurement of toxins

8.1. Handling of samples for microcystins measurements

The samples were handled differently in Flanders and Wallonia. In Blaarmeersen, depending on the bloom density, some scum material (50-250 ml) was taken in a sterile Falcon tube or concentrated on a phytoplankton net (mesh size 10 µm). In all cases, 1 L-samples were taken to the laboratory to collect the algal material on filters. For the Eau d'Heure lakes and the few Walloon BLOOMNET samples, there was no concentration of the algal samples in the field and the water samples were taken to the laboratory for filtrations. In the laboratory, 5 to 250 ml of water were filtered on GF/C filters (Whatman, USA), till the filters were clogged. The filters were folded in two, wrapped in aluminium foil, and frozen at –20°C in the laboratory. Three to four filters were obtained for each sample.

In Flanders, bloom samples (n = 104) from BLOOMNET were collected in the littoral zone with a bucket from which sub-samples for microscopical enumeration and toxin analysis were taken (in general not concentrated). In a few cases, tubes were filled directly from the scum layer. In the laboratory, scum samples and concentrated samples were all lyophilized in Petri dishes with a Lyotrap freeze-drying machine (LTE Scientific LTD.) overnight (about 12 hours). Before freeze-drying, the volume of the samples was noted. Afterwards, the material was weighted, put in Falcon tubes and stored in a deepfreeze at –20°C.

During transport to CRITT-BioIndustries for toxin analyses, the material was kept frozen on dry ice. For the transport from Gent to Namur, it was maintained refrigerated.

8.2. Measurements of microcystins at CRITT-BioIndustries (Toulouse, France)

Both protein phosphatase (PP2A) inhibition assay and measurement of microcystins by HPLC-DAD were carried out. For PP2A and HPLC-DAD, ca 40 mg of lyophilized biomass (or each filter) was extracted three times in methanol/water (75:25) with sonication. The extracts were pooled, filtered (0.2 µm pore diameter), evaporated under nitrogen flux and the dry extract was dissolved in 500 µL of methanol/water (20:80).

For the PP2A assay, the extracts were incubated with phosphatase and the colorless substrate, para-nitrophenyl phosphate. In function of the intensity of the yellow color measured by spectrophotometry at 405 nm, the quantity of inhibitor present was estimated, expressed as equivalent microcystin-LR. This molecule (Alexis Biochemicals, USA) was used to calibrate the test.

The microcystins-LR and -RR were measured by HPLC-DAD. HPLC was carried out according to the method of Lawton et al. (1994). The column Symmetry

C_{18} (5-150 x 3.9 mm, 5 µm) was used (Waters, USA) with a water/acetonitrile gradient, both acidified with 0.05 % TFA (trifluoroacetic acid). The HPLC instrumentation was a Dionex system (USA) with a ASI-100 sample injector, a P680 pump and a UVD 340U detector. The chromatograms were evaluated at 238 nm. The threshold for quantification was 5 µg/g of dry matter.

8.3. Measurements of microcystins at FUNDP

Samples were extracted either from material collected on filters, or from bulk bloom samples, with 80 % methanol. These extracts were evaporated by centrifuge evaporation at 35˚C and re-dissolved with 100 % methanol. Extracts were kept at -20˚C until the assay or the HPLC analysis were carried out.

Both protein phosphatase (PP1) inhibition assays and measurements of microcystins by HPLC-DAD were carried out. The PP1 assay was adapted from the colorimetric method of Heresztyn & Nicholson (2001). Protein phosphatase 1 activity was determined by spectrophotometric measurement of para-nitrophenol from the substrate para-nitrophenyl phosphate.

Prior to HPLC analysis, the extracts were purified using hydrophilic-lipophilic balanced cartridges Oasis HLB (Waters, USA), following Rapala et al. (2002). HPLC analysis of the toxins was carried out according to Lawton et al. (1994). The HPLC instrumentation consisted of a Waters system (USA) with Model 600 solvent pump, Model 717 plus autosampler, and a Model 996 photodiode array detector. Chromatograms were evaluated at 238 nm. Analyses were performed on a Symmetry C_{18} column (250 x 4.6 mm I.D., 5 µm, Waters) with a water/acetonitrile gradient, both acidified with 0.05 % v/v TFA. Microcystin standards (MC-LR, MC-RR and MC-YR) were purchased from Alexis Biochemicals (USA) and standard solutions were prepared with 100 % methanol. The detection limit for lyophilized biomass was ca 5 µg/g of dry matter.

8.3. Analyses of anatoxin-a at CRITT-BioIndustries (Toulouse, France)

40 mg of lyophilized biomass was suspended in 1.5 ml of acetic acid (0.05 M), vortexed 5 times 1 min with glass beads and centrifuged. This extraction was repeated 3 times. All supernatants were pooled and filtered (0.2 µm pore diameter) and stored frozen at –20°C till analysis. The measurement was realised by HPLC-DAD. The instrumentation was the same as for the microcystin measurements. Chromatograms were evaluated at 227 nm. Analyses were performed on a Symmetry C18 column (5-150 x 3.9 mm, 5 µm, Waters) with a gradient of NaH_2PO_4 4.5 mM + H_3PO_4 0.5 mM (pH 3.5)/acetonitrile. The standard was anatoxin-A fumarate from AG Scientific (USA) in water.

9. Data processing: multivariate analyses and artificial neural network

9.1. Prediction of cyanobacteria in Eau d'Heure pre-dam lakes

Data from 2002 to 2004 from the pre-dam lakes of Eau d'Heure were gathered in the final data processing. From measured variables, new descriptors were calculated that could be useful for the prediction of cyanobacteria bloom occurrence (Table 1). Data were validated, and whenever necessary, missing values were interpolated.

Table 1 *– Environmental variables taken into account for global analyses of data from the 3 pre-dam lakes of Eau d'Heure (multivariate analyses and Artificial Neural Network).*

Variables	Codes
Soluble reactive phosphate (µg $P\text{-}PO_4^{3-}$ l^{-1})	SRP
Silica (mg Si l^{-1})	Si
Inorganic dissolved nitrogen [NO_3^-, NO_2^-, NH_4^+] (µgN l^{-1})	DIN
Total phosphorus [SRP, Ppart.] (µgP l^{-1})	TP
Total nitrogen [NO_3^-, NO_2^-, NH_4^+, Npart.] (µg N l^{-1})	TN
Particulate carbon (µg C l^{-1})	Cpart
Photic depth (m)	Zeu
Solar radiation (average on the period 11h-14h of the day ; $\mu Em^{-2}s^{-1}$)	I0
Temperature of the epilimnion (°C)	TempE
Dissolved oxygen (%)	O2
pH (mean of the epilimnion)	pH
Conductivity (mean of the epilimnion ; µS cm^{-1})	Cond
Mixing depth (m)	Zm
Photic depth vs. mixing depth	Zeu/Zm
Number of days with stratification	NJS
Photoperiod (h)	Photop
Air Temperature (average on the week prior to sampling ; °C)	TempO
Difference between maximum and minimum air temperature of the week (°C)	Dif temp
Relative humidity (average on the week prior to sampling ; %)	Hum
Wind speed of the day prior to sampling (ms^{-1})	VVJP
Wind speed (average on the 7 days prior to sampling ; m s^{-1})	WS_7j
Wind speed (average on the 3 days prior to sampling ; m s^{-1})	WS_3j
Number of days without a wind of 4.3 m s^{-1} or higher	NJV<4.3
Number of days without a wind of 5.0 m s^{-1} or higher	NJV<5.0
Rain (sum of the week ; mm)	Précip
Number of large cladocerans (ind l^{-1})	N_Gclado
Number of small cladocerans (ind l^{-1})	N_pClado
Number of calanoids copepods (ind l^{-1})	N_Cyclo

Number of cyclopoids copepods (ind l^{-1})	N_Calan
Number of nauplii (ind l^{-1})	N_Naupl
Number of rotifers (ind l^{-1})	N_Rotif
Number of total zooplankton (ind l^{-1})	N_tot
Filtration rate of the large cladocerans (% j^{-1})	F gd Clado
Filtration rate of the small cladocerans (% j^{-1})	F pt Clado
Filtration rate of the calanoids copepods (% j^{-1})	F Cala
Filtration rate of the cyclopoids copepods (% j^{-1})	F Cyclo
Filtration rate of the nauplii (ml j^{-1})	F Naupl
Filtration rate of the rotifers (% j^{-1})	F rotif
Total filtration rate (% j^{-1})	F tot

9.1.1. Multivariate analyses

Prior to analyses, data were transformed in order to fit as far as possible with a normal distribution. Data processing was carried out using Principal Component Analysis (PCA) and Detrended Correspondence Analysis (DCA), with the Canoco v.4.0 (ter Braak & Smilauer 1998) and the ADE softwares (Chessel et al. 2004). In order to explore relationships between environmental variables and phytoplankton composition, canonical correspondence analyses (CCA) were performed. The biological data matrices included either algal group biomasses (from HPLC analyses; 210 observations) or cyanobacterial species relative composition (116 observations). The environmental data matrix comprised the physical and chemical descriptors, from lake water column and meteorological measurements. A second environmental data matrix included also zooplankton numbers and filtration rates. In this last case, the number of observations was lower, owing to the lower number of zooplankton data (109 and 96 observations, for matrices corresponding to algal groups and relative abundances, respectively).

9.1.2. Artificial Neural Network

Artificial Neural Network models (ANNs) were employed to predict cyanobacterial biomass (expressed in Chlorophyll *a* equivalents) from environmental variables (see Table1). ANNs have proved to be efficient in ecological modelling (Lek & Guégan 1999, Lek et al. 2004) and especially in predicting phytoplankton succession (Olden 2000). The output variables were either total cyanobacterial biomass, or cyanobacteria T1 and T2 biomass.

We used a supervised ANN (Lek & Guégan 1999) with a back propagation algorithm (Rumelhart et al. 1986). The architecture of the network consists of input, hidden and output layers with a one-way flow of information. The input layer of neurons represents the independent environmental variables (Table 1) and the output layer is a single neuron that represents the dependent variable (i.e. cyanobacteria biomass). The number of neurons of the hidden layer (10) and the

number of iterations for the back-propagation algorithm (500) were selected to optimize the accuracy of the model and minimize trade-off between network bias and variance. To standardize the scale of measurement, independent variables were centred and reduced to range between 0 and 1.

Models were validated by the leave-on-out cross-validation test (Efron 1983). This test consists in training k ANN models (i.e. k=number of observations), each time leaving out one observation, and using only the omitted observation to test prediction capabilities of the model. Model predictive efficiency was assessed using regression analysis and especially the correlation coefficient between the observed and estimated cyanobacteria biomass. Finally, the contributions of the environmental variables to the ANN models were determined by using the connection weight procedure (Olden & Jackson 2002, Olden et al. 2004). This ANN application was conducted in collaboration with F. Leprieur, University Paul Sabatier, Toulouse (France).

RESULTS AND DISCUSSION

1. Monitoring of the pre-dam lakes of the "Eau d'Heure" complex

1.1. Cyanobacterial blooms in Eau d'Heure lakes: data analysis and prediction models

Cyanobacterial blooms were regularly observed in the Eau d'Heure lakes during this study. They comprised various taxa, the most important being *Aphanizomenon flos-aquae*, which was particularly well developed in Lake Falemprise. In the other lakes, blooms were generally composed of several coccoid taxa. Details on abiotic conditions and phytoplankton successions can be found in Verniers & Sarmento (2004). Here we focus on the data analysis and on the development of prediction models of cyanobacteria blooms.

1.1.1. Multivariate analyses

The DCA on algal groups and cyanobacteria taxa (not shown) showed the distinction between lakes, with cyanobacteria T2, and mainly *Aphanizomenon*, more characteristic of Falemprise, while cyanobacteria T1, with *Aphanothece*, *Aphanocapsa, Coelosphaerium* and *Cyanogranis liberia* appeared more associated with Ri Jaune.

The PCA did not distinguish the years but clearly showed a temporal seasonal gradient (figure 2). As expected, wind speed was higher in spring, as were dissolved and total nitrogen concentrations. The lakes were well oxygenated in late spring, presenting lower values in autumn, at the time of deepening of the mixing layer. A well illuminated mixed layer was characteristic of early summer, while number of days without strong winds is maximal at the end of summer. Lakes were slightly discriminated according to trophic status (not shown).

As shown in figure 3, variables of importance for phytoplankton composition are related to season and stratification (axis 1: temperature, photoperiod, days without high wind), and to nutrient concentrations (axis 2). Environmental conditions favouring cyanobacteria T1 and T2 differed: both developed at high temperature and stable stratification, but T1 appeared when SRP was relatively low and DIN relatively high, contrary to T2 which developed at higher SRP concentrations but at low DIN. When considering zooplankton (not shown), the number of large cladocerans seemed associated with T2, while high filtration rates of small cladocerans were associated with T1 cyanobacteria. Nevertheless, those results are to be taken with caution due to the small size of data matrices when considering zooplankton.

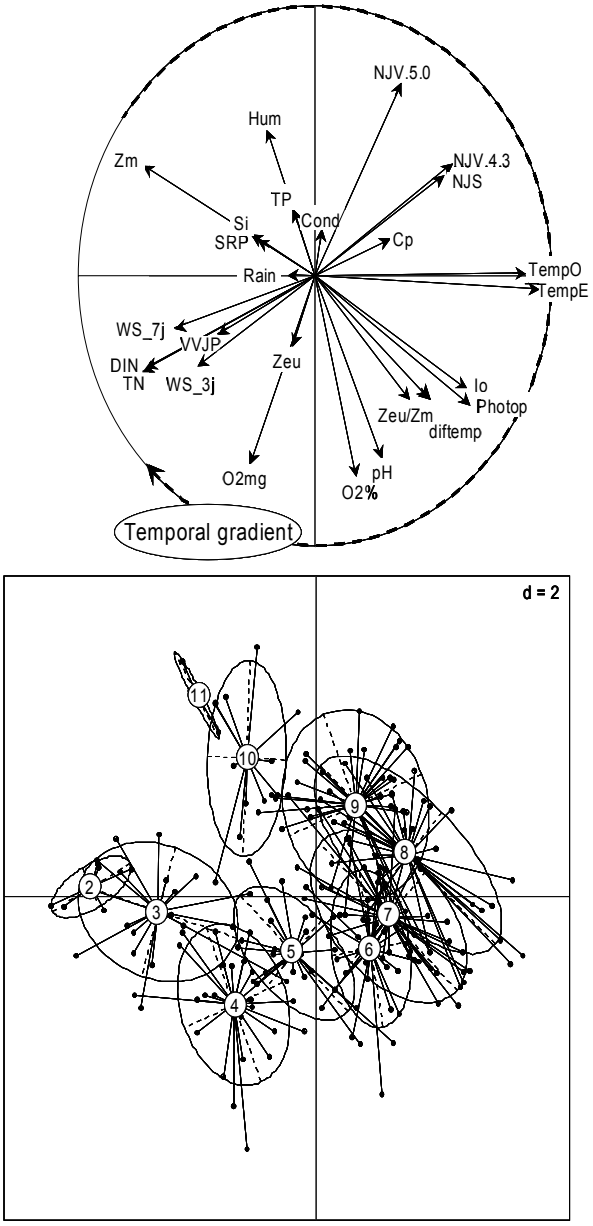


***Figure 2**: PCA on environmental variables (above, without zooplankton) from the 3 pre-dam lakes of Eau d'Heure, from 2002 to 2004. Below: Projection of centroids of months (all years and lakes together).*

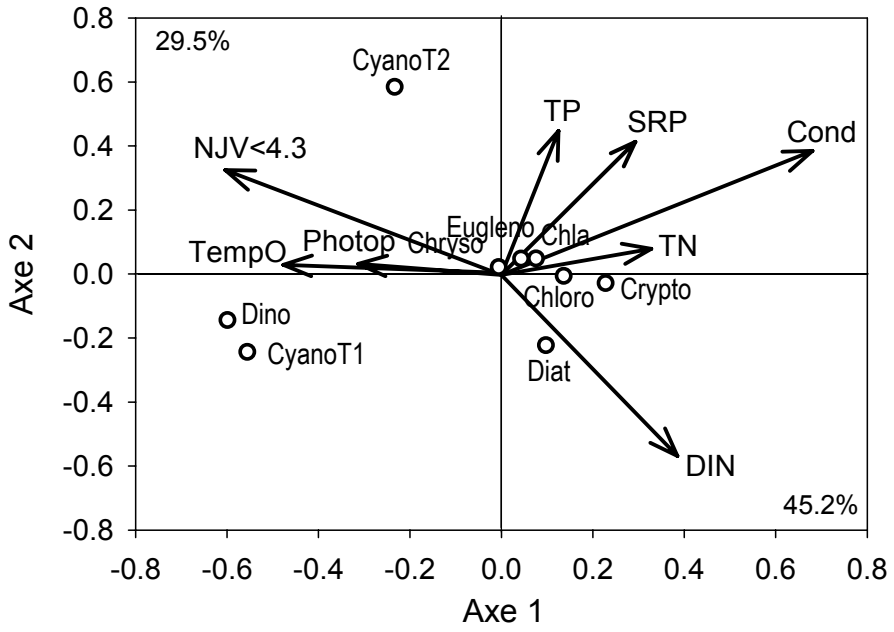


Figure 3: *CCA ordination biplots of the algal groups (dots) at the 3 pre-dam lakes of Eau d'Heure for the growing season of 2002 to 2004. Arrows indicate the environmental variables; legend as in table 1.*

1.1.2. Prediction through Artificial Neural Network

Prediction of total cyanobacteria biomass (expressed in Chl *a* equivalent) gave good results: regression between predicted and observed values, carried out through leave-one-out method, reached a correlation coefficient of 0.95 (data not shown). It is to be noted that the position of the highest observed value, out of the regression line, is to be related to the leave-one-out validation method: when removed from the data matrix, this value is difficult to be predicted by the model as it is out of the data range.

