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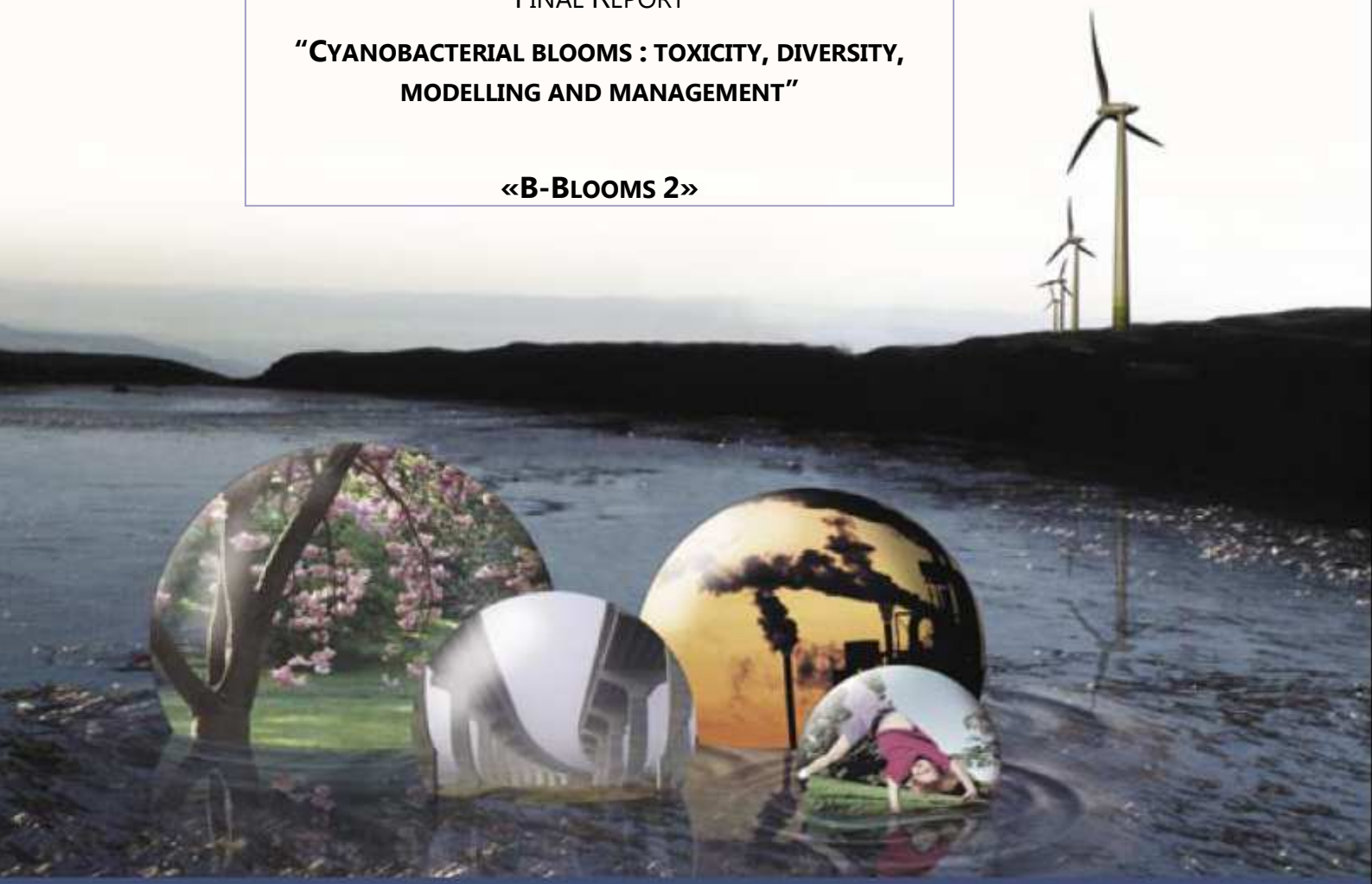
SCIENCE FOR A SUSTAINABLE DEVELOPMENT

FINAL REPORT

"CYANOBACTERIAL BLOOMS : TOXICITY, DIVERSITY, MODELLING AND MANAGEMENT"

«B-BLOOMS 2»



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FINAL REPORT

CYANOBACTERIAL BLOOMS : TOXICITY, DIVERSITY, MODELLING AND MANAGEMENT "B-BLOOMS2"

SD/TE/01B

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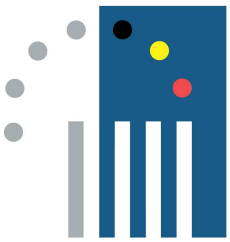

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Table of contents

SUMMARY **7**

ACRONYMS, ABBREVIATIONS AND UNITS 10

LIST OF PARTNERS 10

1. INTRODUCTION 11

2. METHODOLOGY AND RESULTS 14

2.1 MATERIALS AND METHODS **14**

2.1.1. BLOOMNET 14
2.1.2 Sampling of reference lakes 16
2.1.3. Isolation of strains 17
2.1.4. Environmental conditions for reference and BLOOMNET samples 18
2.1.5. Phytoplankton composition and biomass by HPLC analysis of marker pigments and by microscopy 19
2.1.6. Molecular ecology of cyanobacteria 19
2.1.7. Determination of toxin contents 26
2.1.8. Deterministic watershed simulation modelling of Eau d'Heure Lakes 29
2.1.9. Statistical predictive models for Brussels ponds 29
2.1.10. Data base 29

2.2 RESULTS **31**

2.2.1. Ecology 31
2.2.2 Isolation of strains and characterization of their potential toxin production 45
2.2.3. Molecular ecology of cyanobacteria 50
2.2.4. Determination of toxin content 60
2.2.5. Deterministic watershed simulation modelling of Eau d'Heure Lakes 71
Simulations 71
2.2.6. Statistical predictive models for Brussels ponds 75
2.2.7. Data base 81

3. POLICY SUPPPORT 86

4. DISSEMINATION AND VALORISATION 89

4.1 WEB SITE **89**

4.2 COMMUNICATION WITH THE END-USERS COMMITTEE AND WORKSHOPS **90**

4.3 DISSEMINATION TO A WIDE AUDIENCE **93**

5. PUBLICATIONS 93

6. ACKNOWLEDGEMENTS 99

7. REFERENCES 99

SUMMARY

A. Context

Cyanobacterial blooms, mass developments of cyanobacteria floating at the surface of waterbodies, have become a recurrent and increasingly important phenomenon in freshwaters worldwide over recent decades. The formation of such blooms in surface waters is closely linked to water eutrophication (Chorus, 2001). These nuisance blooms represent major potential hazards for human and animal health, and interfere in various negative ways with the sustainable use of surface waters for e.g. drinking water treatment, recreation, irrigation and fisheries. Between 25 and 70% of the blooms are toxic (Sivonen, 1996). The cyanotoxins are mainly released in the water column during collapse of the blooms. Ingestion of, or contact with water containing cyanobacterial cells or toxins can cause health damage (Bell & Codd 1996; Carmichael et al. 2001; de Figueiredo et al., 2004; Dittmann & Wiegand, 2006).

In view of the lack of knowledge about the situation in Belgium, three of the present partners initiated in 2003 the BELSPO project B-BLOOMS1. Thanks to this work, it has been shown that surface waters in Belgium were also plagued by cyanobacterial blooms, particularly in summer and autumn. Eighty % of the blooms contained taxa with the genetic potential to synthetise microcystins, and the presence of this toxin family in the cyanobacterial biomass was shown by HPLC analysis for 40% of the analysed bloom samples.

B. Objectives

The B-BLOOMS2 project aimed to deepen knowledge of cyanobacterial blooms in Belgium, improve the modelling for prediction and early-warning, develop operational monitoring structures and tools, and propose strategies to reduce the impact of cyanobacterial blooms.

From a scientific point of view, the research programme focused on:

- Collection of physical, chemical, biological and meteorological data on selected reference waterbodies plagued by toxic cyanobacterial blooms in Flanders, Brussels and Wallonia;
- Identification and study of the toxigenic cyanobacteria present in the Belgian samples using molecular tools on samples and strains, including genetic diversity and factors regulating their population dynamics;

- Measurement of the major cyanotoxins present in the blooms and water samples by analytical methods;
- Development and test of management scenarios for control or mitigation of cyanobacterial blooms in one reservoir using integrated watershed models;
- Development of a statistical predictive model for a series of urban ponds.

From a practical and science policy point of view, B-BLOOMS2 aimed to:

- Implement a network of samplers based on existing monitoring programmes of surface waters or on collaboration with health authorities or environmental organisations (BLOOMNET);
- Transfer knowledge about methods of monitoring and analysis of blooms to the water/health authorities and environmental organisations by hands-on courses in our laboratories and field sites;
- Reinforce the communication to and with authorities and the general population, to raise public awareness, contribute to future guidelines and risk assessment procedures, and improve monitoring and management.

C. Conclusions

Among the main results of the study, it can be stressed that most of the cyanobacterial blooms consisted of potentially toxic taxa of the genera *Aphanizomenon*, *Microcystis*, *Planktothrix* and *Anabaena*. Improved molecular approaches demonstrated that genetic diversity within blooms can be high and that changes in strain dominance occur and can be caused by strong and specific trophic interactions. Several genes of the *mcy* cluster were regularly detected in bloom samples. The toxin analyses showed the presence of microcystins in all samples tested, the concentration of which exceeded World Health Organization Guideline Values for drinking- and recreational waters on several occasions. These results will help to understand the factors and mechanisms influencing cyanotoxin production, and, from a management point of view, call for measures for improved cyanobacterial bloom monitoring, bloom reduction and for the reduction of public exposure to harmful blooms.

D. Contribution of the project in a context of scientific support to a sustainable development policy

From a management point of view, the project has contributed to the development of measures for improved cyanobacterial bloom monitoring, bloom reduction and for the reduction of public exposure to harmful blooms. Indeed, throughout the project and particularly in the second phase (2009-2010), the teams were actively involved in various information and monitoring activities, in collaboration with water managers and stakeholders throughout the three Belgian regions (Brussels, Flanders and Wallonia). These activities comprised water quality measurements, determination of phytoplankton composition, collection and characterisation of cyanobacterial blooms by different techniques and determination of soluble and particulate microcystins. In the framework of the application of the EU Bathing Water Directive (2006/7/CE), the B-BLOOMS2 teams provided significant help to the authorities in identifying and refining policies and measures related to the risk assessment and management of cyanobacterial blooms.