The contribution of variables to the prediction is shown on figure 4, e.g. low values of TN contribute for 10.3% to cyanobacteria biomass, while high TP contributes for 9.7%. The highest contributions are from chemical and meteorological variables. When considering only the 10 variables contributing for at least 5%, the predicted values reached a correlation of 0.94, when compared to observed values (not shown)

When predicting separately cyanobacteria T1 and T2, the correlations of the relationship between predicted and observed biomass were 0.93 and 0.95, respectively (not shown). It is interesting to note, in this case, the varying contribution of the variables according to the cyanobacterial type (figure 4). As already seen in the CCA, the predictive model confirms the role of light for determining the biomass

of the cyanobacteria T1, while nutrients are the best predictors for cyanobacteria T2; both types need stable stratification and high temperature. Again it is interesting to note that the best predictors are limnological or meteorological variables, which can be monitored easily.

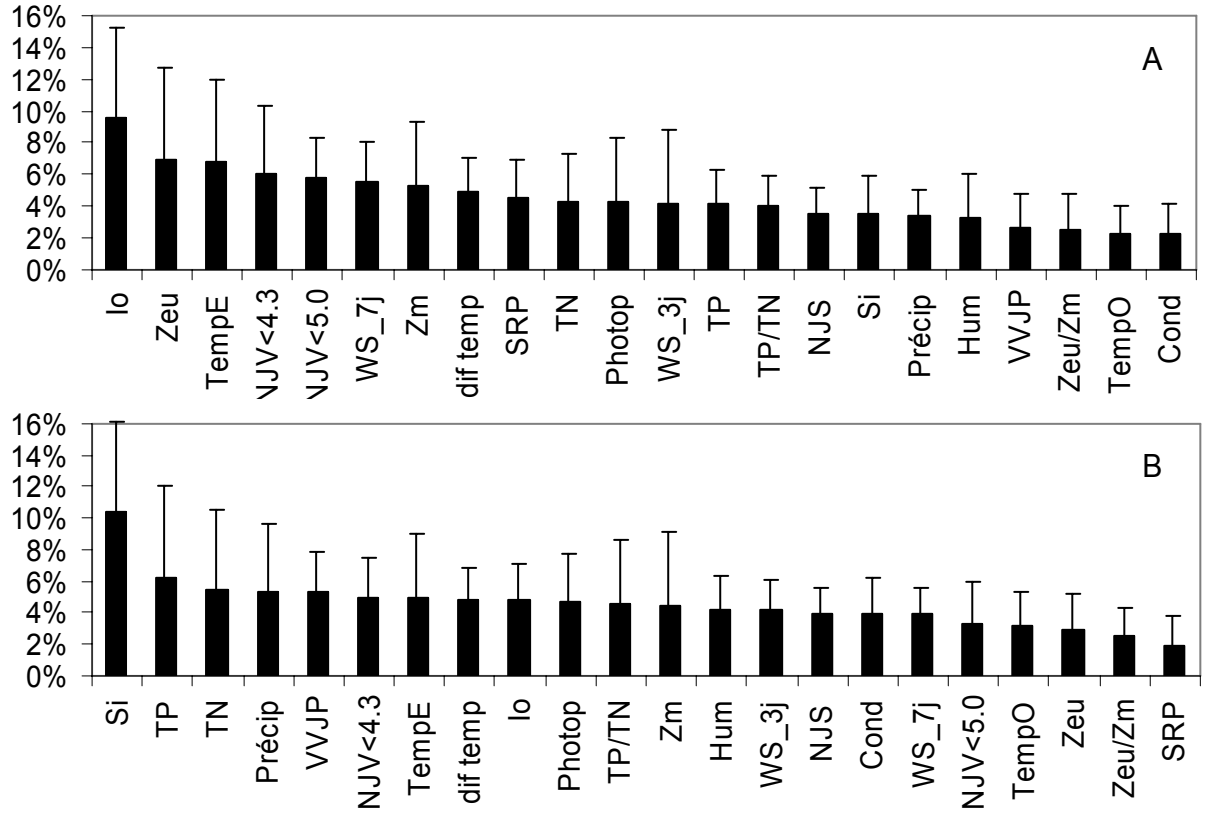


Figure 4: *Contribution of variables to the prediction of (A) cyanobacteria T1 biomass, and (B) cyanobacteria T2 biomass, in the 3 pre-dam lakes of Eau d'Heure, for the growing season 2002 to 2004.*

1.3. Monitoring of Lake Blaarmeersen

1.3.1. The phytoplankton dynamics in Lake Blaarmeersen

Highest concentrations of dissolved nutrients were observed in autumn and winter when organic matter became mineralised and the water column was mixed till the bottom. During summer, dissolved nutrients became depleted in the epilimnion.

In comparison with historical data from 1983 (Geysen & Coppejans 1985), the amount of nutrients has increased, and this has led to a higher algal productivity, also reflected in the pH data which has increased of 1 unit in general. The phytoplankton species composition has also changed over time. Taxa which are more typical for eutrophic conditions became more abundant or replaced the original flora. In general, the abiotic changes were favouring cyanobacteria and green algae.

A redundancy analysis (RDA, figure 5) including as explanatory variables nitrate concentration, temperature, *Daphnia* abundance, Secchi-depth, suspended particulate matter (SPM), cyclopoid copepod abundance and pH explained almost 61 % of the variance of phytoplankton data. Mainly seasonality separates the samples.

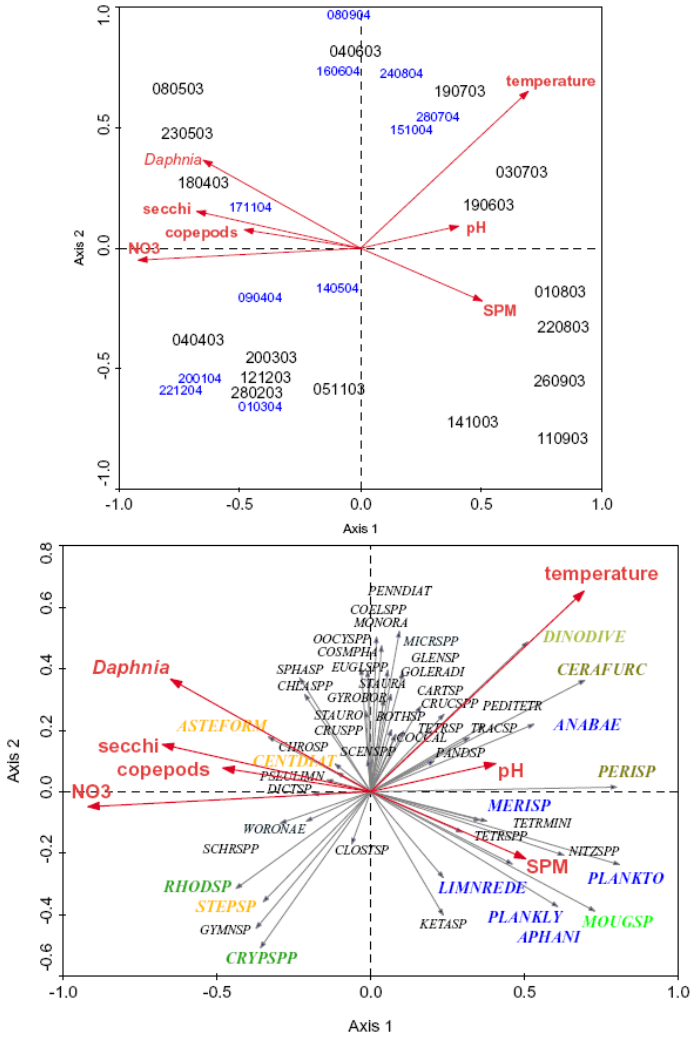


Figure 5: *RDA biplots of phytoplankton data (samples at top, species at bottom) and forward-selected explanatory variables for axes 1 (eigenvalue 0.219) and 2 (eigenvalue 0.098)*.

Winter and spring samples are positioned at the left of the ordination diagram and summer and autumn samples on the right. The summer samples are positively correlated with temperature, pH and SPM, spring and winter samples are positively related with Secchi-depth, nitrate and grazers (*Daphnia* and cyclopoid copepods). Fast-growing cryptophytes (*Rhodomonas*, *Cryptomonas*) and diatoms (*Stephanodiscus*), and large colonial diatoms (*Asterionella*) dominate in spring, during the clear water phase. This typical spring clear water phase, visible when the amount of SPM was lowest and Secchi depth and euphotic depth were maximal, was most probably induced by the high grazing pressure on phytoplankton by large

cladocerans (*Daphnia*) at that time (Lampert et al.1986). Large cell size, colony-forming or high growth rates of phytoplankton are all advantageous in the presence of grazers (Reynolds 1984, Sarnelle 2005). The clear water phase lasted longer in 2004 in comparison with 2003 since a massive development in the whole water column of filamentous green algae (*Mougeotia*) in the extremely warm and dry summer of 2003 changed the zooplankton community in favour of less efficient smaller zooplankton taxa like rotifers and *Bosmina*. Cyclopoid copepods seemed to be important grazers predominantly in winter. *Mougeotia* is typical for light deficient, deep epilimnia (Reynolds et al. 2002). In summer 2004, a different, more diverse phytoplankton community was present. Typical summer taxa were the dinoflagellates *Ceratium* and *Peridinium* and the chrysophyte *Dinobryon.*

1.3.2. Presence of cyanobacteria in Lake Blaarmeersen

Maximal biomasses of filamentous cyanobacteria were encountered during summer (up to 40 % in the epilimnion) in both years and during autumn (up to 60 % in the hypolimnion) in 2003. In summer 2003, during nutrient limitation, N_2-fixing taxa (*Anabaena planctonica*, *Aphanizomenon flos-aquae*) were present (figure 6). In autumn 2003, *Limnothrix redekei*, which is adapted to colder, more turbid conditions, became also important. In summer 2004, picocyanobacteria (only measured in 2004) were mainly responsible for the maximal contribution of cyanobacteria in both epi- and hypolimnion. *A. planctonica* had only a high contribution in October whereas *Ap. flos-aquae* and *L. redekei* were much less important than the year before, possibly due to the less exceptional climatological conditions as in summer 2003. Surface scums of cyanobacteria were never encountered during the sampling period, with the exception of *Anabaena*-scum at one spot in the littoral zone (water polo area), in August 2003. However, no cyanotoxins (microcystins, anatoxin-a) could be detected in a concentrated sample from this small, local surface bloom (see table 2).

In conclusion, although Lake Blaarmeersen is prone to eutrophication (both natural and anthropogenic) as suggested by the higher nutrient levels and changes in the phytoplankton community, the development of (toxic) blooms is not a regular phenomenon (yet). The development of blooms in this lake seems to depend largely on climatological conditions (warm and dry summers) and less from parameters inherent to the system.

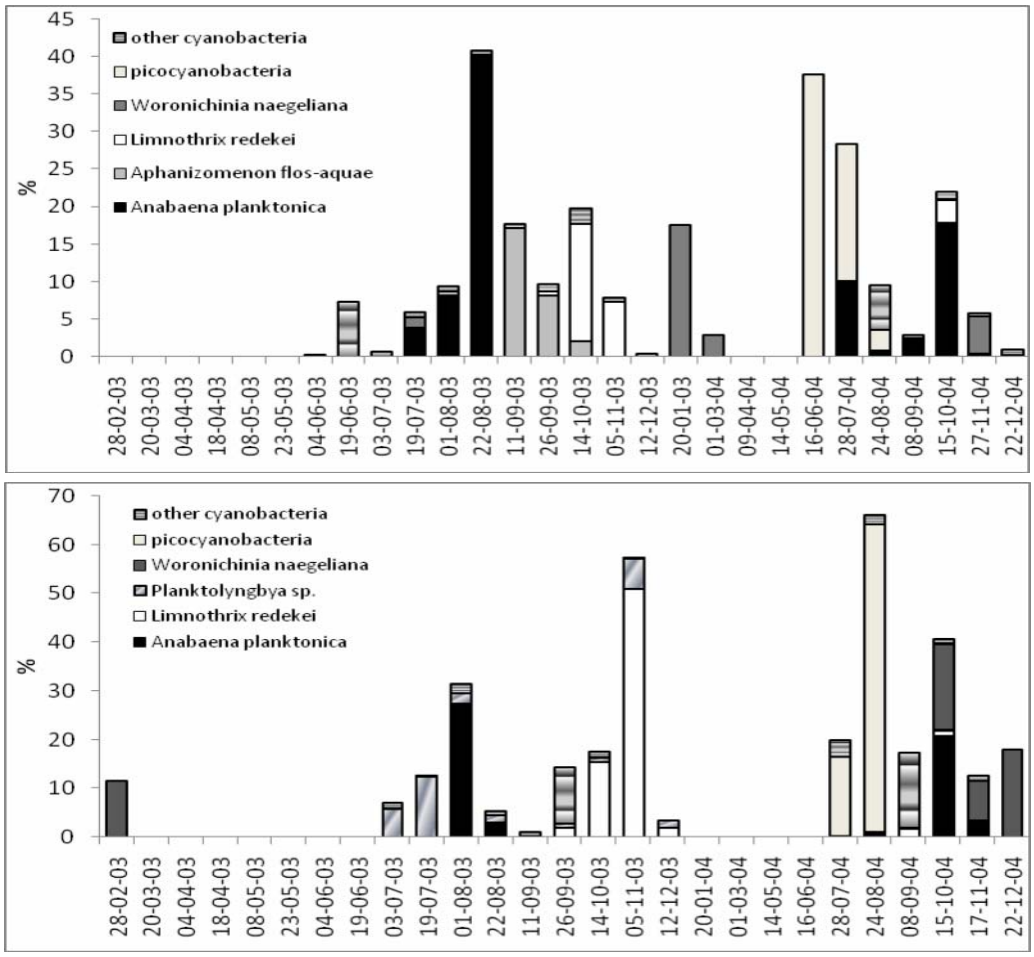


***Figure 6**: Seasonal fluctuation in the relative contribution of different Cyanobacteria to the total phytoplankton biomass in the epilimnion (upper graph) and hypolimnion (lower graph) of Lake Blaarmeersen.*

1.4. Screenings of Belgian lakes for algal blooms by the network BLOOMNET

1.4.1. BLOOMNET samples in Wallonia and Brussels

Twenty-two additional bloom samples were provided by BLOOMNET. They came from the Woluwe ponds (Parc des Sources [TBr] and Parc de la Woluwe [wpk2]), the Museum of Tervuren, the Bambois natural reserve, the harbour of Monsin in Liège, the Grand Large in Péronnes, Clairefontaine in Chapelle-lez-Herlaimont, Cherapont in Gouvy and Renipont . Locations of the sampling points are given on figure 7, and details about algal groups detected on the basis of their pigments (HPLC) are given in figure 8. Dominant cyanobacteria taxa were diverse and are listed in Table 3. *Microcystis*, *Aphanizomenon* and *Planktothrix* were the most frequent genera, without clear dominance of any taxon in general. In addition, 5 other BLOOMNET samples did not contain a cyanobacterial bloom (Table 3).

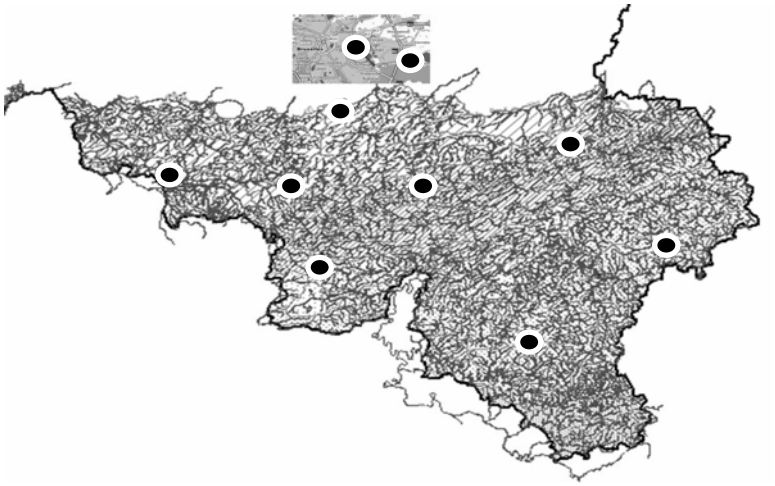


Figure 7: *Distribution of BLOOMNET samples provided or collected in Wallonia and Brussels in 2004.*

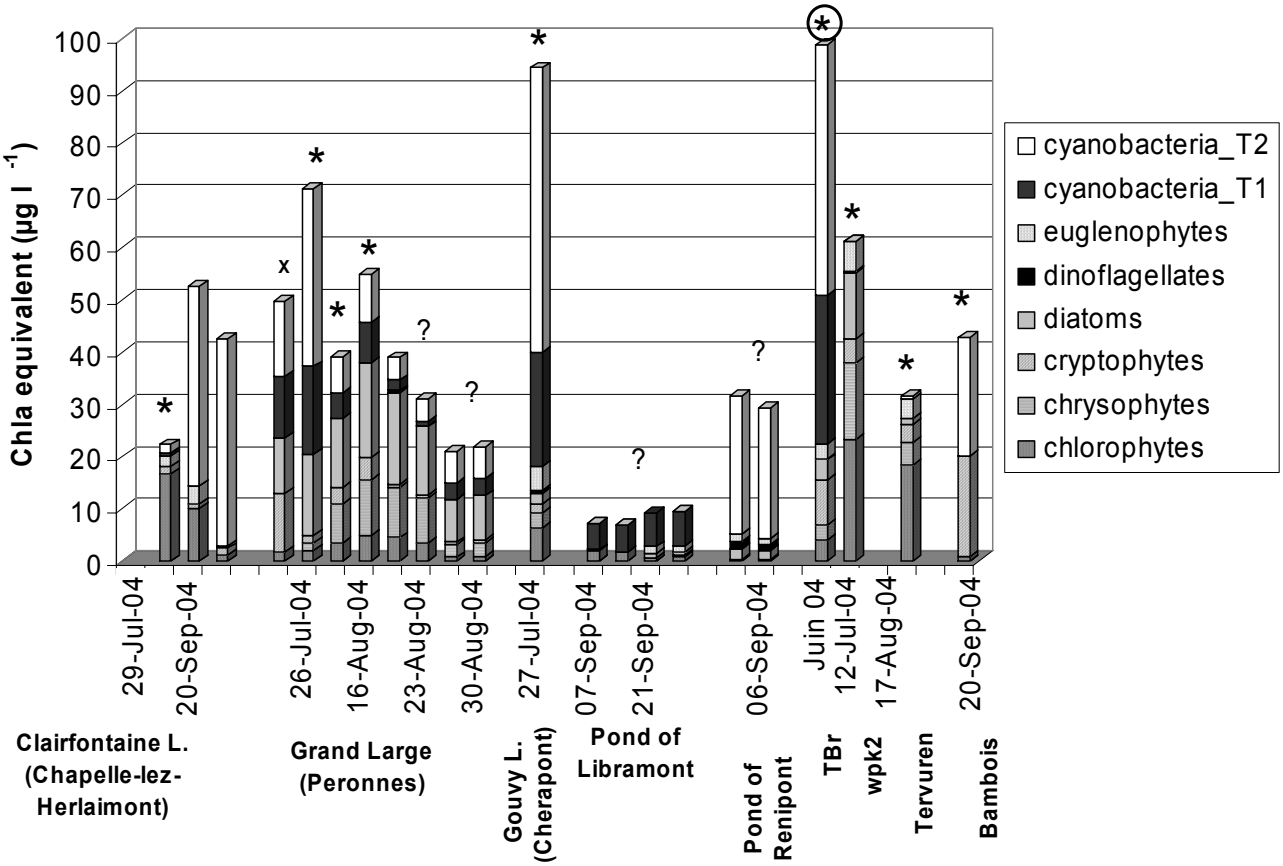


Figure 8 *: Algal composition of the BLOOMNET samples collected in Wallonia and Brussels in 2004, determined by HPLC. When not given, same date as previous one (duplicate samples). *: presence of both mcyB and mcyE genes (toxigenicity markers), x: presence of only one gene, ?: no test of genotoxicity markers, o: microcystin detected by HPLC on filters (table 3).*

1.4.2. BLOOMNET samples in Flanders

Blooms were sampled all over the Flemish territory (figure 9), but the majority of the bloom samples were taken in water bodies close to the city of Gent. Due to this bias, no conclusions can be made about the geographic distribution of blooms in Flanders.

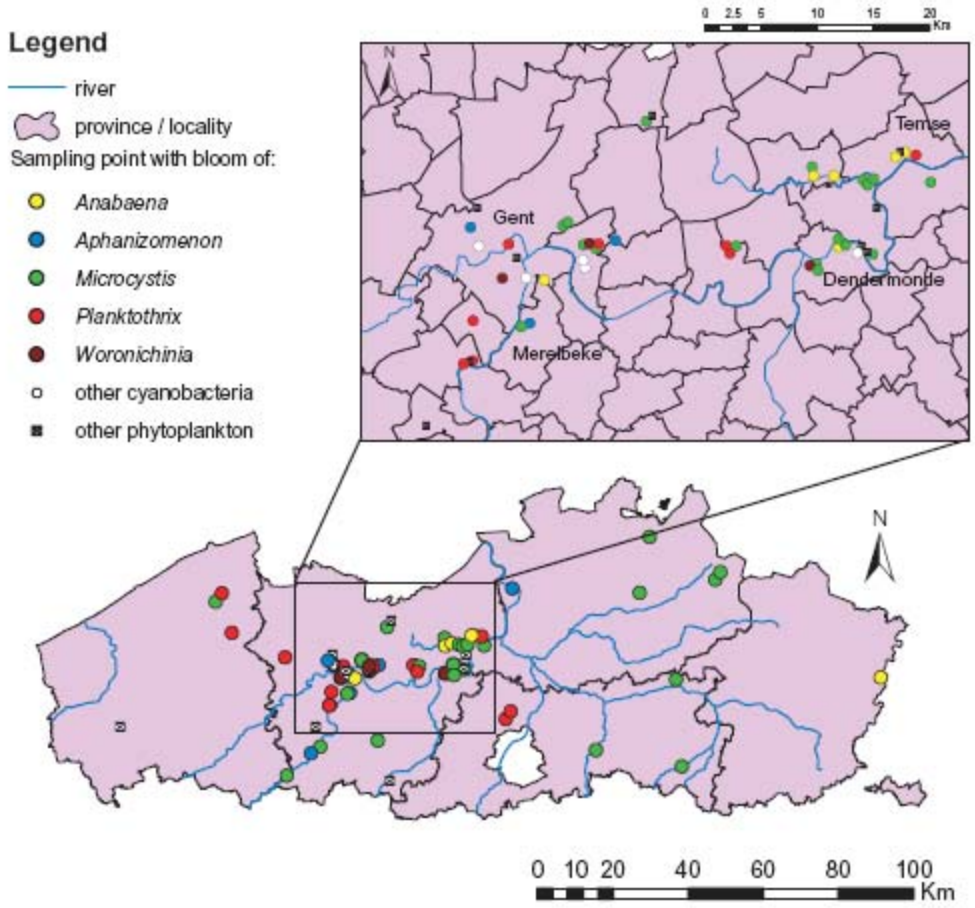


Figure 9: *Map of Flanders showing the localities and nature of sampled phytoplankton blooms in Flemish waterbodies for the period 2003-5.*

The majority (79 %) of the water bloom samples gathered contained a mass development of cyanobacteria (Table 3). However, 21 samples did not contain any blooms (Table 4). Most blooms were observed at the end of the summer, but the observation in March 2005 of a toxic bloom of *Planktothrix rubescens* from a pond in Beernem (Driekoningenvijver) indicated that cyanobacterial blooms may occur at lower water temperatures. Especially *Planktothrix* is adapted to somewhat colder conditions and can form blooms year round in our areas during mild winters (Scheffer *et al.* 1997).

Each year, *Microcystis* was the most important bloom-forming genus (figure 10). It dominated the biomass in 45 % of the sampled cyanobacterial blooms, followed by *Planktothrix* (20%), *Anabaena* (10 %) and *Aphanizomenon* (8 %). Several blooms contained a more or less equal contribution of several cyanobacterial taxa.

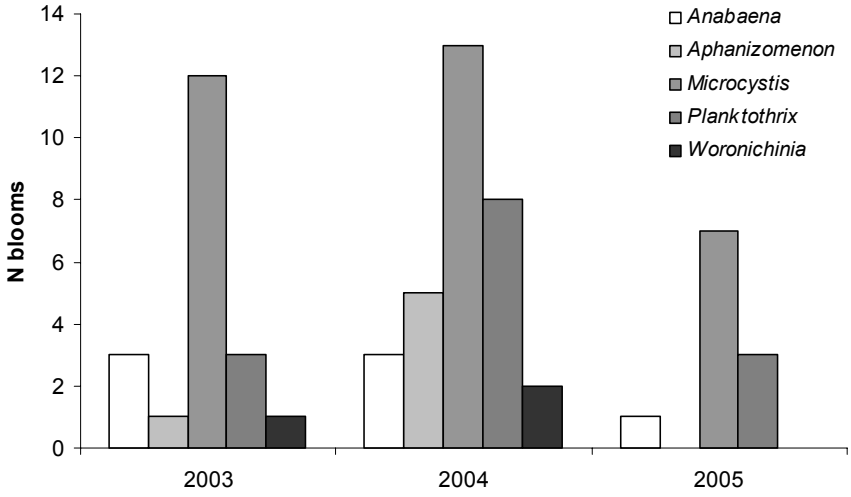


Figure 10: *Dominant bloom-forming cyanobacteria in bloom samples from Flemish water bodies for the period 2003-5.*

Two cyanobacterial blooms coincided with a mass mortality among waterfowl, with paralysis of wings, legs and neck ('limber neck') as the most frequently observed external symptoms. The first abnormal mortality (50 – 100 different birds) was detected in August 2004 in lake Leeuwenhof (Drongen) managed since 1998 as a nature reserve. The most important victims were waterfowl like water ducks and geese but also scavengers like *Pica pica* and *Larus ridibundus* and other birds like *Columba palumbus* were found dead. Dead fish (*Perca fluviatilis* and *Cyprinus carpio*) was visible at the site as well. Microscopical analysis showed a dominance of *Microcystis aeruginosa* and *M. flos-aquae*. Some weeks later, additional flocks of *Aphanizomenon flos-aquae* could be observed in the littoral zone. High concentrations of microcystins were measured in wind accumulated algal biomass from the littoral zone (up to 26 mg microcystins/L bloom material). Autopsies on 5 freshly dead victims were carried out by Dr. P. Tavernier and colleagues (University of Gent, Faculty of Veterinary Medicine). No anatomical abnormalities, which could be linked with other diseases, were observed. Samples from the liver, sent to the Pasteur Institute in Brussels for analysis of botulism toxins, seemed to be positive. Cyanobacterial toxins inside animal tissues could not be analyzed, since the needed technology was not (yet) developed in Belgium.

In July and August 2005, an extensive bloom of *Microcystis flos-aquae* and *Anabaena* sp. coincided again with mass mortalities (100-200) amongst the

waterfowl in the nature reserve Tiens Broek (Tienen). Amongst the victims were *Anas* species (89 individuals), *Fulica atra* (10), *Gallinula chloropus* (10), *Tadorna tadorna* (5), *Larus ridibundus* (20). The autopsy of one victim (*Fulica atra)* with paralysis symptoms was negative for botulism, suggesting cyanotoxin poisoning. Again, high concentrations of microcystins were measured in the accumulated algal biomass in the littoral zone (13 mg microcystins/L bloom material).

Cyanobacterial blooms can lead to mortality among waterfowl, directly by the production of cyanotoxins, which in turn provides dead bodies where *Clostridium botulinum* can proliferate, or indirectly by causing anoxia, which favors the production of botulism toxins. This is supported by a 15 year spanning time series, in which a strong and positive correlation was found between avian botulism outbreaks in Belgium (as percentage of treated birds in wildlife rehabilitation centers) and hot summers with low precipitation, conditions known to favor cyanobacterial blooms (figure 11).

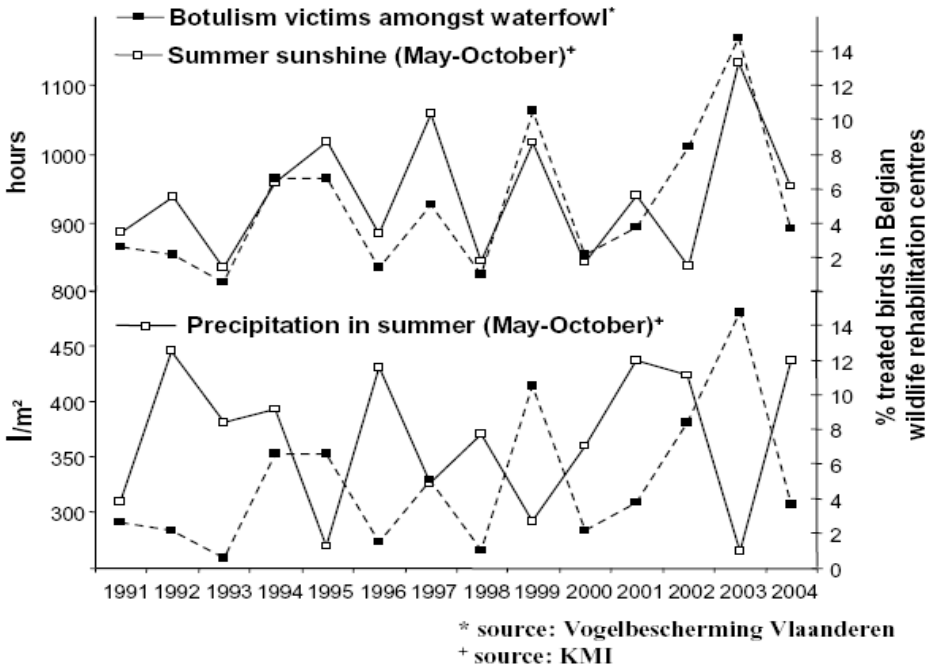


Figure 11*: Relation between avian botulism outbreaks in waterfowl and climatological conditions in Belgium. Botulism outbreaks are presented here as the percentage birds with typical symptoms of botulism intoxication to the total amount of birds that entered Belgian wildlife rehabilitation centres.*

2. Determination of toxigenicity and measurement of toxins

2.1. Determination of the toxigenicity

The presence of the *mcyB* and *mcyE* genes indicates that cyanobacteria possessing the genes involved in the microcystin synthesis were present. It has been shown that strains producing the toxins always express a basic level of expression, though it can be regulated 2 to 4 times by environmental conditions (Kardinaal & Visser 2005). Therefore, this test was used as a pre-screen to avoid analyzing all bloom samples by analytical methods, a costly procedure.

As can be seen in figure 12, the presence of the genes *mcyB* and *mcyE* was quite rare in lake Blaarmeersen (13% of double positives), and detected only during autumn 2003. In this period, *Aphanizomenon* and *Limnothrix* were present (figure 6). In contrast, the genes quite frequently detected in the lakes of d'Eau d'Heure, where toxigenicity was tested when a proliferation of cyanobacteria was visible. This selection of samples complicates the comparison as it probably increased the proportion of positive samples. There were 84% of double positives in Ri Jaune and 67% in Falemprise over the two years. In Féronval in 2003, only 50% of the 8 samples were double positives. No analyses were carried out in 2004 for Féronval, because no proliferation of cyanobacteria was observed.

For the additional bloom samples (Table 3), toxigenicity was tested for 72 Flemish BLOOMNET samples from 63 different lakes. 79% of the samples analysed by PCR were double positive. For the Walloon and Brussels' samples, this was the case in 10/11 blooms (from 7 water bodies). In the 3 Walloon blooms that were also characterized by microscopy, *Aphanizomenon* and/or *Microcystis* were recorded. In the 2 Brussels's ponds, all 4 samples were positive for both *mcy* genes. The Parc des Sources (TBr) contained a bloom of *Planktothrix*, whereas the Woluwe Park (WPk2) had a mixed community with mainly tiny diatoms, and some *Gomphosphaeria, Planktolyngbya* and a few *Planktothrix* (A. Peretyatko, pers. com.).

In some cases, the two tests did not give the same response. The *mcyB* test is targeting the *mcyB* gene of *Microcystis* only, whereas the *mcyE* primers were designed to amplify a portion of the gene in *Microcystis, Anabaena, Planktothrix* and *Nostoc*. Moreover, the sensitivities of the detections have not been compared. Differences in sensitivity can be due to the annealing and polymerisation efficiencies, the length of the amplicon, as well as the PCR conditions. Indeed, the *mcyB* detection was based on two successive PCR reactions whereas only one PCR is used for *mcyE*.

2.2. Identification of the toxin producer by RFLP of the *mcyE* gene

On the basis of the size of the bands, it was possible to detect the organism(s) holding the *mcyE* gene. It was *Microcystis* for GOUDKARPER (6/4/05), SCHU1

(16/8/05), GOVIBE (29/8/05), MOEVI (15/9/05) and SCHU3 (21/9/05) and *Planktothrix* for DRIKOBE (29/3/05 and 4/7/05) and BLAUWPUT (5/4/05). Interestingly, the second sample of DRIKOBE has been taken after the end of the bloom in July, but the presence of a toxigenic *Planktothrix* was still detected, and was confirmed by microscopic observations. A mixture of toxigenic *Microcystis* and *Planktothrix* seemed present in WEERHAAK (14/4/05), whereas *Microcystis* and *Anabaena* were detected in TIEBROE (9/8/05) and PLADILSTO (12/8/05). This determination of the dominant toxigenic cyanobacteria coincided with microscopical observations (Table 2), except in the case of MOEVI and WEERHAAK where no bloom had been observed by microscopy, TIEBROE where *Microcystis* was microscopically dominant and PLADILSTO where *Anabaena* was dominant. Thus, this test seems able to detect the potential toxin producers, even if they are not dominant or have not reached the densities of a bloom visible by microscopy.