E. Keywords

Cyanobacteria, blooms, eutrophic lakes, genetic diversity, monitoring, cyanotoxins, modelling

ACRONYMS, ABBREVIATIONS AND UNITS

DIN : dissolved inorganic nitrogen
LCL : large cladoceran length
LCD : large cladoceran density
SD : Secchi depth
SV : % submerged vegetation cover
T : temperature
SRP : soluble reactive phosphorus
TP : total phosphorus
MD : maximum depth
RDA : redundancy analysis
RT : hydraulic retention time
ITS : Internal Transcribed Spacer region
MC : microcystins
Mcy : microcystin synthetase
PCR : polymerase chain reaction
rRNA : ribosomal RNA
DGGE : Denaturating gradient gel electrophoresis
ELISA: enzyme-linked immunosorbent assay
HPLC: high performance liquid chromatography
WFD: Water Framework Directive

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1. INTRODUCTION

Cyanobacterial blooms, mass developments of cyanobacteria floating at the surface of waterbodies, have become a recurrent and increasingly important phenomenon in freshwaters worldwide over recent decades. The formation of such blooms in surface waters is closely linked to water eutrophication (Chorus, 2001). These nuisance blooms represent major hazards and potential risks to human and animal health, and interfere in various negative ways with the sustainable use of surface waters, for e.g. drinking water treatment, recreation, irrigation and fisheries. Between 25 and at least 70% of these blooms produce potent cyanotoxins (Sivonen, 1996). The cyanotoxins are released in the water column, mainly during collapse of the blooms. The ingestion or contact with water containing cyanobacterial cells or toxins can cause health damage (Bell & Codd 1996; Carmichael et al. 2001; de Figueiredo et al., 2004; Codd et al., 2005; Dittmann & Wiegand, 2006).

Requirements to manage risks presented by cyanobacterial blooms and their toxins, from local to national level, are increasing. Drivers include increasing human population size , geographical spread and seasonal duration of cyanobacterial blooms, recurring associated health incidents, national and international (World Health Organisation) guidelines and legislation in several countries. Also, the implementation of the 2006 EU Bathing Water Directive (Directive 2006/7/CE) requires member states to take measures for detecting blooms, assessing the health risks associated with recreational activities, and for preventing exposure of the human population.

In view of the lack of knowledge about the situation in Belgium, three of the present partners initiated the BELSPO project, B-BLOOMS in 2003. Thanks to this work, it has been shown that many surface waters in Belgium are also affected by cyanobacterial blooms, particularly in summer and autumn. Fifty-four % of the blooms examined contained taxa with the genetic potential to synthesise microcystins, and the presence of this toxin family in the algal biomass was shown by HPLC analysis for 31 % of the analysed bloom samples (Wilmotte et al., 2008). The need of monitoring blooms in Belgium was confirmed in a paper of Willame et al. (2005) where 53% of the analysed bloom samples contained microcystins.

The B-BLOOMS2 project aimed to deepen the knowledge of the cyanobacterial blooms in Belgium, improve the modelling for prediction and early-warning, develop operational monitoring structures and tools, and to propose strategies to reduce their impact.

From a scientific point of view, the research program focussed on:

- measurement of the major toxins present in the blooms and water samples by analytical methods (ELISA, ,High Performance Liquid Chromatography and mass spectrometry methods)
- collection of physical, chemical, biological and meteorological data on selected reference waterbodies plagued by toxic cyanobacterial blooms,
- identification and study of the toxigenic cyanobacteria present in the Belgian samples based on molecular tools on samples and strains, including genetic diversity, and factors regulating toxicity,
- development and test of management scenarios for control or mitigation of cyanobacterial blooms in one reservoir using integrated watershed models,
- development of a statistical predictive model for a series of urban ponds.

From a practical and science policy point of view, the B-BLOOMS2 objectives were to:

- implement a network of samplers based on existing monitoring programmes of surface waters and/or on collaboration with health authorities or environmental organisations (BLOOMNET),
- transfer the knowledge about methods of monitoring and analysis of blooms to be developed, to the water/health authorities and environmental organisations by hands-on courses in our laboratories and at field sites,
- reinforce the communication to and with relevant authorities and the wider population, to raise public awareness, contribute to future guidelines and risk assessment procedures, and improve monitoring and management.

2. METHODOLOGY AND RESULTS

2.1 MATERIALS AND METHODS

2.1.1. BLOOMNET

BLOOMNET is the network of samplers already established under the first B-BLOOMS project (called 'B-BLOOMS1'), that was reactivated and extended under B-BLOOMS2. It is based on existing monitoring programmes of surface waters and on collaboration with health authorities and environmental organisations. As these may differ among regions, regions have been distinguished as detailed below. Contrary to the procedure used in B-BLOOMS1, whenever significant blooms were detected in waterbodies in B-BLOOMS2, the respective partners in each region were contacted by BLOOMNET members and the partners carried out the collection and treatment of samples.

Flanders

In Flanders, 65 phytoplankton blooms from 53 different locations were reported and sampled by the BLOOMNET partners between June 2006 and June 2011, of which 54 contained a mass development of cyanobacteria. Blooms were reported and sampled by the VMM (Vlaamse Milieu Maatschappij, n = 30), UGent (n = 24), VVHV (Vlaamse Vereniging voor Hengelsport Verbonden, n = 9), ANB (Agentschap voor Natuur en Bos, n = 1) and Stad Brugge (n = 1).

The water samples were transported to the PAE laboratory immediately after sampling after which further processing was done by UGent. Next to the immediate determination of temperature, conductivity, oxygen concentration, salinity and pH, samples were taken from these blooms for the measurement of physical and chemical variables (Task 4.1), HPLC pigment analysis (Task 5.1), cyanobacteria and other phytoplankton (Task 5.2), genetic (WP7) and toxin analyses (WP8).

On several occasions, the technical staff of VMM (Vlaamse Milieu Maatschappij), the institution responsible for the monitoring of recreational surface waters in Flanders, was trained by UGent in techniques for bloom sampling. The PAE team was, and remains, in close contact with the Flemish wildlife rehabilitation centres (Frederik Thoelen from the "Natuurhulpcentrum Opglabbeek", Nick De Meulemeester from the VOC Merelbeke) who professionally deal with injured or sick waterfowl. Occasionally, private persons also contacted UGent in the case of problematic blooms, for instance

in private diving- (Lake Dongelberg, Carlo Joseph) or recreational lakes (Boudewijnpark Brugge, Bernard Logghe).

Brussels

The BLOOMNET partner in Brussels consisted essentially of the Brussels Institute of the Environment (BIM/IBGE) and different city districts which reported the occurrence of blooms in the ponds which they managed. The blooms were sampled by the VUB and the results of the sample analyses were incorporated into the B-BLOOMS2 dataset and provided to the respective local authorities. Other projects on pond nutrient fluxes and restoration potential after biomanipulation were carried out simultaneously to the B-BLOOMS2 project, using similar methodology and compatible data collection in 2007-2009. BLOOMNET allowed 48 Brussels ponds to be monitored.

Wallonia

The network was mainly based on the follow-up of bathing areas authorized in Wallonia and surveyed by the SPW (Services Publics de Wallonie : DGARNE –- Direction des Eaux de Surface). ISSeP (Institut Scientifique des Services Publics), in charge of monitoring and analysis of surface waters for the Walloon region, sampled the official bathing areas once a week from June to September, and included counting of cyanobacteria in the regular survey. This close interaction between FUNDP, SPW and ISSeP was maintained throughout the project, so that all blooms detected in the bathing areas monitored by the Walloon authorities were sampled, examined and processed according the B-BLOOMS2 standard procedures (see www.bblooms.be), in order to detect potential and actual cyanotoxin production (screening for *mcy* genes and microcystin analysis, respectively). Additional data were collected thanks to studies carried out for SPW-DEMNA (Département d'Etude du Milieu Naturel et Agricole) in the framework of the determination of the ecological potential of the Walloon reservoirs, in 2009 (Descy et al., 2010a). Moreover, FUNDP contributed to the establishment of the profile of bathing areas in Wallonia in 2010, through a study carried out for PROTECTIS (SPGE – Société Publique de Gestion de l'Eau), for assessing the risks of eutrophication and of development of cyanobacteria in 18 bathing zones in total (Descy et al., 2010b). Finally, a survey of the Virelles Pond, a water body of interest for nature conservation, was conducted in 2009 in collaboration with Aquascope, a nature discovery centre in Virelles. VIVAQUA, which

is responsible for drinking water supply to Brussels, was also regularly involved in BLOOMNET activities with FUNDP.

2.1.2 Sampling of reference lakes

A common protocol (see www.bblooms.be) was defined by the Coordinator, and followed by the different Teams, to ensure, as far as possible, the acquisition of comparable results to be entered into the database. Briefly, field and laboratory measurements were made for acquisition of environmental data (physical and chemical variables of the water bodies), phytoplankton composition and biomass (HPLC analysis of marker pigments for class-level determination, by FUNDP for all samples; microscopy by each Team for identifying dominant taxa), identification and counting of cyanobacteria, quantification of main zooplankton groups, collection of samples for cyanotoxin analysis and *mcy* genes, and collection of samples for subsequent strain cultivation and characterisation.

All data on the reference lakes from the different tYeams, including cyanotoxin gene and toxin data, have been entered into an Excel file using the same format. This file uses the same format as the B-BLOOMS1 database, in order to aid data dissemination and to carry out multivariate analyses, as well as for the design of predictive models of cyanobacterial blooms and of their potential and actual toxicity.

Flanders

The two reference lakes from Flanders (the pond in the 'Westveldpark' in St-Amandsberg and the 'Donkmeer' in Berlare) are situated in the neighbourhood of Ghent and suffer from annual cyanobacterial blooms. Westveldparkvijver is a small parkland pond (*ca.* 2000 m^2) while Donkmeer is a relatively large (*ca.* 86 ha), shallow lake originating from peat-digging and now intensively used for recreation. Samples were taken from 1 May until mid November 2007 in the two reference lakes, once a week in the absence of blooms and twice a week when blooms were present. By the end of November, 52 samplings had been performed in the Westveldparkvijver pond and 43 in Donkmeer. For the second sampling year (2008), sampling began in March and lasted until the end of October 2008. 44 samples were collected for Westveldparkvijver and 23 for Donkmeer. In both lakes, an additional winter sample was taken in January 2008. Each pond was sampled at three locations, after which the water samples were pooled to integrate small-scale spatial variation. During the winter of 2007-2008, surface sediment samples were also taken to determine the

amount and genetic composition of cyanobacteria overwintering in the sediment (an important way of bridging the winter period for several cyanobacteria, see Brunberg & Blomqvist 2003, Verspagen et al. 2004). Additionally, a limited temporal survey (weekly samples, n=10) of an extended *Microcystis* bloom from a moat around Fort Bornem was carried out between August and October 2010 (see below).

Brussels

Two Brussels ponds, IxP1 and IxP2 (étangs d'Ixelles, Place Flagey), selected as reference sites, were sampled on 63 occasions between March 2007 and November 2009, approximately once a week from June to August, and twice or once a month during the colder season. Because of ice cover, the reference ponds were not sampled in January and February 2009.