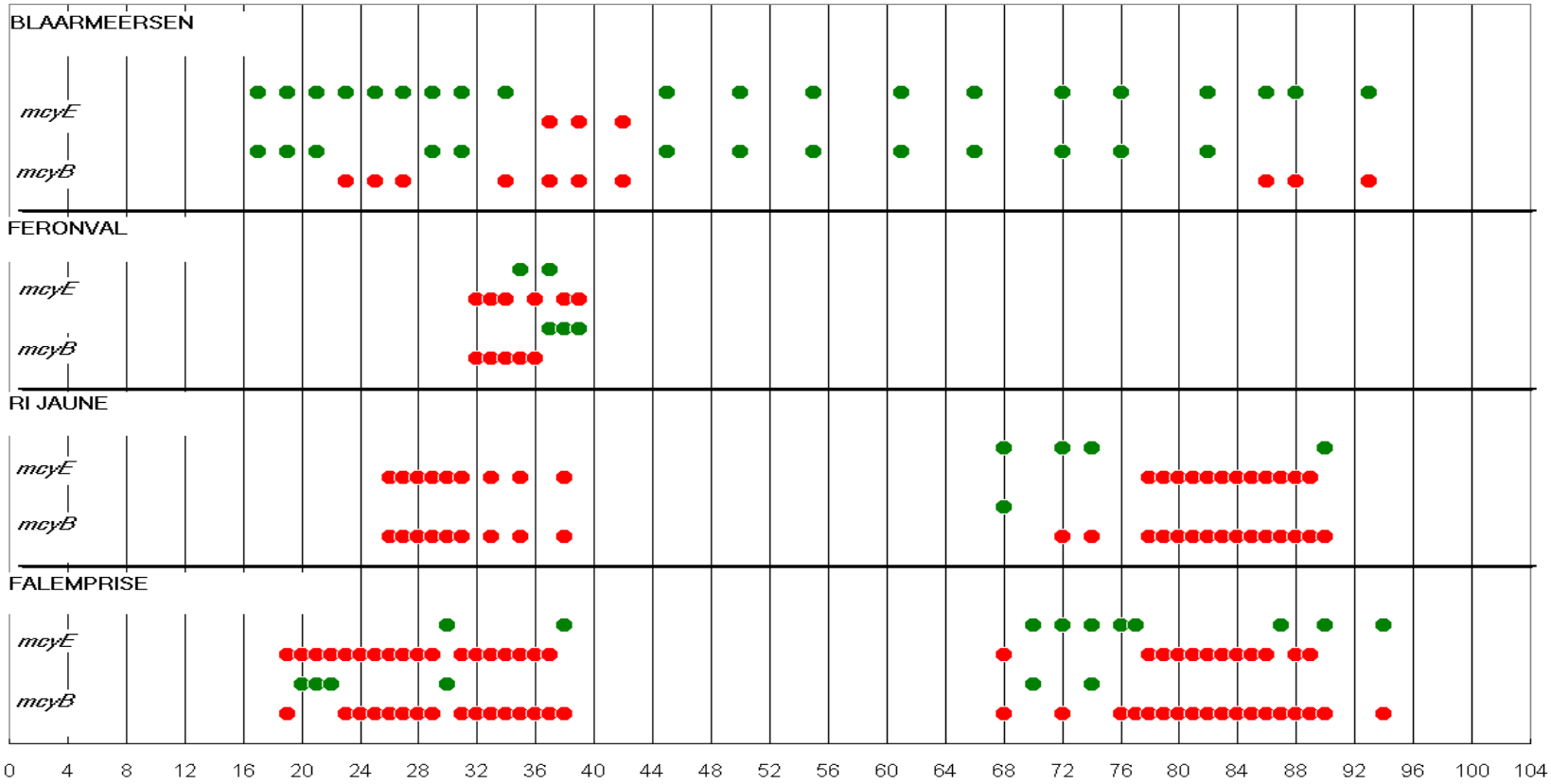


***Figure 12**: Monitoring of the toxigenicity in the four reference lakes from January 2003 till December 2004 (week number indicated). A red dot indicates the presence of a PCR product of appropriate size, whereas a green dot indicates the absence of this PCR product or the presence of a very faint band.*

2.2. Measurement of toxins

The colorimetric protein phosphatase inhibition assays (PP1 or PP2A) were positive for all samples containing cyanobacteria and positive for both *mcyE* and *mcyB* genes, collected either on filters or as bulk samples (not shown). The calibration curve showed a good correlation between microcystin-LR concentrations and inhibition of the enzyme. The assay thus could detect potential toxicity.

Nevertheless, when the samples were tested, there was no correlation between cyanobacterial biomass and the inhibition degree, and positive responses were also recorded for samples negative for one or both genes of toxicity, and for samples without cyanobacteria. In some samples, the presence of dinoflagellates, which produce okadaic acid, another potent phosphatase inhibitor, could have interfered with the evaluation of the inhibition from microcystins. However, samples negative for the toxicity genes and without dinoflagellates also presented a high inhibition of the enzyme. Thus, the PP inhibition assays seemed to respond to a variety of non-cyanobacterial toxins and metabolites and would require additional, more specific analyses of the toxins. It remains, however, a useful screening method (Metcalf et al. 2001). Notably, the concentrations of phosphatase inhibitors were even higher than the highest concentration of the standard used for the calibration curve, and were in the non-linear part of the curve. Dilutions of the extracts would have been necessary to obtain quantitative measurements but would require additional costs, not foreseen in the subcontract.

In agreement with CRITT-BioIndustries, the first HPLC measurements were carried out on filters. For 8 samples from 6 lakes (duplicate to quadruplicate measurements), no microcystins were detected. Then, 7 Walloon samples, 2 Brussels samples and 2 Flemish samples were again analysed using filters. Except in two samples, no microcystins were detected by HPLC, maybe because the amount of algal material on the filters was insufficient for HPLC detection in the conditions of the extract preparation used, due to the relatively low biomass collected (generally 250-500 ml). The two exceptions were the Parc des Sources (TBr) in Brussels and DRIKOBE (Beernem) in Flanders where 78 µg/L of microcystin-RR and 7.9 µg/L of microcystin-LR + 28.4 µg/L of microcystin-RR were found, respectively. In both cases, there was a bloom of *Planktothrix*.

In reaction to this detection problem, it was decided to carry out the HPLC on lyophilized algal material, to avoid being limited by the available biomass. Unfortunately, only the Flemish samples had been taken both as filters and lyophilized biomass. Therefore, no supplementary Walloon sample could be analyzed by CRITT nor FUNDP.

Finally, for the four reference lakes, only 6 samples were used for HPLC analyses (3 on filters and 1 on biomass by CRITT-BioIndustries and 3 on biomass by FUNDP) and all were negative (Table 2).

Forty-six of the 61 bloom samples from Flemish BLOOMNET lakes analysed by HPLC were positive for both *mcy* genes involved in microcystin production (Table 3). Microcystins were detected in 25 out of 61 samples, i.e. 41%. This corresponds to 54% of the samples that were double positive for the *mcy* genes. Total microcystin concentrations in the biomasses ranged from 18 µg/[g DW] to 2651 µg/[g DW] in 20 different Flemish lakes, as multiple samples were taken in several lakes. When the biomass was weighted, it was possible to calculate the total microcystin concentrations per liter of bloom samples. This gives concentrations between 0.35 and 25500 µg/L. These large differences in toxin concentrations are due to the fact that bloom densities varied from a dense bloom to a dilute suspension. To this, we must add the microcystin-LR and -RR found in DRIKOBE in filtered samples (see previous page), giving a total of 21 flemish BLOOMNET lakes with measurable microcystins. *Microcystis* was the dominant cyanobacterial genus in all Flemish samples where microcystins were detected, except in KLOBRVI and DRIKOBE, where it was *Planktothrix*.

A French recommendation of the circular DGS/SD7a-N°2003-270 of 4 June 2003 indicates a threshold value of 25 µg/L microcystin-LR to forbid the access for bathing in recreation waters. In 13 Flemish lakes, STWVPV (23/09/03), HABU1, HABU2, HABU3 (30/09/03), VILEDR (du 24/08/04 au 30/09/04), CENRUVI (28/09/04), KILGRVI (4/10/04), TERELZEN (12/05/05), LENP (19/05/05), SIM (26/07/05), TIEBROE (09/08/05), GOVIBE (29/08/05), SCHU3 (21/09/05), the waters should have been closed for bathing and recreation following the French recommendation. In 7 other lakes, the microcystin-LR values were detectable but still under the threshold. This was the case of KLOBRVI (25/09/04), BAAS2 (30/09/03), LIEOUVI (28/09/04), KWNMEVI (29/09/04), BREZOVI (29/09/04), DRIKOBE (29/03/2005), GOUDKARPER (06/04/2005). However, these were unique samples and it would have been necessary to monitor these waterbodies over time, with multiple sampling sites, to decide on the safety measures. In DRIKOBE 29/03/05, the dominant variant was microcystin-RR for which there is no norm. However, the sum of both variants exceeded the limit of 25 µg/L. Surprisingly, the microcystins were present in spring samples for DRIKOBE and GOUDKARPER, whereas blooms are generally expected in late summer and autumn. In a sample from DRIKOBE taken on 04/07/2005, when the *Planktothrix* bloom had disappeared, the presence of both *mcy* genes was still detected and the RFLP showed that they were produced by *Planktothrix*. *Planktothrix* might cause subsurface blooms, adjusting its depth in the water column thanks to its gas vesicles. Thus, either the bloom had gone deeper, or the few remaining *Planktothrix* filaments still gave a positive signal. Unfortunately, this summer sample was not tested for the presence of cyanotoxins by HPLC. For ROVIWA1, the volume was not measured, and the relation with the dry weight cannot be calculated.

In the case of Walloon BLOOMNET samples, the dataset is small due to the difficulties of CRITT-BioIndustries to measure microcystins with the filters and the lack of lyophilized biomass for additional measurements. Notably, in Gouvy, there was a slight presence of microcystin-LR (1.3 µg/L), that would necessitate some monitoring in the future. In the urban pond Parc des Sources in Brussels, the concentration of microcystin-RR was higher (75 µg/L) than the threshold of 25 µg/L given by the French recommendation for recreation waters. As *Planktothrix* only represented a low percentage of the total chlorophyll *a* (0.77 %), it could be a very toxic strain. However, till now, all norms concern microcystin-LR, and the toxicity of the RR variant is unknown. Anyway, it would have been advisable to forbid fishing and warn the public that dogs should not drink this pond water.

To test the efficiency of the pre-screening, 12 BLOOMNET samples with no or only 1 *mcy* gene were used for HPLC analyses and microcystins have never been detected. In contrast, half of the 46 double positive samples contained detectable microcystins. In 21 cases, double positive tests did not correspond to measurable microcystins but the concentrations could be too low for the HPLC analyses. Thus, the molecular pre-screening seems to allow a selection of samples likely to contain the cyanotoxin.

When the microcystin analyses were carried out by HPLC independently in two laboratories (CRITT BioIndustries and FUNDP), they often agreed on the presence of toxins but the quantification was generally different. This could be due to small differences in the extract preparations and the different instrumentations used.

Anatoxin-a was not detected in any of the samples by HPLC at CRITT-BioIndustries. Filters of Féronval (19/08/03), Falemprise (20/07/04), Bambois (20/09/04), Gouvy (20/09/04), BLODIAL (=BDANLO) (22/10/04) DRIKOBE (29/03/05), and Clairefontaine (26/07/04) did not show any anatoxin-a above the detection level (that ranged from 1.5 to 9 µg/L sample, depending on the filtered volume). In addition, this toxin was checked especially in the lyophilized biomass of 6 Flemish samples (VILEDR, WESGEVI, CENRUVI, BLAUWPUT, TERELZEN, TIEBROE) but no anatoxin-a could be detected above a detection level of ca 50 µg/g dry weight. The same negative data was obtained with a second analysis of 18 bloom biomasses from the Flemish lakes OVDO (21/08/03), BEBR (22/08/03), STWVPV (23/09/03), GEMAIN (23/09/03), BAAS2 (30/09/03), OUSCWE (30/09/03), PLAMEVI (27/09/04), ICCOUVI (28/09/04), DOBEME (29/09/04), KWEMEVI (29/09/04), KWNMEVI (29/09/04), KLOBRVI (25/09/04), LENP (10/05/05), SIM (26/07/05), PLADILSTO (12/08/05), GOVIBE (29/08/05), SCHU3 (21/09/05), and the littoral scum from Blaarmeersen lake (01/08/2003). In addition, Dr. C. Bernard (Systématique et Ecotoxicologie des Microalgues, Muséum National d'Histoire Naturelle, Paris, France) has kindly tested seven samples for anatoxins using the

method of Gugger et al. (2005). However, these samples, selected because of bird casualties, were also all negative (TIEBROE and LEVIDR).

This absence of anatoxin-a in our Belgian samples is in congruence with the fact that, in other european countries, this toxin has always been found at a much lower frequency than microcystins. In the Netherlands, a report on the presence of cyanotoxins in surface waters indicated that anatoxin was only detected in 7/40 samples, whereas microcystin was present at all sites (Hoogenboezem et al 2004). In France, anatoxin has only been found so far in benthic mats in rivers (Gugger et al 2005). In Finland, only 5% and in Spain, 7% of samples were shown to contain anatoxins, whereas the levels were higher (25%) in Germany (Carrasco et al. 2007).

***Table 2**: Results of toxigenicity testing and microcystin (MC) analysis of 7 samples from reference lakes gathered in Flanders and Wallonia for the period 2003-5. nd: not detected, na: not analysed, 1. analyzed by CRITT, 2: analyzed by FUNDP. (F) indicates that the quantification was made on filters.*

Date	Community	code	collector	dominant taxon	toxigenicity *mcyE*	toxigenicity *mcyB*	analyzer	MC-LR µg/ g DW	MC-RR µg/ g DW	MC-YR µg/ g DW
1/08/2003	Gent	BLAARM LIT	UG	*Anabaena*	na	na	1	nd	nd	na
							2	nd	nd	nd
11/09/2003	Gent	BLAARM PEL	UG	*Mougeotia, Anabaena*	+	+	2	nd	nd	nd
15/10/2004	Gent	BLAARM	UG	coccal greens, *Anabaena*	–	+	2	nd	nd	nd
19/08/2003	Eau d'Heure	Féronval	FUNDP	*Aphanizomenon*	+	+	1 (F)	nd	nd	na
20/07/2004	Eau d'Heure	Falemprise	FUNDP	*Aphanizomenon*	+	+	1 (F)	nd	nd	na
31/08/2004	Eau d'Heure	Ri Jaune	FUNDP	coccal	+	+	1 (F)	nd	nd	na

***Table 3**: Results of toxigenicity testing and microcystin (MC) analysis of 93 BLOOMNET samples gathered in Flanders, Wallonia and Brussels for the period 2003-5 (nd: not detected, na: not analysed). 1. analyzed by CRITT, 2: analyzed by FUNDP. In grey, are the samples with detectable amounts of microcystin. (F) indicates that the quantification was made on filters. DW: Dry weight. VVHV: Vlaamse Vereniging van Hengelsportverbonden, PVC: Provinciale Visserij Commissie, VMM: Vlaamse Milieu Maatschappij, INBO: Instituut voor Natuur- en Bosonderzoek, RW: Région Wallonne.*

Date	Community	code	collector	dominant taxon	toxigenicity		analyzer	MC-LR µg/g DW	MC-RR µg/g DW	MC-YR µg/g DW	MC total µg/g DW	MC total µg/l bloom material	MC-LR µg/L bloom material
					mcyE	*mcyB*							
FLANDERS													
5/08/2003	Heusden	HE ST	UG	*Anabaenopsis*	_	_	1	nd	nd	na			
							2	nd	nd	nd			
12/08/2003	Destelbergen	DE HD VV 1	UG	*Microcystis*	_	+	2	nd	nd	nd			
12/08/2003	Damme	WVDAMEX	MANSCAPE	*Euglena, Planktothrix*	–	+	1	nd	nd	na			
21/08/2003	Overmere	OV DO	UG	*Planktothrix*	_	+	1	nd	nd	na			
							2	nd	nd	nd			
21/08/2003	Overmere	BE BR	UG	*Microcystis*	+	+	1	nd	nd	na			
							2	nd	nd	nd			
9/09/2003	Heusden	HE MK P 1	UG	*Aphanizomenon*	+	+	2	nd	nd	nd			
17/09/2003	Waasmunster	RO VI WA 1	UG	*Microcystis*	+	+	2	107	103	36	246	n.a.	n.a.
17/09/2003	Waasmunster	RO VI WA 2	UG	*Anabaena*	+	+	2	nd	nd	nd			
17/09/2003	Wachtebeke	PUY 1	UG	*Microcystis*	+	+	1	nd	nd	na			
							2	nd	nd	nd			
17/09/2003	Waasmunster	OV DU WA	UG	*Anabaena*	+	+	2	nd	nd	nd			
23/09/2003	St-Amandsberg	ST WV PV	UG	*Microcystis*	+	+	1	418	775	na	1193	966	338
							2	517	258	54			
23/09/2003	Gent	GE MA IN	UG	*Woronichinia*	+	+	1	nd	nd	na			
							2	nd	nd	nd			
28/09/2003	Gent	WSPBN	UG	*Planktothrix*	+	+	2	nd	nd	nd			
28/09/2003	Drongen	DR LE GR	UG	*Oscillatoria*	+	_	2	nd	nd	nd			
30/09/2003	Hamme	HA BU 1	UG	*Microcystis*	+	+	2	186	122	35	343	122	66
30/09/2003	Hamme	HA BU 2	UG	*Microcystis*	+	+	2	196	147	28	371	300	158
30/09/2003	Hamme	HA BU 3	UG	*Microcystis*	+	+	2	782	119	54	954	761	623
30/09/2003	Grembergen	GRE 1	UG	*Microcystis*	+	+	2	nd	nd	nd			

30/09/2003	Grembergen	GRE 2	UG	*Anabaena*	_	_		na	na	na			
30/09/2003	Vlassenbroek	VLA 1	UG	> 1 cyanobacteria	+	+		na	na	na			
30/09/2003	Vlassenbroek	VLA 2	UG	*Microcystis*	+	+		na	na	na			
30/09/2003	Baasrode	BAAS 1	UG	*Snowella*	+	+		na	na	na			
30/09/2003	Baasrode	BAAS 2	UG	*Microcystis*	+	+	1	19	145	na	164	2	0.2
							2	158	164	21			
30/09/2003	Bornem	BO WI	UG	*Microcystis*	+	+	1	nd	nd	na			
30/09/2003	Weert	OU SC WE	UG	*Planktothrix*	+	+	1	nd	nd	na			
							2	nd	nd	nd			
1/08/2004	Temse Z		PVC	*Anabaena*	na	na		na	na	na			
1/08/2004	Temse M		PVC	*Anabaena*	na	na		na	na	na			
2/08/2004	Merelbeke	LIEMEVI	UG	*Anabaena*	_	_	2	nd	nd	nd			
9/08/2004	Eke	KRIEKVI	UG	*Planktothrix*	+	_		na	na	na			
16/08/2004	Dendermonde		PVC	*Pseudanabaena*	na	na		na	na	na			
16/08/2004	Dendermonde		PVC	*Microcystis*	na	na		na	na	na			
17/08/2004	Tervuren		FUNDP	*Aphanizomenon*	+	+		na	na	na			
24/08/2004	Drongen	VI LE DR	UG	*Microcystis*	+	+	1	459	nd	na	459	25500	25500
6/09/2004	Drongen	VI LE DR	UG	*Microcystis*	+	+	2	515	0	222	737	1667	1164
16/09/2004	Drongen	VI LE DR PEL	UG	*Microcystis*	+	+	2	403	0	185	589	2	1.4
16/09/2004	Drongen	VI LE DR LIT	UG	*Aphanizomenon*	na	na	2	nd	nd	nd			
16/09/2004	Drongen	VI LE DR SED	UG	*Microcystis*	na	na	2	107	0	98	206	3879	2014
22/09/2004	Gent	UNIGEVI	UG	*Synechococcus*	_	_	2	nd	nd	nd			
23/09/2004	St-Amandsberg	WESGEVI	UG	*Microcystis*	+	+	1	nd	nd	na			
23/09/2004	Heusden	HEUGEVI	UG	*Planktothrix*	+	+		na	na	na			
23/09/2004	Destelbergen	KDAGEVI	UG	*Microcystis*	+	+	2	nd	nd	nd			
24/09/2004	Heusden	POMKLBE	UG	*Oscillatoria*	+	+		na	na	na			
24/09/2004	Destelbergen	DAMGEVI	UG	*Woronichinia*	+	+	1	nd	nd	na			
24/09/2004	Destelbergen	KKDGEVI	UG	*Microcystis*	+	+		na	na	na			
25/09/2004	Brugge	MINBRVI	UG	*Microcystis*	+	+		na	na	na			
25/09/2004	Brugge	KLOBRVI	UG	*Planktothrix*	+	+	1	22	nd	na	22	0.35	0.35
25/09/2004	Heusden	HEUGEVJ	UG	*Microcystis*	+	+		na	na	na			
27/09/2004	Gavere	GRNGAVI	UG	*Planktothrix*	+	_		na	na	na			
27/09/2004	Meise	BOEMEVI	UG	*Planktothrix*	+	_	2	nd	nd	nd			

27/09/2004	Meise	PLAMEVI	UG	*Planktothrix*	+	+	1	nd	nd	na			
28/09/2004	Oudenaarde	LIEOUVI	UG	*Microcystis*	+	+	2	105	126	29	259	1	0.4
28/09/2004	Oudenaarde	ICCOUVI	UG	*Aphanizomenon*	+	+	1	nd	nd	nd			
28/09/2004	Kluisbergen	CENRUVI	UG	*Microcystis*	+	+	1	683	1968	na	2651	76998	19838
29/09/2004	Zottegem	BREZOVI	UG	*Microcystis*	+	+	2	114	44	11	169	2	1.3
29/09/2004	Berlare	DOBEME	UG	*Planktothrix*	+	_	1	nd	nd	na			
29/09/2004	Merelbeke	KWEMEVI	UG	*Aphanizomenon*	+	+	1	nd	nd	na			
29/09/2004	Merelbeke	KWNMEVI	UG	*Microcystis*	+	+	1	149	nd	na	149	6	6
30/09/2004	De Pinte	HAGDPME	UG	*Planktothrix*	+	+	2	nd	nd	nd			
30/09/2004	Drongen	VI LE DR	UG	*Microcystis*	+	+	2	603	0	228	832	843	611
4/10/2004	Dendermonde	FORDEVI	UG	*Microcystis*	+	+	2	nd	nd	nd			
4/10/2004	Dendermonde	DENDEVI	UG	*Woronichinia*	+	+	2	nd	nd	nd			
4/10/2004	Grimbergen	KILGRVI	UG	*Microcystis*	+	+	2	1422	52	19	1493	2051	1953
15/10/2004	Drongen	VI LE DR	UG	*Aphanizomenon*	+	+		na	na	na			
22/10/2004	Antwerpen LO	BD AN LO	UG	*Aphanizomenon*	_	_	1 (F) 2	nd nd	nd nd	na nd			
23/03/2005	Beernem	DRIKOBE	VMM	*Planktothrix*	na	na		na	na	na			
29/03/2005	Beernem	DRIKOBE	VMM	*Planktothrix*	+	+	1 (F)	7.9	28.4	na		36	8
5/04/2005	Bellem	BLAUWPUT	VVHV	*Planktothrix*	+	+	1	nd	nd	na			
6/04/2005	Retie	GOUDKARPER	VVHV	*Microcystis*	+	+	1	24	435	na	459	174	9.1
12/05/2005	Averbode	TERELZEN	VVHV	*Microcystis*	+	+	1	84	1161	na	1245	6568	443
19/05/2005	Poederlee	LENP	VVHV	*Microcystis*	+	+	1 2	22 45	nd 36	na 0	22 82	31 65	31 36
25/05/2005	Nijlen	GM	VVHV	*Planktothrix*	na	na		na	na	na			
04/07/2005	Beernem	DRIKOBE	VMM	*Limnothrix*, rare *Planktothrix*	+	+		na	na	na			
26/07/2005	Merksplas	SIM	VVHV	*Microcystis*	+	+	1 2	18 nd	nd nd	na nd	18	56	56
9/08/2005	Tienen	BEPUTI	UG	*Synechococcus*	_	+	2	nd	nd	nd			
9/08/2005	Tienen	PLABEPUTI	UG	*Synechococcus*	_	+	2	nd	nd	nd			
9/08/2005	Tienen	TIEBROE	UG	*Microcystis*	+	+	1	502	nd	na	502	12938	12938
12/08/2005	Stokkem	PLADILSTO	IN	*Anabaena*	+	+	1	nd	nd	na			
16/08/2005	VMM 452050	SCHU1	VMM	fragments *Anabaena*, *Microcystis*	+	+	2	nd	nd	nd			
29/08/2005	Beringen	GOVIBE	VMM	*Microcystis*	+	+	1	1023	490	na	1513	5600	3786
5/09/2005	VMM 452050	SCHU2	VMM	fragments	na	na	2	nd	nd	nd			

				Anabaena, Microcystis									
21/09/2005	VMM 452050	SCHU3	VMM	*Microcystis*	+	+	1 2	169 441	636 414	na 105	805 961	1294 708	272 325
WALLONIA/BRUSSELS													
09/09/2003	Henry Chapelle		MANSCAPE	picocyanobacteria	na	na		na	na	na			
25/06/2004	Brussels	Parc des sources TBr	VUB	*Planktothrix*	+	+		na	na	na			
28/06/2004	Brussels	Parc des sources TBr	Ulg	*Planktothrix*	+	+	1 (F)	nd	75 (F)	na			0
12/07/2004	Brussels	Parc de la Woluwe wpK2	VUB	*Gomphosphaeria,Planktolyngbya*	+	+	1 (F)	nd	nd	na			
26/07/2004	Péronnes	Le Grand Large E04	RW	*Planktothrix*	_	+		na	na	na			
22/07/2004	Brussels	Parc des sources TBr	VUB	*Planktothrix*	+	+		na	na	na			
26/07/2004	Chapelle lez-Herlaimont	Lac de Clairefontaine E02	RW	*Aphanizomenon*	+	+	1 (F)	nd	nd	na			
27/07/2004	Gouvy	Cherapont GC	RW	na	+	+	1 (F)	1.3	nd	na			1.3
16/08/2004	Péronnes	Le Grand Large M	RW	na	+	+		na	na	na			
17/08/2004	Tervuren		FUNDP	*Aphanizomenon*	+	+		na	na	na			
17/09/2004	Liège	Port Monsin	Ulg	*Microcystis*	+	+	1 (F)	nd	nd	na			
20/09/2004	Bambois		FUNDP	*Aphanizomenon, Microcystis, Anabaena*	+	+	1 (F)	nd	nd	na			
23/09/2004	Liège	Port Monsin	Ulg	*Microcystis*	+	+		na	na	na			

***Table 4**. List of Flemish and Walloon BLOOMNET samples with no cyanobacterial blooms. Abbreviations as in table 3.*

Date	Community	code	collector	dominant taxon
16/07/2003	Zemst	VBZEMIN	MANSCAPE	*Euglena, Trachelomonas*
23/07/2003 *	Sorée	NASORNA	MANSCAPE	*Euglena, Trachelomonas*
23/07/2003 *	Sorée	NASORIN	MANSCAPE	coccal
29/07/2003	Tielt-Winge	VBTWIN	MANSCAPE	*Euglena, Trachelomonas*
1/08/2003	Woumen	WVBLAIN	MANSCAPE	no bloom
4/08/2003	Zottegem	OVZOTEX	MANSCAPE	*Euglena*
12/08/2003	Damme	WVDAMNA	MANSCAPE	*Euglena*
28/08/2003 *	Waillet	LXWAIIN	MANSCAPE	no bloom
4/09/2003	Knokke	WVKNOIN	MANSCAPE	no bloom
11/09/2003 *	Morialmé	NAMORIN	MANSCAPE	*Euglena*
17/09/2003	Wachtebeke	PUY FONT2	UG	*Oocystis*
28/09/2003	Mariakerke	MA SE GR	UG	coccal greens
30/09/2003	Vlassenbroek	VLA GR	UG	coccal greens
1/08/2004	Temse N		PVC	flagellate
22/09/2004	Gent	CONGEVI	UG	flagellate
27/09/2004	Gavere	GREGAVI	UG	*Trachelomonas*
12/04/2005	Kruibeke	SCHELDERUST	VVHV	no bloom
12/04/2005	Ename	ROOSJES	VVHV	no bloom
14/04/2005	Tielen	WEERHAAK	VVHV	no bloom
22/04/2005	Mol	KMZ	VVHV	no bloom
25/07/2005	Baasrode	BAM	VVHV	*Ceratium*
15/09/2005	Moerzeke	MOEVI	VMM	*Euglena*
21/09/2005	Mullem	WAMUL	UG	*Euglena*
21/09/2005	Kanaal Ijzer-Ieper		VMM	*Euglena*
23/09/2005	Geraardsbergen	DEGEJA	UG	*Euglena*

* sample from Wallonia

3. Genotypic diversity of bloom-forming cyanobacteria

3. 1. Cyanobacterial genotypic diversity in lake Blaarmeersen

3.1.1. Denaturating Gradient Gel Electrophoresis

Figure 13 shows the DGGE profile of the cyanobacterial community of the samples taken in lake Blaarmeersen from April 2003 to December 2004 at depths of 0,5 m and 7,5 m (and a few samples at depths of 1,5 m and 3,5 m)(van Gremberghe et al. 2008). Real blooms were not detected in this lake, which is reflected in the DGGE profile by many rather weak bands. The most abundant cyanobacterium in the DGGE profile is *Synechococcus*, represented by three genotypes (bandclasses 10, 14, 18; see table 4). It is known from the literature that the genus *Synechococcus* includes tiny unicellular cyanobacteria belonging to different lineages (Robertson et al. 2001). Consequently, it is very likely that the three genotypes on the DGGE profile represent three different species or genera. The genotype at position 10 was present in Lake Blaarmeersen during the whole sampling period at both depths, whereas the genotype at position 18 was only found in summer and autumn. The genotype at position 14 was only detected in the summer of 2004 at a depth of 7,5 m. So, it seems that these genotypes represent also different ecotypes. A similar observation was made by Zwart et al. (2005) for three different genotypes of *Synechococcus* in Lake Loosdrecht, represented by three bands at different positions in the DGGE gradient. Another abundant cyanobacterium in the DGGE profile was *Limnothrix redekei* (band class 16). This species was found in 2003 in summer at a depth of 7,5 m and in autumn at both depths. In 2004, *L. redekei* was also detected in summer and autumn in agreement with microscopic observations. Previous studies have shown that *L. redekei*, like other species belonging to the *Oscillatoriales*, needs lower temperatures and light intensities than most other freshwater cyanobacterial genera (Mur & Schreurs 1995, Wojciechowska et al. 2004). This can explain the dominance of *Limnothrix redekei* in the hypolimnion during stratification. Furthermore, *L. redekei* was also present in lake Blaarmeersen during periods of mixing (autumn), which confirms that this cyanobacterium grows well in turbulent conditions (Wojciechowska et al. 2004, Zwart et al. 2005).

***Figure 13**: DGGE profile of the cyanobacterial community structure of the samples taken in Lake Blaarmeersen (van Gremberghe et al. 2008). Band classes are indicated at the top, date and depth at the right side. The bands numbered were sequenced.*

***Table 5**. Closest relatives of the sequenced bands from the DGGE profiles from lake Blaarmeersen (for numbers of bands and band classes, see figure 13) (v. Gremberghe et al. 2008).*

Band class	Band number	Sequence length (bp)	Most similar sequence	Accession number	% similarity
7	8	88	plastid (diatom)	AY858017	100
8	13, 14, 15, 17, 21	136	plastid (haptophyte)	DQ363182	100
9	11	137	plastid (diatom)	AY678496	99
10	7, 9, 18	136	*Synechococcus* 0tu28s07	AM259221	100
12	1	114	plastid (cryptophyte)	AY453067	100
14	16, 20	136	*Synechococcus* PS838	AF448068	99.2
15	2, 3, 4	122	chloroplast (diatom)	AF418973	100
16	6, 12, 22	134	*Limnothrix redekei* SAG3.89	AJ544070	100
18	5, 19	120	*Synechococcus* MH301	AY224199	100
19	10	121	*Aphanizomenon flos-aquae* PCC7905	AY038035	100

The nitrogen-fixing cyanobacteria *Anabaena* and/or *Aphanizomenon* were detected in lake Blaarmeersen in late summer 2003 at 0,5 m (band class 19). By sequencing a short fragment of the 16S rRNA, it was not possible to distinguish between those two genera (Gugger *et al*. 2002). However, microscopic determination confirmed that both genera were present in the lake in summer (figure 6) though during a longer period. It is known from the literature that the genera *Anabaena* and *Aphanizomenon* sometimes form blooms at the water surface in high summer when the water column is stable (Salmaso 2000). At the end of the summer, the concentration of dissolved nitrogen is low and nitrogen-fixers can dominate under these circumstances. The higher abundance of *Anabaena* and *Aphanizomenon* in 2003 compared to 2004, is possibly due to the warmer weather conditions in 2003.

Statistical analyses were carried out on the standardized community composition of the taxa with the highest biomasses: *Synechococcus* (band class 10, 14 and 18), *Limnothrix redekei* and *Anabaena/Aphanizomenon*. Analyses of Similarity (ANOSIM, Primer 5, Clarke and Gorley 2001) revealed significant differences in the cyanobacterial community structure between the 'growing season' (late spring, summer and early autumn) and 'winter season' (late autumn, winter and early spring) ($R = 0,47$; $p = 0,001$) and significant differences in the cyanobacterial community structure between depths of 0,5 m and 7,5 m ($R = 0,239$; $p = 0,001$) during the growing season, while such a difference with depth was not found in winter (v. Gremberghe et al. 2008).