Wallonia

Lake Falemprise is one of the Eau d'Heure Lakes (surface area 47 ha), and is an official bathing area in the Walloon Region. Therefore, this lake is monitored for bacteriological contamination by the Walloon authorities. Sampling of Lake Falemprise was performed near the dam where the depth is maximal (5m). This started at the beginning of May and extended to late October in 2007; samples were taken weekly (24 samples). In 2008, sampling began in April, at different depths, as this lake stratifies from late spring to early autumn (25 samples). All parameters were measured according to the analysis protocol; weather data were acquired in situ with a Davis Vantage Pro (mod 6150 C) meteorological station, and irradiance was recorded with a surface LICOR sensor LI-190 SB connected to a data logger. Limnological profiles were acquired using a YSI 6600 or Hydrolab DS5 multiprobe.

2.1.3. Isolation of strains

At UGent, individual colonies or filaments of cyanobacteria were picked out on several occasions using sterile glass Pasteur pipettes under a dissecting microscope to establish monocyanobacterial cultures which were grown in WC-medium in a culture room (12:12h L:D, 23°C, ca. 20 µE m^{-2} s^{-1}) from Driekoningenvijver, Donkmeer, Donkvijver Oudenaarde, Fort Bornem, Leeuwenhofvijver, Schulensmeer, Tiens Broek, Vijvers Zonhoven, Westveldparkvijver.

At ULg, fresh samples were inoculated onto agar plates and into liquid medium containing 500 µg /mL of cycloheximide to inhibit the growth of eukaryotic algae. BG11, B110, Z8, and WC media were used to increase the diversity of the

cyanobacteria cultured and the number of strains of toxic and non-toxic genotypes. Cultures were transferred several times from liquid to solid media until a monocyanobacterial stage was reached. They were checked by epifluorescence microscopy to investigate contamination by picocyanobacteria. At ULg, a total of 42 fresh samples were inoculated in BG11, $BG11_0$, and Z8 media under a neon light source. Samples were taken from Falemprise, Féronval, Ixelles pond I and II, Neerpede 4, Rénipont, Ri Jaune, Schulensmeer, Zonhoven P5, P6 and P7. Genomic DNAs were extracted using DNeasy Plant mini kits (Qiagen), partial sequences of 16S rDNA/ITS were amplified using the cyanobacteria-specific primers CYA359F/23S30R and sequenced using primers CYA359F and 979F for eleven strains. Sequences were assembled and analysed using the BLASTN algorithm from NCBI.

2.1.4. Environmental conditions for reference and BLOOMNET samples

Limnological variables and chemical parameters were analysed as described in a standard protocol for sampling, sample treatment and analyses, as mentioned above. Weather data were collected either by an *in situ* meteo station (Lake Falemprise) or from the closest station of the RMI (Royal Meteorological Institute). Zooplankton was collected from each lake, using buckets or a Schindler-Patalas plankton trap, and enumerated using a dissecting microscope for the larger forms (cladocerans, copepodites and adult copepods) and an inverted microscope for the smaller forms (nauplii, rotifers). In Brussels ponds, 10 sub-samples of 1 L taken with a plastic tube sampler were combined in the field, filtered through a 64 µm mesh net and preserved in 4% v/v formaldehyde before being identified and counted using an inverted microscope. Different levels of identification were used: cladocerans were identified to genus; copepods were divided into cyclopoids, calanoids and nauplii; rotifers were not discriminated. For the analyses, cladocerans were divided into two groups: 'large' (*Daphnia* spp., *Eurysercus* spp., *Sida* spp. and *Simocephalus* spp.) and 'small' (*Acropercus* spp., *Bosmina* spp., *Ceriodaphnia* spp., *Chydorus* spp., *Moina* spp. and *Pleuroxus* spp.). Predator cladocerans, *Leptodora* spp. and *Polyphemus* spp., which feed mainly on other zooplankters, were not included in the group of large cladocerans. The individual size of the large zooplankton taxa was also measured. The length of large cladocerans was also determined and used as a proxy for grazing intensity and size-selective predation (Pinel-Alloul, 1995; Carpenter et al., 2001). As far as possible, all abiotic and biotic parameters were also collected for BLOOMNET samples taken between 2007 and 2010.

2.1.5. Phytoplankton composition and biomass by HPLC analysis of marker pigments and by microscopy

Samples for Chl a and secondary pigment analysis followed a procedure described in Descy et al. (2000): a water volume was filtered through Macherey-Nägel (Düren, Germany) GF/3 filters until filter-clogging. Pigment extraction was carried out in 8 ml 90 % HPLC grade acetone. After two 15-min ultrasonications, separated by an overnight period at 4°C in the dark, HPLC analysis was carried out using the Wright et al. (1991) gradient elution method, with a Waters 600E multisolvent delivery system comprising a Waters 996 PDA detector and a Waters 470 fluorescence detector. Calibration was made using commercial external standards (DHI, Denmark). Carotenoids not present in the standards were quantified against fucoxanthin, using as relative response to the ratio of the specific absorbance coefficients at 440 nm (Jeffrey et al., 1997) in methanol. Identification of pigments was checked against a library of pigment spectra, obtained by diode array acquisition of chromatograms from pure pigment solutions and from acetone extracts of pure cultures of algae and cyanobacteria. Chromatogram processing was done with the Waters Empower software. Abundances of algal classes were determined from HPLC algal pigment measurements using CHEMTAX, a matrix factorisation programme, which estimates the contribution of each specified phytoplankton pigment class to the total Chl a concentration in a water sample (Mackey et al., 1996). A unique initial ratio matrix was used for all lakes and CHEMTAX processing was run until stability of the pigment ratios in the output ratio matrix was reached. This processing allowed estimation of the Chl a biomass of various algal classes, including two types of cyanobacteria with distinct pigment signatures.

In 2007, all samples from Falemprise (FUNDP), Donkmeer and Westveld (UGent) were analysed using this technique. In 2008, additional samples from the Ixelles ponds (VUB) were analysed with this technique, as well as those from most BLOOMNET samples.

2.1.6. Molecular ecology of cyanobacteria

In this section, the molecular techniques used are detailed according to the different observation scales, in order to: (i) determine the cyanobacterial diversity in reference lakes, (ii) detect the presence of potentially toxic genotypes and (iii) analyse the dynamics of toxic and non-toxic genotypes.

Brussels and Walloon reference lakes and additional BLOOMNET samples were filtered through 0.2 µm pore size membranes of 0.47 mm diameter and stored at -20°C. DNA extraction was performed as described by Boutte et al. (2006) (see Annex II for list of samples). In Flanders, from each sampling of the reference lakes and each BLOOMNET sample, a sample was collected on a 0.2 µm pore size GSWP (Millipore) filter for DNA extraction and frozen at -80°C.

Molecular diversity of cyanobacteria in reference lakes

During recent decades, intensive efforts have been devoted to the identification and characterisation of freshwater cyanobacteria. Analysis of 16S rRNA gene sequences has been shown to be an efficient phylogenetic marker for prokaryotic classification (Rossello-Mora and Amann, 2001). This method is now widely used for prokaryotic identification (Ouellette and Wilhlem, 2003) and 16S rRNA gene amplification from freshwater samples is now used worldwide. Cyanobacterial sequences can be obtained by different techniques, with or without cultivation (e.g. clone library, DGGE) and then compared to sequences from the global database, Genbank. This first comparison generally allows for a rapid identification of the cyanobacterial genera present in the sample. Moreover, the phylogenetic analyses give a schematic representation of the cyanobacterial evolution and diversity.

During this first phase of the B-BLOOMS2 project, a 16S rRNA gene sequence database of cyanobacteria present in Belgian waterbodies was created. The aims of this database were to: (i) make an inventory of potentially toxic and non-toxic cyanobacteria present in the freshwater reservoirs under study; (ii) observe diversity changes in reference ponds and lakes or in sites that were repeatedly sampled during recent years; (iii) detect potentially invasive cyanobacterial species including the subtropical *Cylindrospeyrmopsis raciborskii* in Belgian freshwaters.

ARB database: Belgian freshwater cyanobacteria

We collected 272 cyanobacterial sequences from cyanobacterial strains isolated from Belgian freshwater samples to create the 16S rRNA database. Cyanobacterial and chloroplast sequences from the SILVA rRNA database (SILVA 98 release) were retained in an ARB database and other sequences were deleted. Then, each Belgian sequence was imported into ARB, aligned and inserted into the phylogenetic tree by ARB parsimony application. Their number is still quite limited, and the B-BLOOMS1

project has been the major source so far. The origins of the Belgian sequences are listed in Table I.

Table I. Belgian cyanobacterial 16S rRNA database composition

Sequence origin	Methodology	Number of samples	Number of sequences
Willame et al., 2006	Cultivation approach	22	26
B-BLOOMS1	Clone library	5	201
B-BLOOMS2 (2007)	DGGE analysis	32	45
-	Clone library	1	9

16S rRNA-DGGE analysis

For the Brussels and Walloon samples, the genomic DNA was extracted from 32 filtered samples from the 2007 survey, 21 from the Falemprise Lake, 9 from Ixelles Pond II, and 2 from additional lakes (quarry of Ecaussines 2007 and Lake Chérapont 2007). These were used as templates for PCR. We used the cyano-specific PCR primers designed by Nübel et al. (1997) and Taton et al. (2003) to amplify the 16S rRNA gene and ITS sequences. A second semi-nested PCR was performed for DGGE analysis as described by Boutte et al. (2006). Bands were excised from gels and reamplified using the primers CYA 359F/CYA781R. PCR products were purified with GE Healthcare GFX PCR product purification kits and sent for sequencing.

Genotypic analyses of toxic and non-toxic cyanobacteria

The *mcy* gene cluster encodes the genes involved in microcystin biosynthesis. They belong to the families of non-ribosomally-encoded peptides (NRPS), polyketide synthesis (PKS) and fatty acid synthesis (Fischbach and Walsh, 2006). Sequences of *mcy* genes determined in *Anabaena*, *Microcystis* and *Planktothrix* (Tillet et al., 2000; Christiansen et al., 2003; Rouhiainen et al., 2004) have provided specific PCR-based *mcy* gene detection assays (see Ouellette and Whilhem 2003 for review). Recent findings showed that deletions and insertions can occur in these genes. For example, deletion of the *mcyT* region in the *Planktothrix mcy* operon resulted in a non-microcystin producing strain (Christiansen et al., 2008). In such cases, positive PCR detection is not concomitant with microcystin-based toxicity. On the other hand, deletion of other regions might have no consequence for microcystin production but

could hinder detection of *mcy* genes. In the genus *Anabaena*, deletions in the N-methyltransferase domain in *mcyA* genes were found in microcystin-producing strains (Fewer et al., 2008). In this case, the deletion is responsible for the production of a different variant of microcystin. Nevertheless, it was previously shown that the presence of the *mcyA,B,D,E,F* and *mcyH* genes is necessary for microcystin production (Dittmann et al., 1997; Tillet et al., 2000; Nishizawa et al., 2001; Pearson et al., 2004).