In conclusion, the cyanobacterial community in lake Blaarmeersen, investigated by DGGE, differed from season to season and from year to year. During the stratification period, there was also a difference between the community in the epilimnion and hypolimnion.

3.1.2. Clone library of a sample of Blaarmeersen

For the clone library from **Blaarmeersen 18/07/03**, 36 clones were obtained and appear all related to organisms commonly found in freshwater lakes in Europe and Asia (table 6). The coverage index (Good 1959) was 97.2%. Eighteen clones were related to sequences of the genus *Anabaena* (e.g. *Anabaena spiroides* strain PMC 9403) that dominate the clone library, five were related to unicellular cyanobacteria corresponding to *Synechococcus* sp. (*Synechococcus* sp. PS717) and eight to another cluster identified as the same genus (*Synechococcus* 1tu14s11), one clone is related to *Aphanothece sacrum* and four to the genera *Limnothrix/Pseudanabaena* (e.g. *Limnothrix redekei* CCAP 1443/1). This confirms and extends the diversity found with DGGE. The sequences related to *Anabaena* represent 50% of the clone library but did not appear in the sequenced DGGE bands. However, they might belong to a band class for which no sequences were obtained.

***Table 6**. Most similar strain sequence for the Blaarmeersen clones*

Number of clones	Most similar strain sequence	Accession number	% similarity	Origin
18	*Anabaena spiroides* PMC9403	AJ293116	98-100	French lake
5	*Synechococcus* PS717	AF216953	99-100	Japanese lake
8	*Synechococcus* 1tu14s11	AM259272	97-99	Finnish lake
1	*Aphanothece sacrum*	AB094350	99	Japanese spring water pond
4	*Limnothrix redekei* CCAP1443/1	AJ580007	99-100	German lake

3.2. L' Eau d'Heure complex

3.2.1. Denaturating Gradient Gel Electrophoresis

Figure 14 shows the DGGE profile of the cyanobacterial community of the samples taken in the pre-dam lakes Falemprise and Ri Jaune of the 'Eau d'Heure complex' in spring and summer 2003 and spring and summer 2004 (see also table 7). In the DGGE profile, there were several strong bands representing blooms. There was a bloom of *Anabaena/Aphanizomenon* (*Aphanizomenon flos-aquae* according to the microscopic determination) on 15-6-04 in Falemprise, and a bloom of *Planktothrix* on 21-06-04.

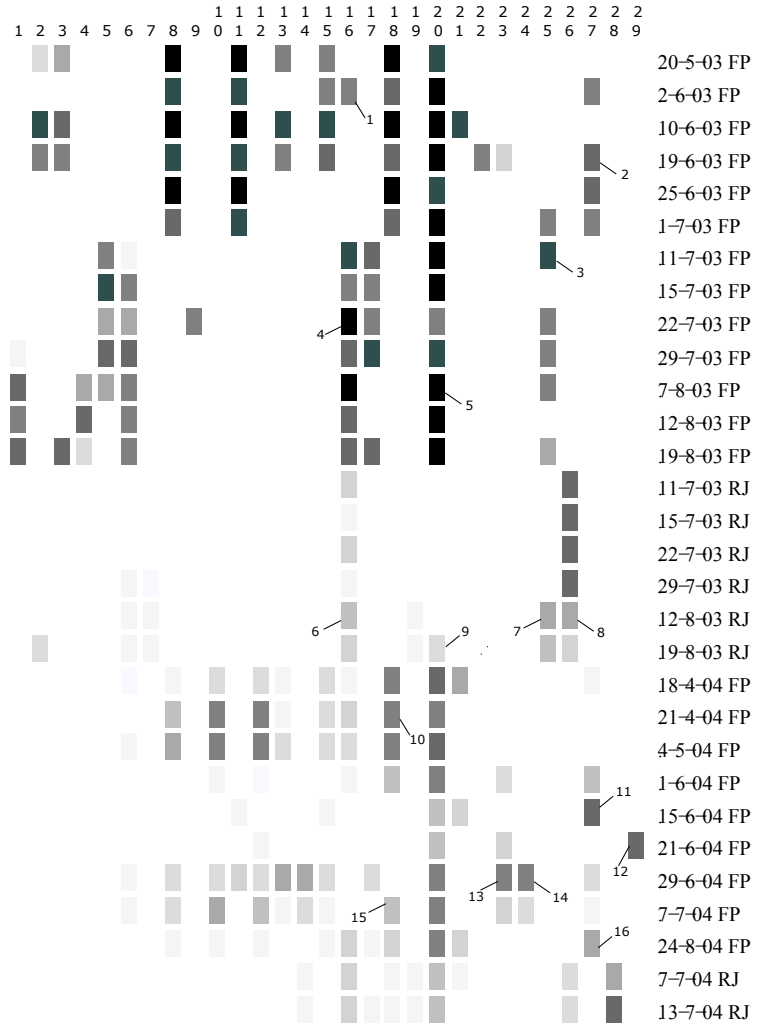


***Figure 14**: DGGE profile of the cyanobacterial community structure of the samples taken in Falemprise (FP) and Ri Jaune (RJ) Band classes are indicated at the top, date and sample name at the right side. The bands numbered were sequenced.*

This short-time shift was possibly due to changing weather conditions which caused a lot of turbulence in the lake Falemprise due to its low depth. These conditions could have favoured the growth of *Planktothrix* instead of heterocystous taxa. The nutrients released when the *Aphanizomenon* bloom collapsed, might also have stimulated the growth of *Planktothrix*. In Ri Jaune, there was a dominance of *Synechococcus* in both years. Furthermore, *Synechococcus* was also detected in Féronval (data based on three samples in 2003, not shown).

Table 7. *Closest relatives of sequenced bands from DGGE profiles of Falemprise and Ri Jaune (for numbers of bands and band classes, see figure 14).*

Band class	Band number	Most similar sequence	Accession number	% similarity
18	10, 15	clone cyanobacterium lac Erie	AY858021	99
20	5, 9	*Synechococcus* sp. MH305	AY224198	100
23	13	*Anabaena* sp 7VI37S1	DQ264177	98
24	14	*Aphanizomenon gracile* HEANEY/Camb 1986 140	AJ630444	99
25	3, 7	*Microcystis viridis* KCTC AG10198	AY121357	100
26	8	*Synechococcus* sp. RS9920	AY172830	100
27	2, 11, 16	*Aphanizomenon flos-aquae* Aph Ku	AY196085	100
29	12	*Planktothrix rubescens* PMC11002	AJ544068	99

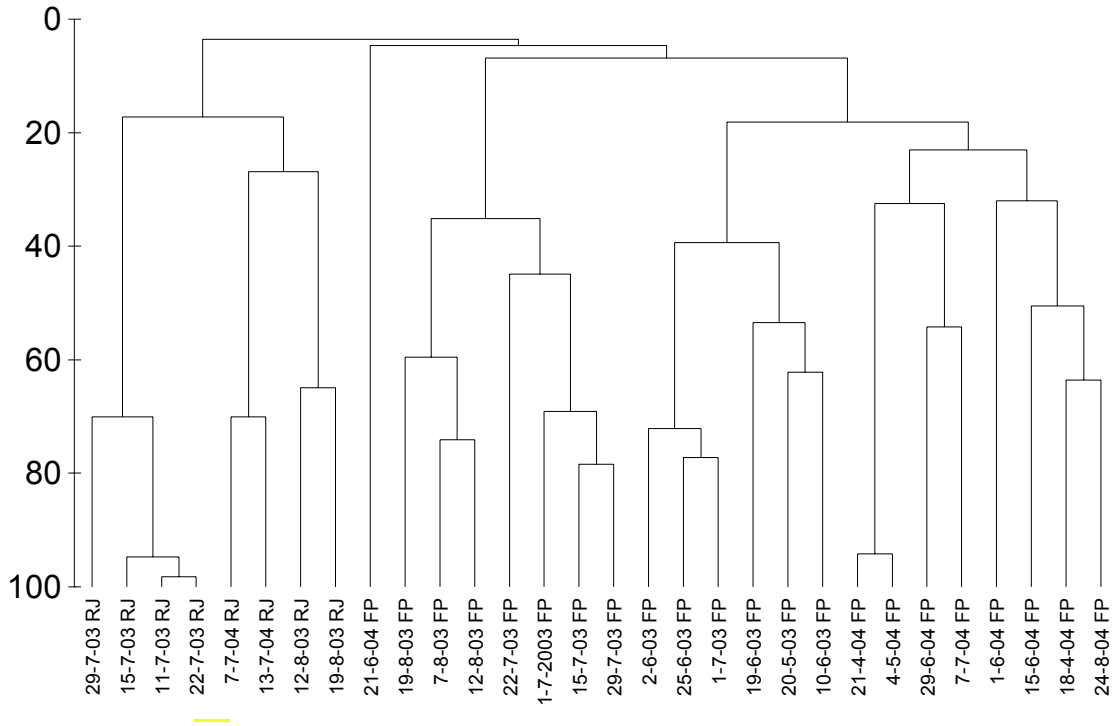


Figure 15: *Bray-Curtis cluster of the cyanobacterial community structure of the samples from Falemprise and Ri Jaune.*

A Bray-Curtis cluster-analysis on the dataset containing the samples from Falemprise and Ri Jaune (see figure 15) clearly shows that the cyanobacterial community differed between the two lakes and between the two years. The sample characterized by a *Planktothrix* bloom (21-06-04 FP) clusters separately from the other samples of 2004.

According to the ANOSIM-algorithm (Primer 5), the community structure differed significantly between Falemprise and Ri Jaune (R = 0,713; p = 0,001). A Simper-analysis (Primer 5) revealed the discriminating cyanobacteria responsible for the difference between the two lakes. The most important are *Anabaena/Aphanizomenon* in Falemprise and *Synechococcus* in Ri Jaune.

3.2.2. Clone libraries of the Eau d'Heure lakes

For the clone library of **Falemprise 01/07/03**, 74 clone sequences were obtained (table 8). According to the BLAST analysis, these sequences were all related to sequences of *Aphanizomenon/Anabaena* that dominated the clone library. The most similar strain sequences were from freshwater or brackish biotopes. Two clusters could be distinguished, related to *Aphanizomenon flos-aquae* PMC9707 or strain 'BC aph' 9601. The sequence of clone B10 is distinct, due to 12 substitutions in a segment of 59 positions. However, no database sequence shares all the same polymorphic positions and therefore, a chimeric origin cannot be proven. Considering B10 as real, the coverage index (Good 1959) was 98.6%. The comparison with the DGGE data shows that bands belonging to *Microcystis* and *Synechococcus* did not have counterparts in the clone library.

***Table 8**. Most similar strain sequences for the Falemprise clones*

Number of clones	Most similar strain sequence	Accession number	% similarity	Origin
69	*Aphanizomenon flos-aquae* PMC9707	AJ293130	99-100	French dam
4	*Aphanizomenon* sp. 'BC aph' 9601	AJ245457	99	Baltic Sea
1	*Aphanizomenon flos-aquae* PMC9707	AJ293130	97	French dam

For **Féronval (19/08/03),** 52 clone sequences were obtained, from which 3 chimera were removed. The coverage index (Good 1959) was 98%. The phylogenetic analysis showed they all belonged to three lineages in the genera Aphanizomenon/Anabaena. The difference between the clusters II and III was small (ca 2.5 % sequence similarity), but a bootstrap value of 95% supported the distinction of lineage II. The closest related strains came from freshwater lakes or the brackish waters of the Baltic Sea (table 9).

***Table 9**. Most similar strain sequences for the Féronval clones*

Number of clones	Most similar strain sequence	Accession number	% similarity	Origin
44	*Aphanizomenon issatchenkoi* 0tu37s7	AJ630446	99-100	Finnish lake
1	*Aphanizomenon* sp. 'BC-Aph' 9601	AJ245457	99	Baltic Sea
4	*Anabaena spiroides* PMC9403	AJ293116	99-100	French lake

For **Ri Jaune (31/08/04),** 39 clone sequences were obtained, including 1 bacterium sequence related to *Myxobacterium* AT1.01 and two plastid sequences related to green algae. Four chimeras were detected and discarded. Whereas three of them were mosaics of sequences already found among the clones, the fourth one contained a segment of 200 bp that matched at 98 % similarity with a clone from a salt pan in the Bahamas (DQ424724). The coverage index of the library (Good 1959) was 87.5%, showing that the analysis of more clones could have revealed some additional diversity.

The most similar strains for all cyanobacterial clusters came from freshwater ponds. The sequences were distributed in 7 clusters (table 10). Cluster I corresponded to the genus *Microcystis*. Cluster II contained sequences related to *Woronichinia*, a colonial coccoid planktonic cyanobacterium (Rajaniemi et al. 2005b) that dominated the clone library. Cluster III contained one sequence quasi-identical to the one of *Aphanizomenon flos-aquae* var Klebahnii 21. The four last clusters all corresponded to sequences assigned to *Synechococcus*, but with some sequence polymorphisms enabling a separation. Cluster IV contained one sequence identical to the one of the marine strain MBIC10007, surprisingly. Cluster V included a sequence similar to PCC7920. Cluster VI contained a sequence related to the one of PS717 and cluster VII gathered 5 sequences related to CENA108 and LBG2. This sample was not used for DGGE (table 10).

Whereas the samples of Féronval and Falemprise appear each dominated by one single genotype, different for each lake, the clone library of Ri Jaune has a more balanced composition with 7 genotypes, of which 3 are more abundant. It was also the only lake where *Microcystis* was found in the clone libraries.

***Table 10**. Most similar strain sequences for the Ri Jaune clones*

Number of clones	Most similar strain sequence	Accession number	% similarity	Origin
4	*Microcystis wesenbergii* TC7	AB035553	99	Thailand
19	*Woronichinia naegeliana* 0LE35S1	AJ781043	98-100	Czech pond
1	*Aphanizomenon flos-aquae* var Klebahnii 218	AJ293123	100	Danish lake
1	*Synechococcus* MBIC10007	AB058205	100	Pacific Ocean
1	*Synechococcus* PCC7920	AF216948	99	Finnish pond
1	*Synechococcus* PS717	AF216953	99	Japanese lake
5	*Synechococcus* CENA108	EF088334	97-99	Brazilian waste pond

3.3. BLOOMNET lakes and ponds

3.3.1. Cyanobacteria detected in the BLOOMNET lakes and ponds by DGGE

Figure 16 shows the DGGE profiles of the cyanobacterial communities of the samples taken in the extra lakes and ponds containing cyanobacterial blooms (2003: 24 ponds from Flanders; 2004: 25 ponds from Flanders, 6 ponds from Wallonia and 2 ponds from Brussels; 2005: 17 ponds from Flanders). From four lakes (Leeuwenhofvijver [VILEDR], Schulensmeer [SCHU], Driekoningenvijver [DRIKOBE] and Parc des Sources [TrBr]), two or three samples were taken during one summer period. Samples from the pond in the 'Westveld park' (STWVPV/WESGEVI) were taken in two consecutive years (2003 and 2004). The most important blooming genus was *Microcystis*, followed by *Planktothrix* (Table 7). On the DGGE profiles, it is visually very clear which band corresponds to the blooming cyanobacteria, because most of the lakes have one very strong band and only a few weak bands.

ANOVA (Analysis of Variance, Statistica 6.0) was done on the samples from Flanders taken in 2003 and 2004 to look for environmental conditions that stimulate the growth of certain taxa. These analyses revealed that *Planktothrix* was found at significant lower temperatures and at significant higher concentrations of nitrate in comparison to *Anabaena* and *Aphanizomenon*. *Planktothrix* was also found at significant higher concentrations of ammonium in comparison to *Microcystis*.