During this project, we tested the detection of *mcyA, mcyB* and *mcyE* with primers described in the literature.

Cyanobacterial species composition in lakes is relevant to the production of different microcystin variants and their concentrations (Kardinaal and Visser, 2005). A combination of PCR and Restriction Fragment Length Polymorphism (RFLP) analysis gives the opportunity to differentiate potential microcystin-producing genera and species in a sample (Hisbergues et al., 2003; Dittmann and Börner, 2005). To quickly identify the potential microcystin-producers present in our samples, we used the RFLP analysis of *mcyE* that had been designed during the BELSPO project B-BLOOMS1.

It is generally impossible to distinguish toxic from non-toxic cyanobacteria based only on morphological criteria and 16S rRNA sequences. Therefore, a genotypic analysis is required on the basis of different DNA sequences.

Previous studies of *Microcystis* population dynamics based on ITS-DGGE have shown a succession of different genotypes during a bloom. This succession suggested a relation with microcystin concentration (Kardinaal et al., 2007). Therefore, we have used the ITS-DGGE technique to determine whether the occurrence of particular *Microcystis* or *Planktothrix* genotypes can be associated with environmental factors.

Microcystin and/or *mcy* gene detection in single colonies or filaments of cyanobacteria has previously shown that field populations can be composed of both toxigenic and non-toxigenic genotypes (Janse et al., 2004; Via-Ordorika et al., 2004). It was not possible until recently to quantitatively measure cyanotoxin concentrations in these individual colonies or filaments. However, Young et al. (2008) (B-BLOOMS2 U. Dundee partner) demonstrated that it is now possible to quantify microcystins in single *Microcystis* colonies. This provided an excellent opportunity in B-BLOOMS2 to combine, for the first time, a quantitative microcystin analysis (U. Dundee) with a genetic analysis (ULg) for the same single colonies of *Microcystis* from environmental

sources. Genotypic analysis of single colonies/filaments requires a large amount of DNA. So far the genotypes of environmental single colonies of *Microcystis* have been characterized on the basis of one or two PCR products. In this project, we have developed a new approach that allows a Multi Locus Sequence Analysis (MLSA) to be performed on a single colony or filament. Moreover, the morphology of the colonies is recorded because it has been suggested that the *Microcystis* morphotypes are indicative of microcystin production.

The *mcy* gene detection strategy and RFLP of *mcyE* analysis

Three different PCR protocols were used to detect the *mcyA* (Hisbergues et al., 2003), *mcyB* (Nonneman and Zimba, 2001) and *mcyE* genes (Rantala et al., 2006). These primer pairs have different targets. The *mcyA* (mcyA-CdF/ mcyA-CdR) and *mcyE* (mcyEF2/ mcyER4) primer pairs were based on an alignment of the *Anabaen*a, *Microcystis*, *Planktothrix* and *Nostoc* microcystin synthetase sequences. As a result, these primer sets were able to amplify the *mcyA* and *mcyE* partial sequences in these 4 genera. Thus, we have used these primers to detect the presence of potential toxigenic genotypes from several microcystin-producing genera in a single reaction. In addition, the *Microcystis*-specific primers *mcyB* (mcyF1 and mcyR1/mcyR2) were used in two successive PCRs (nested strategy) to detect potential microcystin-producing *Microcystis* genotypes in our samples. Additionally, a *mcyE* RFLP analysis was performed on Lake Falemprise samples 2007/2008 and additional samples from Ixelles pond II 2007/2008, Lake Chérapont 2007, the quarry of Ecaussines, 2007/2008, Virelles pond 2008, Lake Féronval 2008, Lake Bambois 2008, and Ixelles pond I 2008. The *mcyE* PCR products (Rantala et al. 2006) were digested with the restriction enzyme *AluI*. The PCR programmes were performed as described in Table II.

Table II. PCR programme description

Target gene	Targeted genera	Primers	Programs				
mcyA	All genera	Cdf/CdR	Cycle 1 (1X)		Cycle 2 (35X)		Cycle 3 (1X)
			94°C-5min	94°C-1min	54°C-1min	72°C-1min	72°C-7min
mcyB	*Microcystis*	mcyF1/mcyR1	Cycle 1 (1X)		Cycle 2 (28X)		Cycle 3 (1X)
			94°C-3min	94°C-30sec	57°C-45sec	72°C-1min	72°C-7min
		mcyF1/mcyR2	Cycle 1 (1X)		Cycle 2 (35X)		Cycle 3 (1X)
			94°C-3min	94°C-30sec	57°C-45sec	72°C-1min	72°C-7min
mcyE	All genera	mcyEF2/mcyER4	Cycle 1 (1X)		Cycle 2 (35X)		Cycle 3 (1X)
			94°C-3min	94°C-30sec	57°C-45sec	68°C-1min	68°C-10min

ITS-DGGE analysis

We have used ITS-DGGE to determine the occurrence of particular genotypes in relation to environmental factors. In parallel, we have compared this succession with the microcystin concentrations measured in our bloom samples.

In Flanders, from each sampling of the reference lakes and several BLOOMNET samples, filters were used for DGGE of PCR-amplified 16S-23S rDNA intergenic spacer (also called ITS) sequences to investigate the genetic structure of the populations of the dominant cyanobacteria *Microcystis* and *Planktothrix* (Janse et al., 2003; 2004). For the monoclonal cultures, a small amount of concentrated material was used for the same purpose. A specific nested-PCR protocol based on the protocol described by Janse et al. (2003) was developed to amplify only *Microcystis* and *Planktothrix* ITS sequences from the water samples. In a first PCR, a specific 16S rDNA primer for *Microcystis* and *Planktothrix* (Rudi et al.,1997) was used as forward primer and a general 23S rDNA primer (ULR) was used as reverse primer (Janse et al., 2003). The resulting PCR product was purified using a QiaQuick PCR purification kit (QiaGen), diluted, and used as template for a second PCR with the cyanobacterium-specific 16S rDNA primer GC-CSIF in combination with primer ULR (Janse et al., 2003).

Single filaments/colonies approach

Colonies and filaments were isolated and washed under a dissecting microscope at ULg, starting from fresh environmental samples sent by FUNDP, UGent and VUB. Single colonies/filaments were observed and photographed under the microscope with 400X magnification. They were stored at -20°C until required. Samples were then resuspended in BG11 medium and heated in a boiling waterbath for 1 min. The resulting cell suspension was divided into 2 equal portions to be used for genetic and microcystin analyses, respectively. For the genetic analyses, 0.5 µl of the boiled lysate mixture was used as a template. A Repli-g mini kit (QiaGen) was used to amplify the whole genome of cells in the mixture by Multiple strand Displacement Amplification (MDA) with the phy29 polymerase. MDA products were subsequently used as template for PCR reactions specific for cyanobacteria or *Microcystis*. Aliquots (9.5 to 19.5 µl) of the remaining boiled cell suspensions were sent to the U. Dundee for quantitative ELISA assays for microcystins. This was done in triplicate according to Metcalf et al. (2000).

Genotypic analysis of Microcystis individual colonies

Eleven individual colonies of *Microcsystis* were isolated from Lake Falemprise, Ixelles pond I, Tervuren and Westvelpark ponds. Eight of the colonies were photographed. The housekeeping genes *ftsZ*, *gyrB*, *recA* were sequenced after amplification as described in Tanabe et al. (2007). The presence of a microcystin synthetase genes cluster was detected by PCR of *mcyA, mcyB* and *mcyE* as described in the *mcy* genes detection strategy section (above). In parallel, quantitative ELISA for microcystins was performed.

Genotypic analysis of Woronichinia individual colonies

We performed MDA reactions on the boiled cell suspensions from 6 individual colonies of *Woronichinia*. Then, the 16S rRNA gene was amplified with the ITS region using primers 359F/23S30R. Partial sequences of the gene *rpoC1* were amplified using primers RF and RR as described in Rantala et al. (2004). The *mcyA*/E genes were amplified as described in the previous section. Twelve clone libraries were constructed to determine the presence of NRPS and PKS-like sequences in *Woronichinia* colony genomes. PCR for amplification of NRPS-like sequences was performed using the primers MTF2/MTR of Neilan et al. (1999). PCR for amplification of PKS-like sequences was performed using the primers DKF/DKR designed by Moffitt & Neilan (2004). Sequence analyses of NRPS and PKS were carried out with the software Geneious (Drummond et al., 2009). The sequences were aligned using the algorithm MUSCLE and manually corrected (Edgar et al., 2004). Phylogenetic trees were constructed using the Neighbor joining method with the MEGA software. The Neighbor joining trees were constructed with a Poisson correction model. We performed 1000 bootstrap replicates for each tree. In parallel, the second halves of the boiled cell suspensions of 6 *Woronichinia* individual colonies were used for quantitative microcystin ELISA.

2.1.7. Determination of toxin contents

Partners collected and processed environmental samples throughout the 2007 and 2008 bloom seasons for the analysis of microcystins by HPLC and immunoassay (ELISA). This involved sample filtration to provide a particulate (cyanobacteria-containing) fraction and recovery of soluble (extracellular phase) microcystins by solid phase extraction. All fractions were stored at -20°C by the different partners until analysis by the U. Dundee. Analysis was carried out by HPLC and by immunoassay (ELISA) as described below, the majority of the toxin analyses being carried out by

both methods for samples from 2008 and 2009, whereas the samples from 2010 were only analysed by ELISA.

HPLC detection of cyanotoxins

Measurements of intracellular and extracellular microcystin concentrations by HPLC with photodiode array detection (HPLC-DAD) were carried out from selected bloom samples, collected from the reference lakes and from waterbodies sampled by BLOOMNET partners. Samples were analysed on a Waters HPLC-DAD system consisting of a Waters 600 E solvent delivery system, a 717 WISP autosampler and a 991 photodiode array detector. The 2 eluents were Milli Q water plus 0.1% (v/v) trifluoroacetic acid (TFA) and acetonitrile with 0.1% TFA. Separation was via a Waters Symmetry C18 Cartridge column (3.9 x 150 mm, particle size 5µm) using a linear gradient (water-acetonitrile) of 70%-40% over 30 min. Based on earlier experience that these genes are useful to select samples likely to contain microcystins, only bloom samples having the two microcystin synthase genes, *mcyE* and *mcyB*, were processed. Toxin concentrations were expressed in µg toxin / g dry weight and, if sampling volume had been recorded, as µg toxin per L water. If necessary, mass spectrometry was used to verify toxin identification and to identify substances presenting similar UV absorption spectrum as microcystins, often found in the chromatograms. Similar techniques were used at FUNDP to detect other toxins in Belgian blooms, in particular anatoxin-a.

Quantitative immunoassay of microcystins in environmental and other water samples

Water samples collected from Belgian waterbodies were aliquotted to provide subsamples for quantitative immunoassay and comparison with HPLC. Immunoassay was by enzyme-linked immunosorbent assay (Metcalf et al., 1990) using antibodies produced in Dundee versus microcystin-LR. Water samples were analysed for: (a) total microcystin, by pretreatment of 1 ml samples for 1 minute in a boiling water bath (Metcalf and Codd, 2000) to release intracellular microcystins. The resulting analyte was therefore the sum of the intra- and extracellular toxin pools; (b) extracellular microcystin, by centrifuging 1 ml samples at 10000 rpm in a microcentrifuge for 10 minutes and taking the supernatant for analysis; (c) intracellular microcystin concentrations were estimated by determining the difference between (a) and (b) (Metcalf and Codd, 2000).

Quantitative immunoassay of microcystins in single colonies and filaments of cyanobacteria

Single filaments and colonies were removed using Pasteur pipettes from water samples under a dissecting microscope, washed by serial transfer through 3 drops of sterile water, dimensions measured by microscopy (filament length, colony area) and total microcystin content per colony or filament determined after boiling to release the toxins (Metcalf and Codd, 2000; Akcaalan et al., 2006; Young et al., 2008).

Quantitative immunoassay of microcystins associated with other isolated aquatic organisms

In 2007, 2008 and 2010, bloom samples from Donkmeer and Westveldparkvijver were filtered on a nylon net with meshsize of 125 µm and rinsed with freshwater WC-medium to remove all unicellular cyanobacteria. From the concentrate, specimens of various planktonic organisms (*Daphnia*, copepods, rotifers, testate amoebae, ciliates) were picked out with a micro-pipette in freshwater WC-medium and rinsed 3-5 times by transferring them in several drops of medium before bringing them each separately in a sterile Eppendorf vial which was stored at -20°C before toxin analysis. In 2010, bloom samples from Donkmeer and Fort Bornem were filtered on a nylon net with meshsize 100 µm and rinsed with distilled water. From the concentrate, specimens of various planktonic organisms were picked out with tweezers in de-ionized water and stored for 1-2 hours in a small glass beaker (one for each species) filled with de-ionized water and the bulk was filtered and rinsed again with distilled water on a nylon net of 100 µm meshsize. From the net, individual specimens were picked out and blotted dry on a sheet of Kleenex, counted and stored together in an Eppendorf vial (each containing several specimens of one taxon) at -20°C before toxin analysis.

Production of quantitative analytical standards for microcystin analysis

Microcystin-LR was purified by HPLC from mass-cultured *Microcystis* PCC 7813, quantitated by gravimetric analysis using a microbalance, aliquotted in glass vials and lyophilized at the University of Dundee for use by all partners as required throughout the project according to Fastner et al. (2002).

Measurements of anatoxin-a and saxitoxins

Samples for analyses of anatoxin-a and saxitoxins were sent to Dr C. Bernard at the Museum of Natural History in Paris (Unit Ecosystems and Toxic Interactions,

USM0505). Analyses were carried out by HPLC-DAD followed by ESI-MS-MS (Gugger et al., 2005).

2.1.8. Deterministic watershed simulation modelling of Eau d'Heure Lakes

The existing model of the Eau d'Heure basin was developed in the framework of a partnership between the public Water authority INTERSUD/IGRETEC and two universities (FUNDP and ULg), in connection with local and regional authorities (Verniers & Sarmento, 2004).

The model is derived from the PEGASE model (Smitz *et al*., 1997; Deliège *et al*., 2009) and comprises several coupled sub-models, representing the functioning of the soil, groundwater and surface water. In addition, a wastewater sub-model allows to link human activities and input of pollutants in soil and water. The coupled model then represents the main quantities and fluxes in the global system, as well as water quality variables. Further developments of the model have dealt with the representation of the phytoplankton to specifically include cyanobacteria. The model has allowed running simulations of various management scenarios. In the B-BLOOMS2 project, the model was improved thanks to the data on lake monitoring (including those from B-BLOOMS1). The model was used in this study to simulate cyanobacteria in Lake Falemprise and to assess the effects on various management scenarios.

2.1.9. Statistical predictive models for Brussels ponds

The data from the 22 Brussels ponds sampled monthly from May to September in 2007 and 33 and 29 ponds sampled in May, July and August in 2008 and 2009 respectively were combined with the data from the same or other Brussels ponds, acquired between 2003 and 2006 according to the same methodology, into a single matrix. The resulting data matrix comprises 48 ponds sampled on more than 500 occasions. The combined dataset was analysed using multivariate and univariate statistical techniques and incorporated into a probabilistic model based on conditional probability calculation. The output of the probabilistic model was verified by the Classification Trees (Breiman et al., 1984) to make sure that it was not an artefact of a particular methodological approach.

2.1.10. Data base

The B-BLOOMS2 data base (see www.bblooms.be) includes the monitoring data collected at the reference lakes according to the standard protocols, i.e. mainly the

data collected in Phase 1. This file contains environmental data (from water analysis and from the collection of weather data), quantitative composition of phytoplankton and zooplankton, relative percentage of cyanobacteria, total and soluble microcystins, and the presence-absence of *mcy-genes*. In addition, samples from BLOOMNET have been added, when the records were complete for all variables. For the samples collected in 2009 and 2010 through BLOOMNET, microcystins were systematically measured, and the dominant cyanobacterial taxa were identified, although only some environmental data were recorded.

The processing of these data was made using a multivariate regression technique: "boosted regression trees" (BRT). BRT is a form of logistical regression using decision trees and a boosting algorithm (Elith et al., 2008). This method allows identification of the best predictors (e.g. among environmental variables) to a dependent variable (e.g., relative abundance of a given taxon in blooms). The technique does not require data transformation prior to analysis, but can fit complex non-linear relationships and automatically handles interactions between predictors. The analysis was applied to various parts of the B-BLOOMS2 data base, essentially comprising the Walloon and Flemish reference lakes, totalling *ca.* 200 samples, for building predictive models of total cyanobacterial biomass, occurrence of the main taxa found in the blooms and microcystin concentrations. All analyses were run in the R environment (R Development Core Team, 2006) by F. Leprieur, PhD., ECOSYM (Ecologie des Systèmes Marins Côtiers), Université de Montpellier II, France.

2.2 RESULTS

2.2.1. Ecology

Flanders

Nine BLOOMNET-samples did not reveal cyanobacteria, but contained flagellated (*Tetraselmis*, *Chlamydomonas*) or filamentous (*Spirogyra*) green algae, euglenophytes (*Euglena*) or heterotrophic (purple) bacteria. Nineteen percent of the cyanobacterial blooms from 2006 - 2011 were dominated by *Anabaena spp.,* 17% by *Aphanizomenon spp.*, 7 % by *Planktothrix rubescens* and 43 % by *Microcystis spp*. Some blooms were particularly important because of their socio-economic consequences:

- In spring 2007, a massive bloom of *Aphanizomenon flos-aquae* in the Gavers in Harelbeke coincided with the mortality of big carp and some waterfowl. However, no anatoxin-a or saxitoxins (analysis done at Institut Pasteur, Paris) nor microcystins could be detected in the samples (see Table VI) and the reasons for the high mortalities remain unknown. The lake was closed for recreation for several weeks.
- In Schulensmeer (Lummen), every year since 2005, a massive bloom of *Woronichinia naegeliana* and *Microcystis* spp. developed in autumn. Microcystin concentrations exceeded 20 µg MC-LR equivalents L^{-1} every year (see Tables VI and VII) and sometimes reached very high levels (8600 µg L^{-1} in October 2007), on the basis of which the local government closed the lake to the public for several weeks for safety reasons.
- In February 2008, a massive bloom of *Planktothrix rubescens* developed in a quarry in Dongelberg (Jodoigne). ELISA-tests at UGent revealed a microcystin concentration of 525 µg MC-LR equivalents L^{-1}. Due to this problem, this lake could not be opened by the owners for recreation (diving).
- A bloom of *Aphanizomenon flos-aquae* caused the closure of the large recreational lake Dikkebussevijver (Ieper) in the summer of 2009.
- In two lakes in Zonhoven, the invasive and potentially toxic *Cylindrospermopsis raciborskii* was detected by J. Van Wichelen on 26 August 2009. This was the first signalled occurrence of this species in Belgium (Van Wichelen 2009) and this observation confirms its northward spread (Wiedner *et al.* 2007). In a recreational lake nearby (De Vijvers, Averbode) a bloom of the same species

was detected on 31 August 2009 and because the water contained 27 µg MC-LR equivalents L^{-1}, the lake was closed to the public.

- The Paalse Plas (Beringen) was closed in July 2010 due to a bloom of *Anabaena flos-aquae,* and a microcystin concentration >80 µg MC-LR equivalents in the water.
- A massive and toxic bloom of *Microcystis* spp. in early September 2010 in the large recreational Lake Donkvijver (Oudenaarde) also caused the closure of this lake to the public.
- Detection of cyanobacteria also resulted in the closure of the recreational lakes De Plas/Ter Heide (Rotselaar) in July 2009 and Ekerse Plassen (Muysbroek, Ekeren) in September 2010 which both reached the press (De Morgen 29/07/2009, 09/09/2010), indicating an increased awareness of the public and stakeholders.
- Toxic blooms are not necessarily limited to the summer season in our area. Indeed, cold water-adapted toxic *Planktothrix rubescens* blooms were recorded in Dongelberg (Jodoigne) in February 2008 and in Boudewijnpark (Brugge) from December 2010-May 2011. In both cases, recreation was not allowed for several months due to microcystin concentrations reaching 70 and 3000 µg MC-LR equivalents L^{-1}, respectively. Since Boudewijnpark is a family park and situated in a heavily populated area, this phenomenon received much press attention (Het Nieuwsblad 18/03/11, 03/06/11).
- A BLOOMNET sample from August 20th 2010 revealed a dense and toxic bloom of *Microcystis* spp. (mainly *M. aeruginosa* and *M. flos-aquae*) in the moat surrounding a pre WWII fortification at Bornem (Fort Bornem) which coincided with the presence of similar *Microcystis*-eating amoebae as observed in Westveldpond (*Vanella* sp., *Korotnevella* sp.) (see further). Monitoring of the *Microcystis* surface bloom was carried out weekly until the bloom vanished at the end of October in order to document possible effects of amoebae grazing on the *Microcystis* population. The population density of the amoebae in this surface bloom varied between 600 and 5300 ind. ml^{-1}, much lower than the peak densities observed at Westveldpond (see further) and as a result no clear changes in *Microcystis* community structure and biomass were detectable

during this bloom until its disappearance in autumn, presumably due to low temperature.