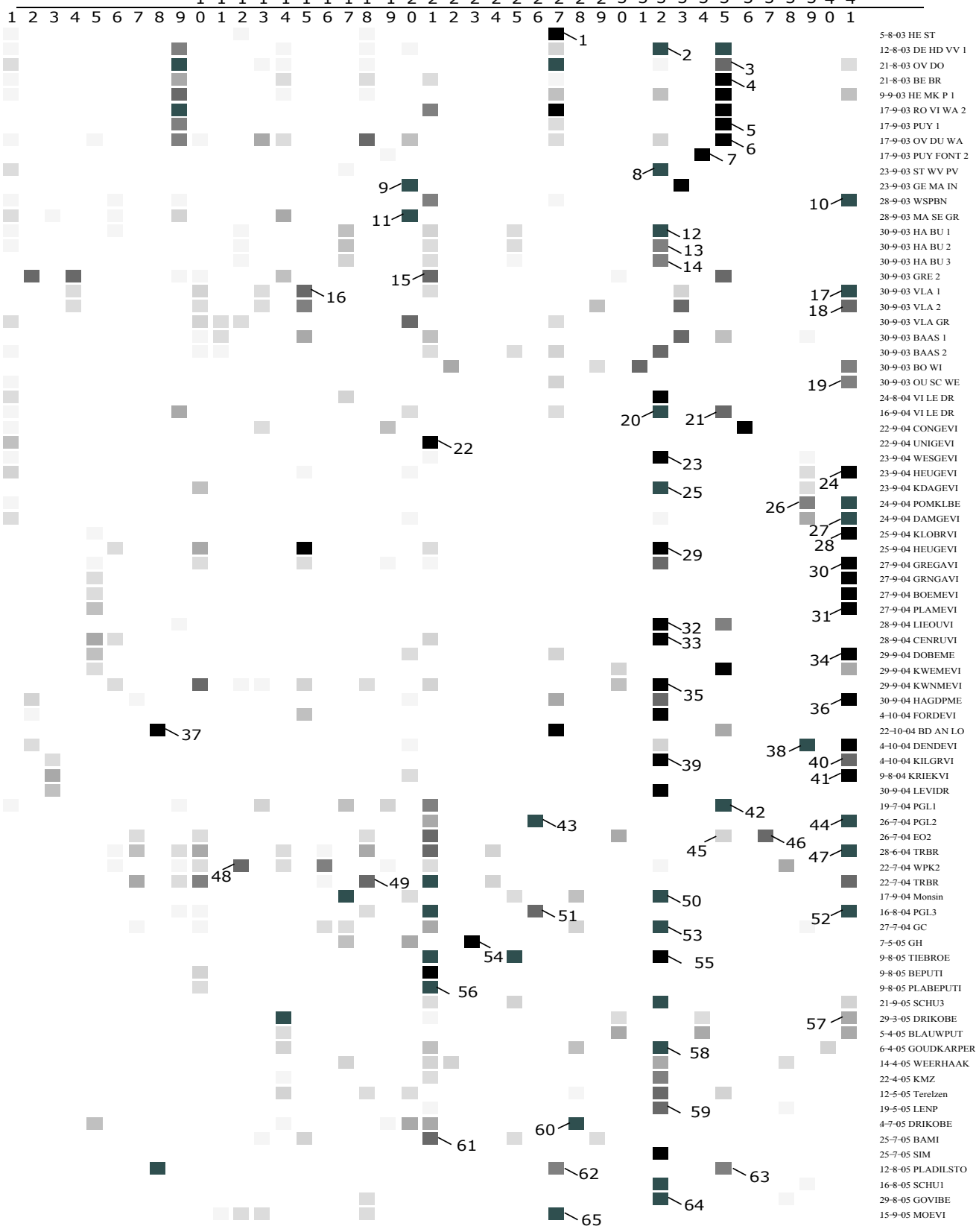


Figure 16: *DGGE profile of the cyanobacterial community structure of the BLOOMNET samples taken in the additional lakes and ponds*

***Table 11**: Closest relatives of sequenced bands from the DGGE profiles of the BLOOMNET lakes and ponds. For number of bands and band classes, see figure 16.*

band class	number band	closest relatives	accession number	% similarity
8	37	*Anabaena variabilis* A514	EF488831	99
12	48	clone cyanobacterium lake Erié	AY858017	100
15	16	plastid (diatom)	Z77548	99
18	49	clone cyanobacterium lake Erié	AY858021	99
20	9, 11	*Microcystis* 1tu31S06	AM259270	100
21	15	*Synechococcus* sp. MH305	AY224198	100
21	22	clone *Synechococcus* aerosol	AY436570	98
21	56	*Synechococcus* sp. CC9605	CP000110	100
21	61	*Synechococcus* sp. MW73B4	AY151250	100
23	54	*Aphanothece sacrum*	AB116658	100
26	43, 51	clone cyanobacterium salt marsh	AY712382	99
27	1	*Aphanizomenon issatschenkoi* TAC419	AY196087	100
27	62, 65	*Anabaena spiroides* NIES79	AY701564	100
28	60	*Limnothrix redekei* SAG3.89	AJ544070	100
32	2, 8, 12, 13, 14, 20, 23, 25, 29, 32, 33, 35, 38,50, 53, 55, 58, 59, 64	*Microcystis aeruginosa* KCTC AG10159	AY121356	100
35	3, 4, 5, 6, 21 42, 45, 63	*Anabaena spiroides* PMC9702	AJ293118	100
37	46	*Synechococcus* RS9920	AY172830	100
39	26, 38	*Woronichinia naegeliana* 1ES42S1	DQ264221	98
41	10, 17, 18, 19, 24, 27, 28, 30 31, 34, 36, 40, 41, 44, 47, 52, 57	*Planktothrix agardhii* PMC7502	AJ544065	100

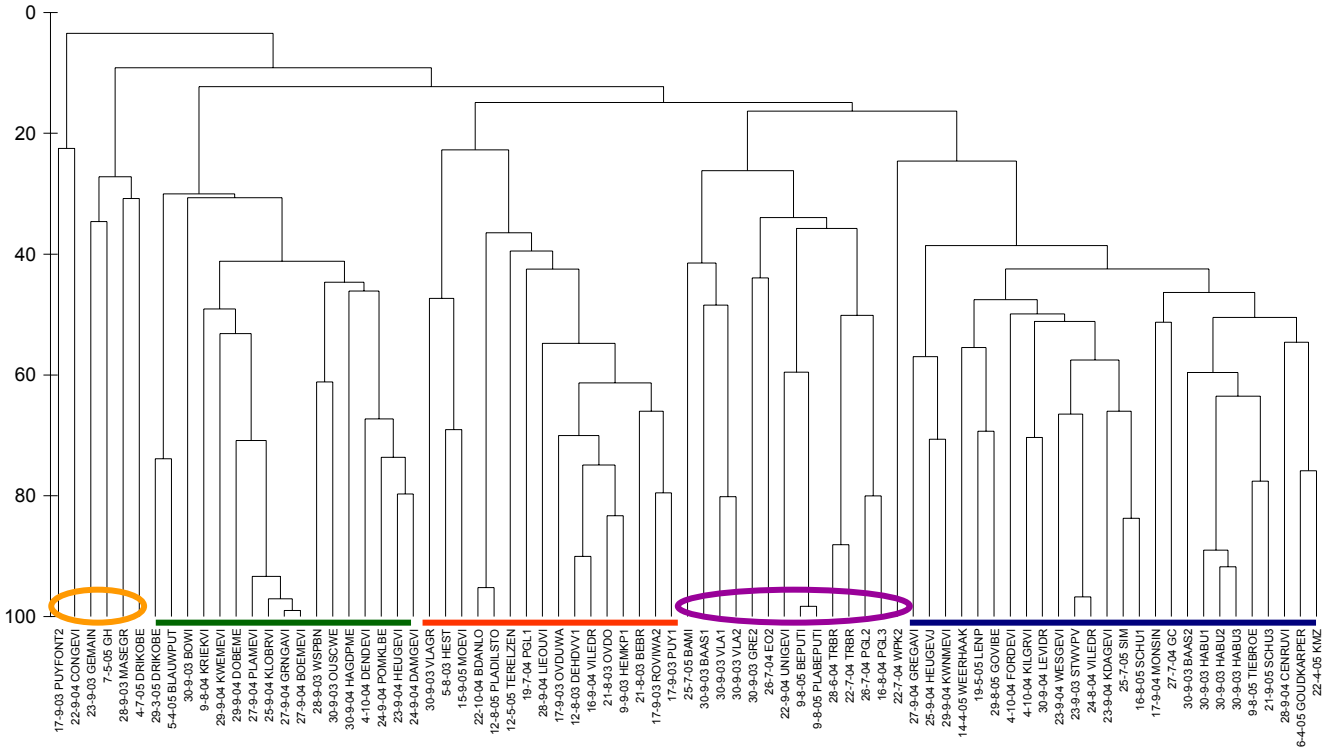


***Figure 17**: Bray-Curtis cluster of the cyanobacterial community structure of the samples from the extra lakes and ponds (green: Planktothrix, red: Anabaena/Aphanizomenon, blue: Microcystis, yellow: other blooms, purple: mixed blooms).*

In conclusion, *Anabaena* and *Aphanizomenon* seem to have a competitive advantage when the nitrogen concentrations are low (because they can fix nitrogen) whereas *Planktothrix* has a competitive advantage when the temperature is relatively low. *Microcystis* seems to have a competitive advantage when the ammonium concentration is low.

3.3.2. Clone library of the sample of Parc des Sources

For the clone library built with DNA extracted from the sample from **Parc des Sources (22/07/2004)**, 15 sequences have been obtained: 13 sequences related to *Planktothrix rubescens* (BC-Pla 9402) and 2 to *Aphanizomenon* 'BC-Aph 9601' (table 12). The large dominance of *Planktothrix* and the limited diversity in the clone library is in agreement with the microscopical observations that showed a bloom of *Planktothrix* sp.

Because this was a BLOOMNET sample for which no measurement of environmental parameters, we cannot make any link with ecological factors.

***Table 12**. Most similar strain sequences for the Parc des Sources clones*

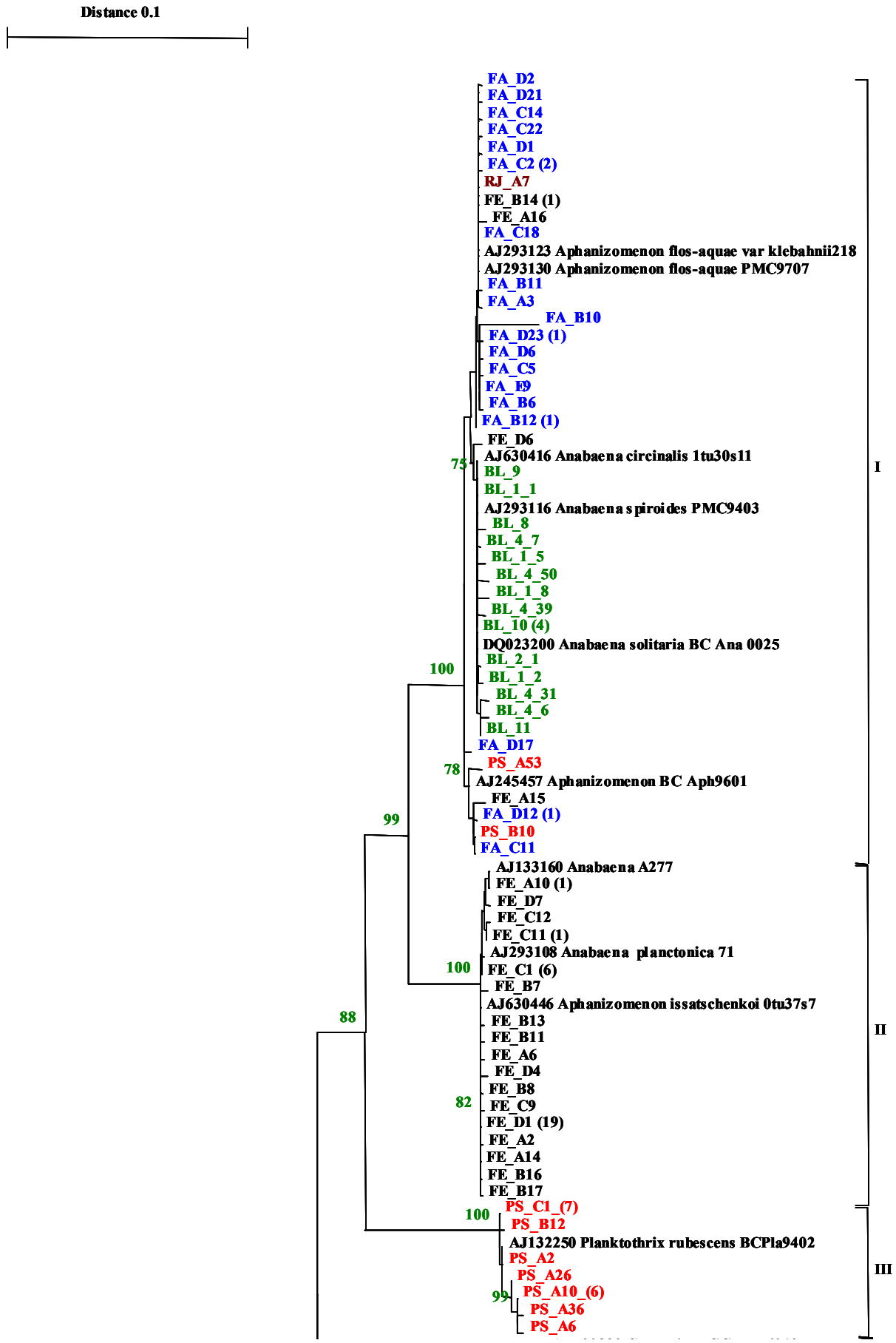
Number of clones	Most similar strain sequence	Accession number	% similarity	Origin
13	*Planktothrix rubescens* BC-Pla 9402	AJ132250	99-100	Swiss lake
2	*Aphanizomenon* sp. 'BC_Aph' 9601	AJ245457	99	Baltic Sea

3.4. Global tree based on the five clone libraries

A tree with all the clone sequences from the 5 samples was constructed on the basis of partial 16S rRNA sequences (583 positions). The OTUs were defined as groups of sequences sharing at least 97.5 % sequence similarity and calculated by DOTUR (Schloss & Handelsman 2005). According to Stackebrandt and Göbel (1994), sequences belonging to different OTUs are surely different at the species level, using the bacterial species definition. However, each OTU might correspond to one or more species. Thus, this is probably an underestimation of the species diversity.

The sequences appeared to be distributed in 9 OTUs (figure 18). The heterocystous *Anabaena/Aphanizomenon* seemed to dominate numerically the populations of Féronval, Falemprise and Blaarmeersen, if the clone libraries reflect the sample structure. They are distributed into 2 OTUs, where OTU II only includes sequences from Feronval, whereas OTU I contains sequences of the three lakes. In the clone library of Ri Jaune, *Woronichinia* appeared most numerous and Parc des sources clearly corresponded to a bloom of *Planktothrix*. The clone library of Blaarmeersen appeared most diverse, as this lake is less eutrophic than the Eau d'Heure lakes, and less prone to blooms. The lineage identified as *Synechococcus* also includes two different OTUs (IV and V), where sequences from Blaarmeersen lake and Ri Jaune are distributed.

For five reference strains of *Anabaena/Aphanizomenon* used in the phylogenetic analysis, it is known that they are non-toxic (218, PMC9707, PMC 9403, A277 and 71) but the information is not available for the other *Anabaena*, *Aphanizomenon*, *Planktothrix* and *Microcystis* strains (Rantala et al. 2006). Till now, no toxic strains were found in the lineages of *Synechococcus*, *Limnothrix* and *Woronichinia* to which our sequences belong (e.g. Rajaniemi et al. 2005b). However, the presence of *mcy* genes was detected in all samples, except for Blaarmeersen. This could be due to low concentrations of the well-known toxic genera that were not detected by microscopy, or to not-yet-detected toxicity of new taxa as the list of toxin-producers is still increasing.


Distance 0.1
FA_D2
FA_D21
FA_C14
FA_C22
FA_D1
FA_C2 (2)
RJ_A7
FE_B14 (1)
FE_A16
FA_C18
AJ293123 Aphanizomenon flos-aquae var klebahnii218
AJ293130 Aphanizomenon flos-aquae PMC9707
FA_B11
FA_A3
FA_B10
FA_D23 (1)
FA_D6
FA_C5
FA_E9
FA_B6
FA_B12 (1)
FE_D6
AJ630416 Anabaena circinalis 1tu30s11
BL_9
BL_1_1
AJ293116 Anabaena spiroides PMC9403
BL_8
BL_4_7
BL_1_5
BL_4_50
BL_1_8
BL_4_39
BL_10 (4)
DQ023200 Anabaena solitaria BC Ana 0025
BL_2_1
BL_1_2
BL_4_31
BL_4_6
BL_11
FA_D17
PS_A53
AJ245457 Aphanizomenon BC Aph9601
FE_A15
FA_D12 (1)
PS_B10
FA_C11
AJ133160 Anabaena A277
FE_A10 (1)
FE_D7
FE_C12
FE_C11 (1)
AJ293108 Anabaena planctonica 71
FE_C1 (6)
FE_B7
AJ630446 Aphanizomenon issatschenkoi 0tu37s7
FE_B13
FE_B11
FE_A6
FE_D4
FE_B8
FE_C9
FE_D1 (19)
FE_A2
FE_A14
FE_B16
FE_B17
PS_C1_(7)
PS_B12
AJ132250 Planktothrix rubescens BCPla9402
PS_A2
PS_A26
PS_A10_(6)
PS_A36
PS_A6
75
100
78
99
100
88
82
100
99
I
II
III

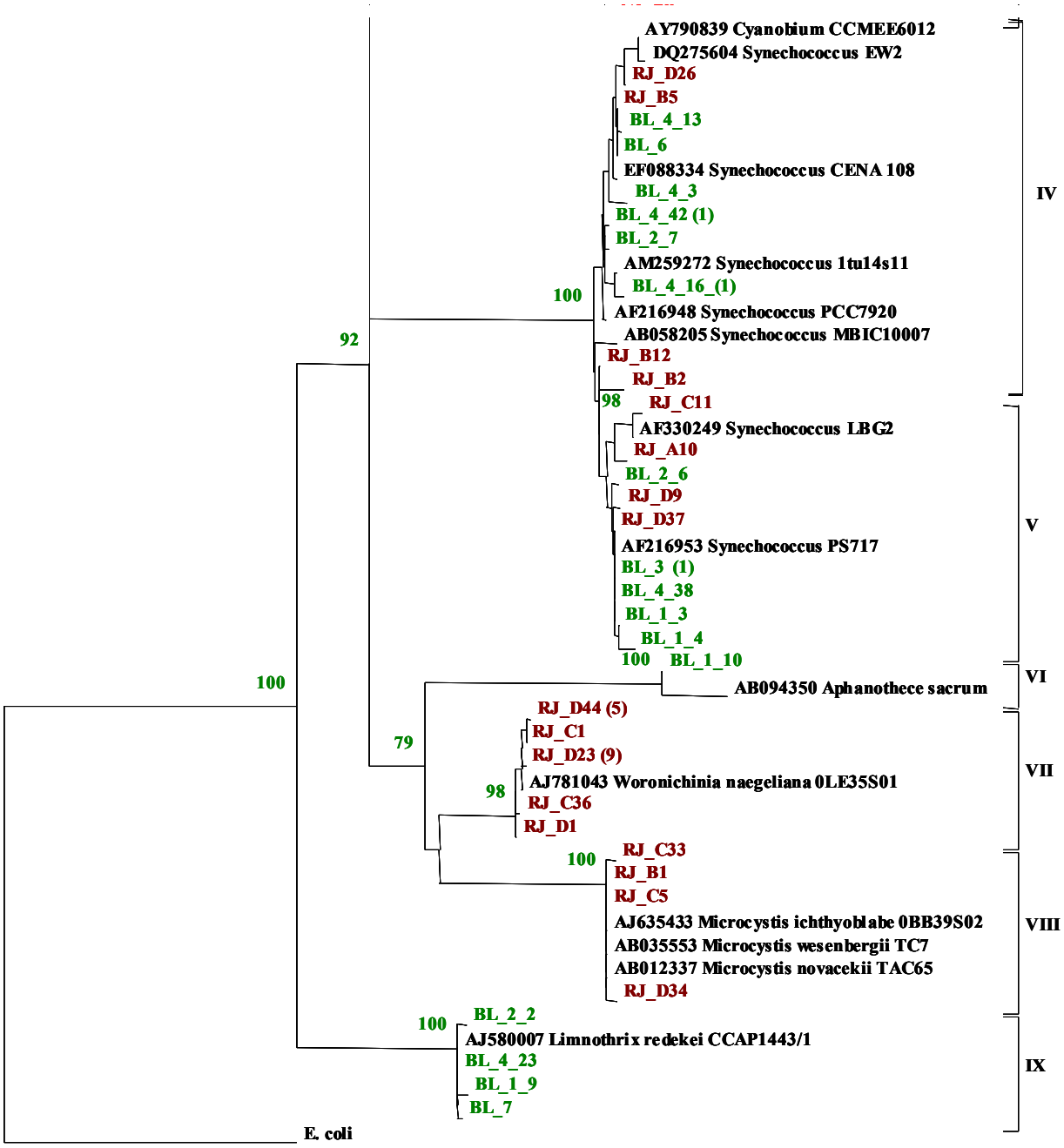


Figure 18: *Neighbor-joining tree for the five clone libraries of B-BLOOMS samples, constructed on 583 16S rRNA positions (E. coli positions 379-959), with the Jukes and Cantor correction for multiple mutations and a bootstrap analysis involving 500 replicates. The bootstrap percentages higher than 70% are indicated besides the concerned nodes. When duplicate sequences are present, their number is indicated between parentheses besides the name. BL: Blaarmeersen, FA: Falemprise, FE: Féronval, PS: Parc des Sources, RJ: Ri Jaune.*

3.4. Comparing DGGE and clone libraries with microscopic determinations

The results from the molecular analyses and the microscopic determinations were compared for lake Blaarmeersen, l'Eau d'Heure complex and the extra lakes and ponds. Each technique has its strength and limits (Boutte et al. 2005). Artefacts that could influence the diversity observed with molecular methods have been listed by Witzingerode et al. (1997).

For lake Blaarmeersen, there are some differences between the molecular methods and the counts. First, the presence of *Synechococcus* was underestimated in the microscopic counts. *Synechococcus* can easily be missed because of its small size and unspecialized form (it does not form colonies). Moreover, in most of the samples of 2003, *Synechococcus* was not counted. However, by DGGE, *Synechococcus* was detected in all the samples from lake Blaarmeersen and three different genotypes were distinguished. The clone library on a July sample in 2003 also detected this unicellular diversity, that corresponded to two different taxonomic units on the basis of 16S rRNA gene similarities. Second, the genera *Anabaena* and *Aphanizomenon* were counted in summer and autumn of 2003 and 2004, while they were detected as one cyanobacterial genus '*Anabaena/Aphanizomenon*' in three samples in the summer of 2003 by DGGE and clone library, due to the fact that the 16S rRNA sequence does not coincide with these morphological types (Rajaniemi et al. 2005a) (see also 2.1.1.) Moreover, there appear two different OTUs in the cluster '*Anabaena-Aphanizomenon'* in the tree based on 16S rRNA gene sequences, hinting to a certain genetic diversity. Third, *Limnothrix redekei* was detected earlier in the season (summer) in the hypolimnion by DGGE compared to the microscopic countings (late-summer and autumn). Furthermore, the genera *Merismopedia*, *Planktolyngbya* and *Planktothrix* and the species *Woronichinia naegeliana* were not detected by DGGE, while they were determined by microscopy. In addition, the sequence related to *Aphanothece sacrum* was only observed by cloning.

Not all bands in the DGGE profiles were sequenced; it is thus possible that the corresponding bands were not detected. In fact, the bands from 10 band classes could be obtained, but only 5 corresponded to cyanobacterial sequences. Moreover, few 16S rRNA-sequences from *Merismopedia* and *Woronichinia naegeliana* and no sequences from *Planktolyngbya* are present in GenBank. These genera are underrepresented in the database, which can eventually cause misleading determination.

Considering l'Eau d'Heure complex, the data obtained from DGGE and microscopic determination show some differences. For instance, strong bands of *Synechococcus* are visible in the DGGE profiles of the samples from Falemprise, whereas these genera were not detected by microscopy for the same reasons as in Blaarmeersen. In Ri Jaune, microscopy revealed a dominance of *Aphanothece* and *Anabaena*, whereas blooms of *Synechococcus* were detected by DGGE. In the

August sample analysed by clone library, the sequences assigned to *Synechococcus* were also abundant, but *Woronichinia* sequences dominated, whereas *Microcystis* and *Anabaena/Aphanizomenon* were also observed. The closest sample analysed by DGGE was taken 12 days earlier (19/08/03) and bands corresponded to *Synechococcus* (20, 26), *Microcystis* (25) and 5 enigmatic band classes. Two different OTUs were present, that included *Anabaena/Aphanizomenon* sequences, but this does not fit with the morphological identifications.

For the extra lakes and ponds with cyanobacterial blooms, the results from the microscopic countings and DGGE were very similar. However, there were some differences generally when there were several dominant genera. In these cases, both techniques complement each other. The drawback of DGGE for identification was that not all bands could be extracted and identified. Thus, 26/41 classes remain enigmatic. This is not the case with clone libraries, where a long sequence can always be determined (between 600 bp to almost complete sequences) and allows an adequate phylogenetic analysis. On the other hand, DGGE is fast and allows to quite easily obtain community fingerprints that can be combined with environmental parameters for multivariate analyses to extract the factors explaining the ecological distribution of genotypes.

Molecular techniques based on the 16S rRNA sequence appear more sensitive to detect taxa that have such small size that they were overlooked by microscopy. The PCR based on *mcy* genes appear also more sensitive to detect toxigenic taxa that were not yet abundant enough to be counted. On the other hand, not all cyanobacterial taxa are represented in the databases, and thus, their sequences cannot be identified. However, this problem is only transitory, as the taxonomic coverage of the databases is steadily increasing.

In conclusion, molecular and microscopic determinations are complementary and both are useful to obtain a complete picture of the cyanobacterial community composition.

GENERAL CONCLUSIONS AND RECOMMENDATIONS

This first study of algal blooms in Belgium has shown that the phenomenon was quite common when meteorological and environmental conditions were favourable. It has also demonstrated that **54 % of the Flemish BLOOMNET blooms, showing the presence of two genes responsible for toxin production, contained measurable concentrations of microcystins, a potent hepatotoxin**. **If the percentage of microcystin-containing samples is calculated on the total number (81) of Flemish blooms, the proportion of toxic samples amount to 31% and corresponds to 21 different waterbodies out of 69 in total.** The sampling and toxin measurements were biased towards recreational and fishing ponds in Flanders, but sporadic blooms in Wallonia and Brussels also showed some toxicity. Therefore, the development of a model and the design of a toolbox for detection of the toxic blooms and some early-warning system appear useful and timely.

The monitoring for 2 years of a limited number of reference lakes provided the environmental and biological data to assess the feasibility of developing **predictive models** of cyanobacterial blooms. As expected, the different lakes had distinct phytoplankton communities and dynamics, depending on their trophic status and physical characteristics. In all lakes, there was a strong seasonal change related to temperature, stratification, and daylight duration. Environmental conditions favouring cyanobacteria were systematically high temperature, high pH and stable stratification, but potentially toxic taxa required high dissolved phosphorus, with relatively low inorganic nitrogen. Number of days without strong winds was also a factor favouring cyanobacteria blooms, as was the abundance of the larger filter-feeders (*Daphnia*, cyclopoid copepods) in the zooplankton. Indeed, these large filamentous or colony-forming forms have an advantage in the presence of efficient grazers (Sarnelle 2005), which select for small phytoplankton (essentially the nanoplankton, i.e. with size between 2 and 20 µm, and the small microplankton, up to about 50 µm). According to all these factors, maximal biomass of filamentous cyanobacteria was most of the time encountered during summer and early autumn. The particular warm conditions of the summer 2003 did not result in larger development of cyanobacteria, except in Blaarmeersen, where N_2-fixing taxa (*Anabaena*, *Aphanizomenon*), favoured by low N concentration, appeared.

The prediction of total cyanobacteria biomass using **Artificial Neural Networks** (ANN) gave good results, despite the relatively low numbers of samples in the available data: regression between predicted and observed values reached a correlation coefficient of 0.95. The ranking of the best predictive variables identified the prominent role of nitrogen (TN) and phosphorus (TP), along with the meteorological variables. This points to the importance of physical factors in bloom development: if specific conditions of temperature (hence stratification), light and

wind are not met, cyanobacteria do not develop significantly, despite favourable nutrient concentrations (Maberly et al. 1994, Reynolds 1998). In other words, if nutrient concentrations are among the best predictors for the potentially toxic cyanobacteria, meteorological and limnological factors are of primary importance and account for most of the variability in the observations. This implies that key conditions for bloom prediction can be monitored relatively easily using meteo stations and water sensors connected to data loggers. However, the prediction models have to be developed from much larger data bases, from monitoring a larger set of lakes, before they become reliable for the surveillance of eutrophic lakes.

The project used a combination of approaches to monitor the **diversity and toxigenicity** of the blooms, including microscopic counts and identification, pigment analyses, and molecular tools to detect the toxigenicity on the basis of the presence of genes involved in the synthesis of microcystin, the most current cyanotoxin in Belgium. This was tested on the four reference lakes regularly monitored, but also on the sporadic blooms samples that were obtained from the partners or BLOOMNET members.

Seventy-nine percent of the BLOOMNET samples tested by PCR harboured **toxigenic taxa** (double positives for the *mcyB/E* genes). The toxigenicity tests by amplification of *mcyB/E* genes appeared adequate to select samples that could be submitted to an analytical measurement of microcystins by HPLC. However, the latter measurements were not carried out on all selected samples, due to the high costs of the analyses. In addition, technical difficulties were unexpectedly encountered by the subcontractor CRITT-BioIndustries when using the filters, and some negative results (e.g. in Eau d'Heure lakes) could simply be due to this problem. In later analyses, cell biomass was used instead of filters to measure microcystins at CRITT and FUNDP. However, these additional analyses were limited to Flemish samples where biomass had been taken in parallel to the filters (Todorova 2006).

Therefore, the most representative dataset to calculate the percentage of **toxic blooms** comes from the Flemish BLOOMNET samples. Combining the data from CRITT-BioIndustries and FUNDP, 54% of the 46 bloom samples positive for the two *mcy* genes actually contained **total microcystins**, in a range of concentration from 18 µg/[g DW] to 2651 µg/[g DW]. In 13/21 Flemish lakes, the microcystin-LR concentrations are higher than the threshold of a French recommendation used to forbid the access to the recreation waters. Therefore, the situation warrants a more detailed monitoring of these waterbodies in the future. Indeed, exposure to cyanotoxins during water recreation and sport seems to be more relevant than contamination by drinking water in our country, where water reservoirs are quite rare. In addition to have a higher probability, it is known that recreational exposure can have adverse effects on human health (Stewart et al. 2006a) and cause deaths of

animals. *Microcystis* was the dominant cyanobacterial genus in all samples where microcystins were detected. In a few cases, toxic *Planktothrix* were observed.

Anatoxin-a was not detected in any of the 38 samples selected for analysis of this toxin. This coincides with the fact that, in other European countries, this neurotoxin has always been found at a much lower frequency than microcystins. However, it should still be included in the toxins to monitor, especially as they also were found in benthic cyanobacterial mats in French rivers (Gugger et al. 2005). In addition, cylindrospermopsin is a recently detected problem in Middle Europe, and should be looked for.

The **protein phosphatase inhibition assays** used for detection and analysis of cyanobacterial hepatotoxins did not provide satisfactory results in this study. It seems that they produced false positives, probably because the targeted enzyme system also responds to a wide variety of noncyanobacterial toxins and metabolites.

The **diversity** of the cyanobacteria present in the samples was estimated by a polyphasic approach, using both microscopic countings and molecular methods (DGGE and clone libraries). The small-sized genera appear to escape the microscopic observations, but were well detected by molecular methods. In addition, a cryptic genetic diversity appeared hidden behind these simple morphologies, like the one of the genus *Synechococcus*. Other genera like *Anabaena* and *Aphanizomenon* can be recognized on the basis of their morphology, but their 16S rRNA sequences do not match these classical designations, and show an unrecognized genetic diversity. DGGE gave a fast survey of the diversity but a quite large proportion of bands could not be sequenced, and the sequences were too partial for an adequate phylogenetic analysis. On the other hand, the banding patterns could well be used to correlate the genetic diversity with environmental factors (van Gremberghe et al. 2008). The clone libraries were more time-consuming but gave 16S rRNA sequences of better quality for phylogenetic analyses. A combination of approaches, depending on the particular purpose, seems the most useful strategy. The use of a less conserved molecular marker, like the Internal Transcribed Spacer (ITS) between the genes coding for the 16S and 23S rRNA genes, would bring more resolution to the molecular taxonomic study.

Though the isolation and purification of **strains** could not be accomplished in the 2 years of activity, this would be an important step to better characterize the link between the taxonomic and the toxin diversity, and the ecophysiology of toxin synthesis.

The building of an **active BLOOMNET network** that could obtain samples when blooms are occurring is of great importance. In this project, a good start was taken by making first contacts, organising workshops and making a bilingual informative video. However, it should be continued and improved. Kardinaal & Visser (2005) have shown that the largest proportion of toxic genotypes was present at the

beginning of the blooms in Dutch lakes, and that this proportion was declining with the age of the bloom. One hypothesis is that non-toxic genotypes are better competitors for light. For the Walloon samples, we were far from ideal conditions. The SWDE that was doing weekly monitoring of the bacteriological quality of recreation waters for the Region Wallonne was only taking BLOOMNET samples when there was a coloration of water the week before. Thus, the blooms were already quite old. In other countries, like in The Netherlands or Finland, public authorities are more involved in bloom observations and this facilitates the timely measurements of toxins.

As explained in the text, the study was hindered by technical problems concerning the measurement of cyanotoxins. It is necessary that Belgium can build an adequate **infrastructure to measure the diverse cyanotoxins** efficiently in the water, in field blooms, but also in the tissues of dead animals that could be victims of cyanotoxins.

A recent **report** of French Agencies (AFSSA/AFSSET 2006) has dealt with several topics concerning toxic cyanobacterial blooms in drinking and recreational waters, including the French situation, the sampling and measurement methodologies, the evaluation of risks and the strategies to minimize exposures of the population. Their recommendations, that also would be useful for the Belgian waters, concern the evaluation of the risks linked to the cyanotoxins, the prevention and control of those risks, and special sanitary monitorings in recreational waters, including a determination of the sites at risk and public alert strategies.

In the context of european and international cooperations, it is interesting to note that Belgium has already participated to the inquiry held by **CYANONET** (part of UNESCO's International Hydrology Programme – VI) (Codd et al., 2005b) and should continue to participate to this network addressing the problems of cyanotoxins in water resources and the risks these present to health.

A last remark is that the **public** is still largely unaware of the potential risks of cyanotoxins in Belgium, and that initiatives should also be taken to spread information. A first step has been the design of an informative website (www.bblooms.ulg.ac.be). In the future, enough attention should be given to the transfer of knowledge.

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