In both reference ponds in Flanders, cyanobacterial blooms were detected with contrasting dynamics and cyanobacterial species, as revealed by qualitative microscopy examination of the phytoplankton communities. In Donkmeer, more or less the same pattern was seen in 2007 (Figure 1) and 2008. In spring and early summer, cyanobacterial blooms consisted mainly of *Anabaena* spp. (mainly *A. subcylindrica*, *A. flos-aquae*) and *Aphanizomenon flos-aquae* (with some *Microcystis* spp. and *Planktothrix agardhii*), which regularly formed surface scums in parts of the littoral zone. In August, *Planktothrix agardhii* formed a bloom suspended in the water column throughout the lake, with occasional local surface scums. *Planktothrix* remained dominant in the phytoplankton community until the end of October when they were replaced by cryptomonads and/or coccoid green algae. A diverse zooplankton community was present with rotifers, the small cladoceran *Bosmina* and cyclopoid copepods dominating, during the *Planktothrix* bloom periods and large *Daphnia* in between.These dynamics are a recurrent phenomenon in Donkmeer: every year the multi-genera (*Anabaena-Aphanizomenon-Microcystis*) surface blooms in the littoral zones and the suspended *Planktothrix agardhii* blooms in the pelagic can be observed in summer (last documented on 19.08.2010).

In Westveldparkvijver, in 2007 at the beginning of May, a *Euglena* bloom was present, which was replaced by *Microcystis* after two weeks (Figure 2). In 2008, the bloom of *Microcystis* increased later. The *Microcystis* bloom was associated with a constant extremely low water transparency (Secchi depths often <15 cm) and lasted uninterrupted until the end of October. It was mostly suspended in the water column, but occasionally surface scums were observed. Accompanying phytoplankton taxa consisted mainly of euglenophytes and green algae (*Desmodesmus, Pediastrum*) in 2007, and euglenophytes, green algae (*Desmodesmus*) and cyanobacteria other than *Microcystis* (*Limnothrix* and *Anabaena planctonica*) in 2008. In 2007, by the end of October, the *Microcystis* bloom declined and phytoplankton dominance was taken over by chlorococcal green algae. In 2008, a bloom of *Anabaena planctonica* was seen in late summer.

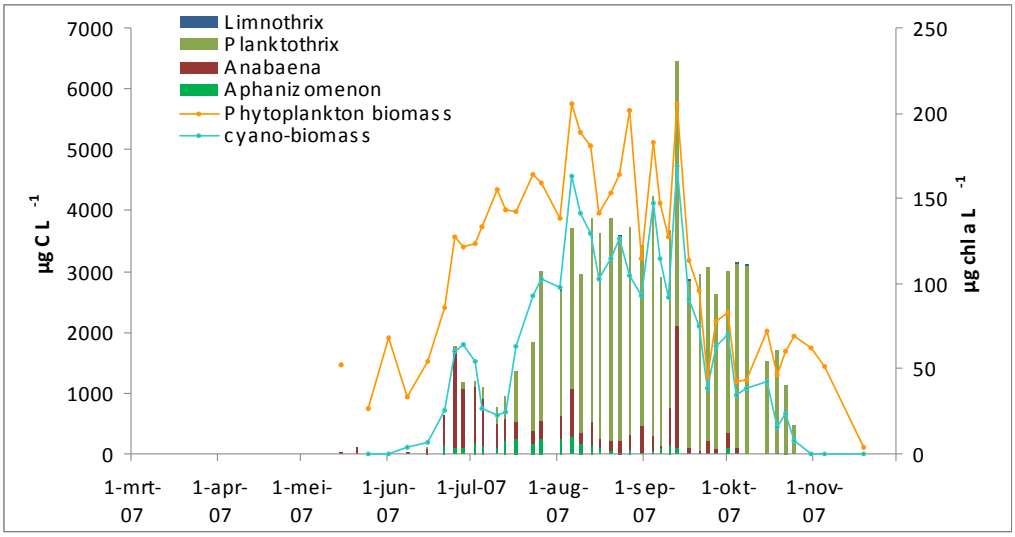


Figure 1: Cyanobacterial bloom dynamics in the reference lake Donkmeer (only results for 2007 are shown), as revealed by microscopy counts, and a comparison with estimates of total and cyanobacterial biomass based on HPLC pigment analysis (performed by FUNDP, Namur).

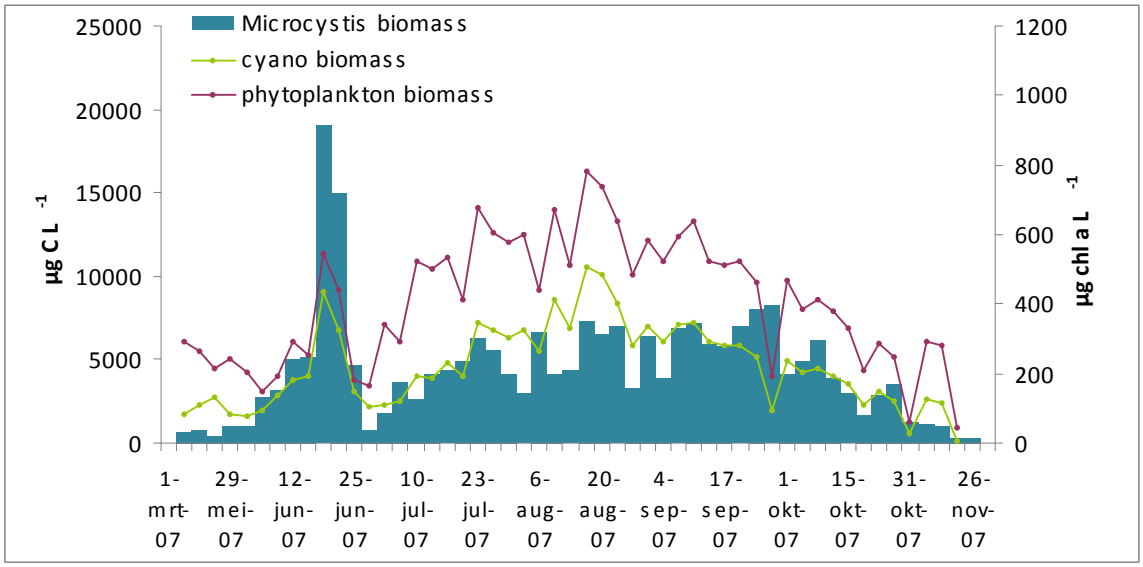


Figure 2: Cyanobacterial bloom dynamics in the reference pond Westveldparkvijver (only results for 2007 are shown), as revealed by microscopy counts, and a comparison with estimates of total and cyanobacterial biomass based on HPLC pigment analysis (FUNDP, Namur).

Microscopic examination of the samples from Westveldparkvijver revealed the presence of two *Microcystis* morphotypes, corresponding to *M. aeruginosa* and *M. viridis*. In 2007, the first had a maximal abundance in early summer but suddenly the population collapsed and was quickly replaced by *M. viridis* (Figure 3). At precisely this moment, the *Microcystis* population became heavily infected by naked amoebae

(*Korotnevella* sp. and *Vanella* sp.), which mainly infected the *M. aeruginosa* colonies (as seen by the percentage infected colonies of each morphotype), and therefore presumably caused the rapid decline of this morphotype in favour of *M. viridis*, after which the amoeba numbers rapidly declined. Peak densities reached 67,000 amoebae ml^{-1}, among the highest ever reported (Van Wichelen *et al.* 2010). This suggests an important role for the amoebae in causing a major and extremely rapid shift in *Microcystis* population structure and a temporal biomass decline. This contrasts with the zooplankton, consisting almost exclusively of rotifers, which had an abundance peak before the bloom maximum and were present only at low densities throughout the bloom period. A chytrid fungus, *Chytridium microcystidis*, also reached high infection frequencies (up to 75% infected colonies) in both *Microcystis* morphotypes but was apparently incapable of controlling *Microcystis* biomass and remained present throughout the bloom period. In 2008, a *M. aeruginosa* bloom was present again in summer and a peak of amoebae coincided again with the end of the bloom (although later in summer), but was this time not followed by a bloom of *M. viridis*. The observed community structure corresponded with the DGGE analyses. The magnitude of amoebae grazing and the food preference of these zooplankton were further investigated experimentally. All amoebae tested were capable of reducing growth of the majority of *M. aeruginosa* strains isolated from Westveld pond while none of the tested amoebae were capable of reducing growth of *M. viridis* strains (Van Wichelen *et al.* 2010). Microcystins were apparently not responsible for the observed food preference since all strains tested from both morphotypes produced these toxins. Subsequent growth experiments indicated that colony morphology (differences in the thickness of the mucilage matrix) was not responsible for the observed preference but instead other biochemicals probably played an important role in the protection of *Microcystis* strains against amoebae grazing (van Wichelen *et al.*, submitted to Aquatic Ecology).

In April 2009, Westveld pond was restored, as commissioned by the City Council of Ghent. The pond was drained and the fish (estimated at about 500 kg ha^{-1}) and sludge layer (around 530 m^3) were removed. The middle of the pond was deepened, shores were re-profiled using local sand and planted, and most of the semi-domestic waterfowl were captured and removed. The restoration measures seemed successful: four sampling campaigns (June 10th, August 24th 2009, January 26th, June 6th 2010) could not reveal a single *Microcystis* colony. Instead a bloom of euglenophytes was

present in June 2009 with *Trachelomonas* appearing in the water column and *Euglena* forming a small surface layer, while the zooplankton community was characterized by a high density of rotifers. In August 2009, only a surface layer of the flagellated chlorophyte *Chlamydomonas* was present while a very high density of large *Daphnia* was observed in the water. In 2010, a mixed phytoplankton community with chlorophytes, cryptophytes and euglenophytes, all at very low densities, was observed.

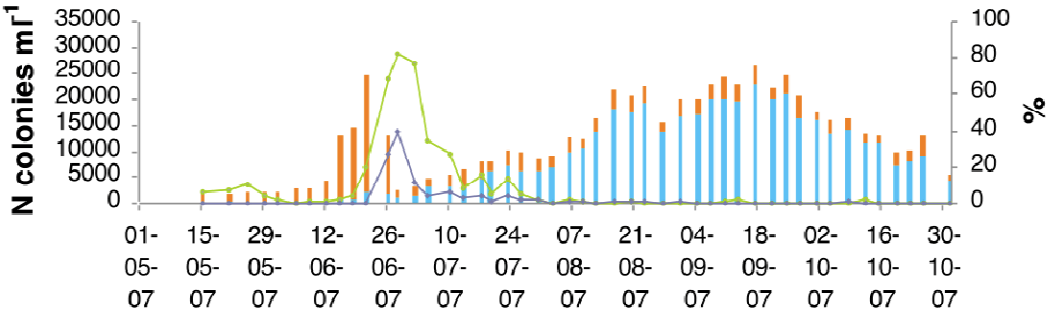


Figure 3: *Microcystis aeruginosa* and *M. viridis* population density in Westveldparkvijver in 2007 and the percentage of amoebal infestation (green line) during the same period.

Brussels

The two reference ponds from Brussels (étangs d'Ixelles, hereafter IxP1-upstream and IxP2-downstream) are rich in phosphorus with mean TP concentrations exceeding 0.1 mg L^{-1}. The TP level in IxP2 was about twice as high as that of IxP1 (Figure 4, a). This was reflected in total phytoplankton biomass which was significantly higher in IxP2 than in IxP1. Higher phytoplankton biomass in the former was generally associated with higher pH that was significantly different from that of the latter. SRP concentrations were significantly higher in IxP2, whereas in IxP1 they were often below 0.003 mg L^{-1} suggesting concurrent phosphorus limitation (Reynolds, 2006). Conversely, IxP1 was significantly richer in DIN, mainly due to elevated NOx concentrations, whereas ammonium, except for a few spikes in IxP2 during the cold season 2007-2008, was comparably low in both ponds. The DIN/SRP ratio was below 7 (Redfield N/P ratio; Reynolds, 2006) in 8% of the samples in IxP1 and in 71% of the samples in IxP2.

The zooplankton communities of both ponds studied were dominated by rotifers and small cladocerans, with occasional rises in densities of cyclopoid copepods. Although subdominant, large cladocerans were constantly present in IxP2, where they often occurred at markedly higher densities than in IxP1. Besides numerical superiority,

large cladocerans in IxP2 were also generally larger in size than those in IxP1. Phytoplankton communities of the two ponds were represented mainly by cyanobacteria, chlorophytes, diatoms, cryptophytes and, to a lesser degree, dinophytes. Despite a substantial overlap in phytoplankton composition at genus level, the two ponds often differed markedly in terms of assemblage dominance. The phytoplankton assemblage of IxP2 was mostly populated by large eukaryotic phytoplankters, and large filamentous or colonial cyanobacteria including *Aphanizomenon* spp., *Planktothrix* spp. and *Woronichinia* spp.. However, IxP1 was predominantly populated by smaller eukaryotes and smaller cyanobacteria including *Gomphosphaeria* spp., *Snowella* spp., *Chroococcus* spp. and *Aphanocapsa* spp.

Redundancy analysis (RDA; ter Braak & Smilauer, 2002) allowed the variables having the strongest relationship with the phytoplankton groups to be identified. The first two RDA axes explained 33% of the variation in the phytoplankton data. As indicated by the Secchi depth arrow, the first axis corresponds to a phytoplankton biomass gradient (r=0.79; Figure 4). Chl a and Secchi depth showed highly significant relationships with phytoplankton biovolumes ($p<0.01$), suggesting that the latter give a reasonable estimation of phytoplankton biomass and that turbidity in the ponds studied was mostly phytoplankton-induced. To avoid the blurring effect of chlorophyll a and Secchi depth on the other environmental variables, they were excluded from the model in forward selection analysis. After their exclusion, 8 variables showed significant a relationship with phytoplankton. These were DIN, TP, temperature, pH, large Cladocera length, Rotifera density, NH4 and SRP. They explained 39% of the total variance in the phytoplankton data explained by the model. The relationships between phytoplankton and the other zooplankters were not significant.

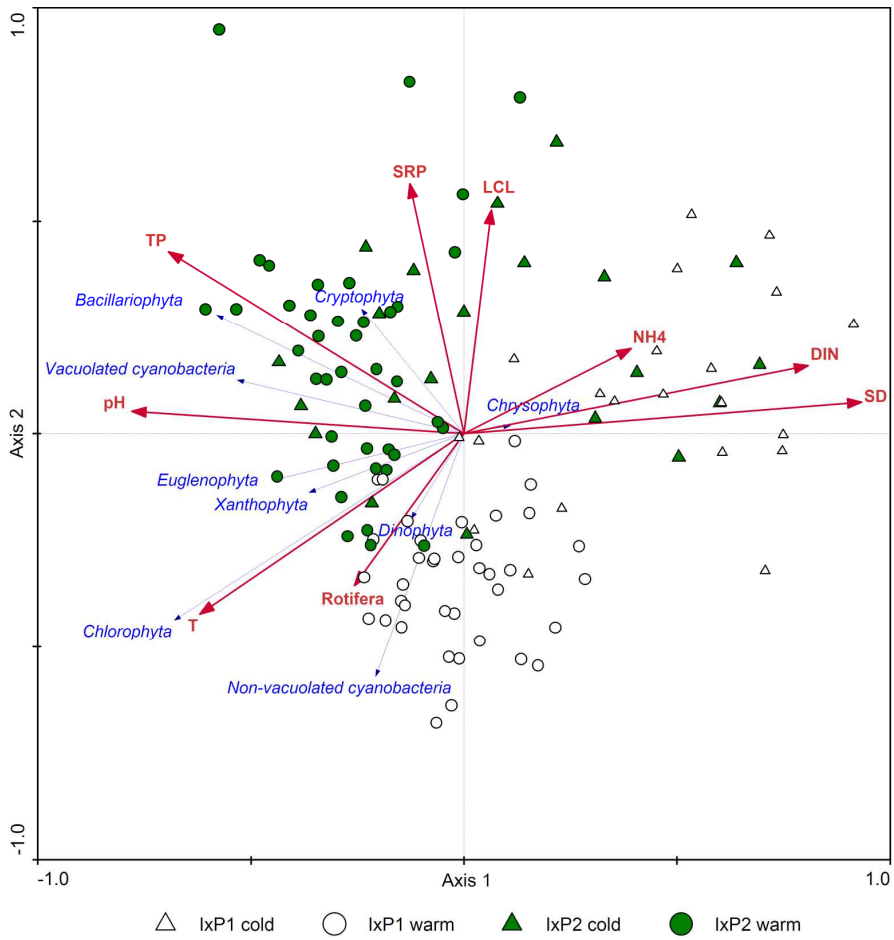


Figure 4: RDA triplot based on phytoplankton and environment data from the Brussels ponds (IxP1 and IxP2) in cold and warm seasons (see symbols) Phytoplankton biovolumes are aggregated to division level. Only environmental variables having significant relationships with phytoplankton are shown. TP – total phosphorus, SRP – soluble reactive phosphorus, LCL – large Cladocera length, DIN – dissolved inorganic nitrogen, SD – Secchi depth, T – temperature.

Continuous monitoring of the ponds allowed seasonal periodicity in the dynamics of phytoplankton, zooplankton and environmental conditions to be elucidated. Predictably, seasonality appeared to have a strong effect on phytoplankton biomass and composition. Phytoplankton biomass was markedly higher during the warm months in both ponds, except for springs 2007, 2009 and autumn 2009 in IxP2 where phytoplankton biomass was exceptionally high for the season (Figure 5). This was mainly due to cyanobacterial blooms dominated by *Aphanizomenon* spp. in spring and *Leptolyngbya* spp. in autumn (Figure 5). During the warm season, cyanobacteria of IxP2 were dominated by *Planktothrix* spp. and/or *Woronichinia* spp. with *Limnothrix* spp. and *Aphanizomenon* spp. often being important subdominants.

In IxP1, elevated cyanobacterial biomass was confined to the warm season when it was dominated mainly by *Gomphosphaeria* spp. associated with a variety of other

small non-gas vacuolate cyanobacteria and occasional rises in biomass of *Planktothrix* spp. and *Aphanizomenon* spp.

Phytoplankton biomass was roughly mirrored by TP and pH that also markedly increased during the warm months (Figure 5). Except for NOx in IxP1 that showed the opposite trend, dissolved nutrients showed lesser dependence on seasonality, with rather erratic rises in concentrations of SRP and ammonium, particularly in 2007 and the first part of 2008. This may have been related to storm water overflow into these ponds during heavy rains and suggests that IxP2 was visibly more affected. This possibility is supported by the occasional abrupt changes in phytoplankton biomass and composition in this pond after heavy rains.

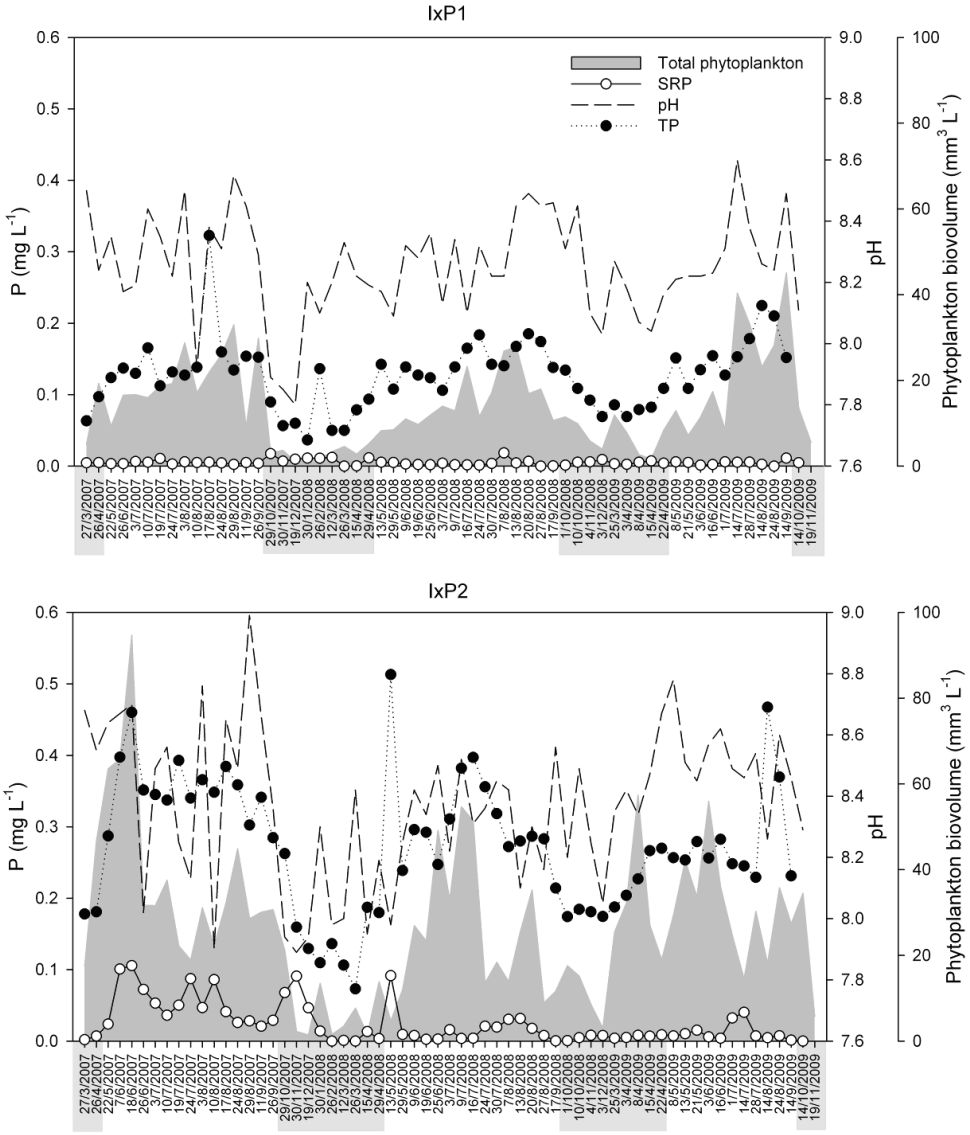


Figure 5: Temporal dynamics of total phytoplankton biomass in the two Brussels ponds (IxP1 and IxP2) in relation to pH, TP and SRP concentrations. Cold months are shaded grey.

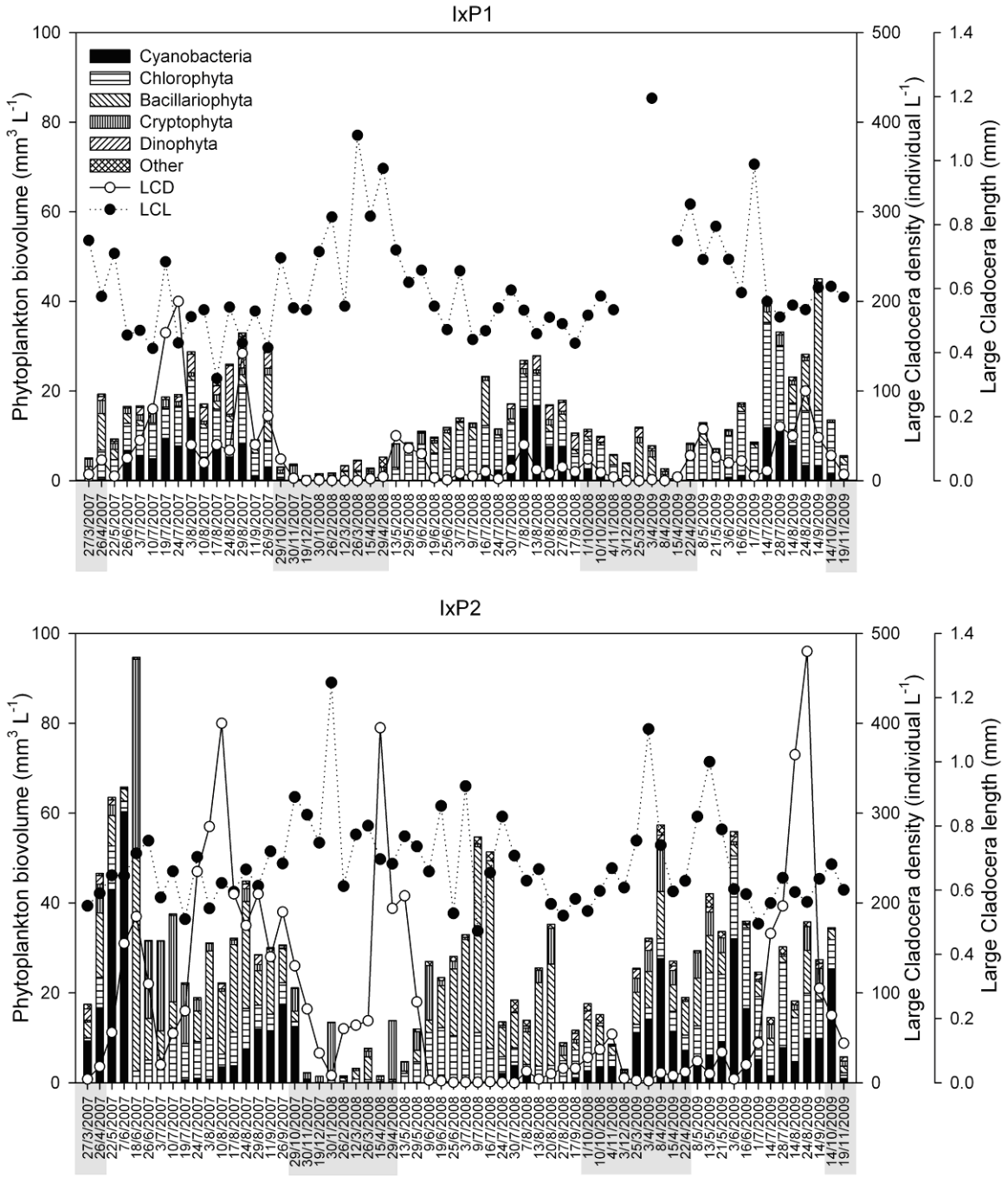


Figure 6: Temporal dynamics of phytoplankton biomass and composition in the two Brussels ponds (IxP1 and IxP2)studied in relation to large Cladocera density and length. Cold months are shaded grey. LCD – large Cladocera density, LCL – large Cladocera length.

Zooplankton densities, composition and size structure were also markedly affected by seasonality. During the cold season, zooplankton communities were mainly represented by rotifers. Cladocerans were markedly more common during the warm season (Figure 6) and in IxP2 high densities of large cladocerans were occasionally associated with a visible increase in water transparency. Although mostly less abundant, large cladocerans were generally bigger during the cold months. Increase in population density during the warm months usually coincided with a marked decrease in large cladocera size in both ponds (Figure 6), prompting increased predation pressure by fish as reported by Pourriot (1995). Lower density and size of large cladocerans in IxP1 suggest that the fish community in this pond was more biased towards planktivorous fish than in the IxP2 pond.

Thus three years of continuous monitoring of two turbid ponds in close proximity showed that despite apparent similarities in size, shape, depth, substrate and surroundings, they differ considerably in their phytoplankton dynamics. Total phytoplankton biomass appears to have been determined mainly by nutrient concentrations. IxP2, the pond with markedly higher phytoplankton biomass, also showed significantly higher TP and SRP concentrations. Phytoplankton in this pond was mainly nitrogen-limited as indicated by the generally low DIN concentrations. Conversely, frequent SRP depletions in IxP1 suggest that the phytoplankton was mainly phosphorus-limited in this pond. This is supported by the DIN/SRP ratio that was mostly above 7 in IxP1 and mostly below 7 in IxP2, prompting potential phosphorus-limitation in the former and nitrogen-limitation in the latter (Capblancq & Décamps, 2002).

Besides total phytoplankton biomass level, the difference in phosphorus and nitrogen availability seems to have also affected the composition of the phytoplankton assemblages in the 2 ponds . Thus IxP2, characterised by low DIN concentrations, was more affected by cyanobacterial blooms than IxP1 that was generally much richer in DIN. Cyanobacterial assemblages of the 2 ponds were also markedly different, with IxP1 being dominated by smaller non-gas vacuolate cyanobacteria while IxP2 by larger gas vacuolate species. Low DIN concentrations in IxP2, prompting competitive advantage to cyanobacteria capable of nitrogen-fixation (Reynolds, 1998, 2006) were, however, more often associated with elevated biomass of non-heterocystous taxa, mainly *Planktothrix* spp., *Woronichinia* spp. and *Limnothrix* spp., than the heterocystous *Aphanizomenon* spp. or *Cuspidothrix* spp., suggesting that low

combined nitrogen concentrations favoured not only heterocystous cyanobacteria, capable of nitrogen-fixation, but also gas vacuolate cyanobacteria in general. This supports the idea of Ferber et al. (2004) that nutrient limitation renders gas vacuolate cyanobacteria more competitive than eukaryotic phytoplankters due to their ability to regulate their position in the water column, thereby enabling them to access nutrients released from the sediment. The strong migratory abilities of most of the gas vacuolate genera which made up cyanobacterial assemblages in the ponds studied are well documented (Hyenstrand et al., 1998; Graham & Wilcox, 2000; Ferber et al., 2004; Reynolds, 2006). Thus, reaching abundant benthic nutrients and bringing them stored to the surface for photosynthesis can give cyanobacteria a strong advantage over eukaryotic phytoplankters which are unable to regulate their position in the water column. Frequent mixing owing to the low depth of the ponds can considerably reduce this advantage of cyanobacteria by facilitating access of eukaryotic algae to benthic nutrients.

Generally high pH values in IxP2, mostly above 8.3 and indicative of carbon-limitation for many phytoplankters (Reynolds, 2006), also favoured bloom-forming cyanobacteria known to have a strong affinity to elevated pH due to superior CO_2 fixation kinetics (Shapiro, 1973, 1997; Peretyatko et al., 2010). The significantly higher pH in IxP2 than in IxP1 was probably responsible, at least in part, for the difference in the frequency and magnitude of cyanobacterial blooms between the two ponds. The dominance of cyanobacterial assemblages of IxP2 by large filamentous and colonial forms , in contrast to smaller cyanobacteria prevalent in IxP1, is consistent with the difference in the population density and size of large cladocerans.

Occasional abrupt changes in the biomass and composition of phytoplankton in IxP2 as compared to smoother, more predictable phytoplankton dynamics of IxP1 support the idea that the two ponds differ in hydraulic regime, with the former being more affected by storm water overflows.

Thus, contrasts in local factors including trophic state, zooplankton community structure, nitrogen availability and hydraulic regime determined marked differences in the phytoplankton dynamics of the two apparently similar turbid ponds exposed to the same meteorological conditions. This underlines the importance of local factors for phytoplankton dynamics in general and cyanobacterial bloom development in particular and implies that appropriate management of local factors can considerably reduce the risk of noxious bloom occurrence. Besides immediate improvement in

ecological quality, this might counter the future effects of global warming, increasingly evoked as a reason for the increase in the frequency and magnitude of cyanobacterial blooms.

Wallonia

In Lake Falemprise, the reference lake in Wallonia, cyanobacterial blooms occurred every year during the project, but with variable intensity. These blooms were much less developed than those which occurred in summer 2002, when blooms of *Aphanizomenon flos-aquae* dominated the phytoplankton from May through August (Verniers et al., 2005). By contrast, total phytoplankton biomass was similar from year to year, with maxima often exceeding 100 µg chla L^{-1}. This illustrates the variability in this lake, which is eutrophic (mean total phosphorus 55 µg L^{-1}), with oxygen-depletion below 3 m when stratified. However, it is relatively shallow (mean depth 2.6 m), so that the stability of summer stratification is relatively weak and easily disrupted during cool and windy weather episodes. In general, *Aphanizomenon flos-aquae* developed during periods of warm, stable weather, with a variable contribution of other phytoplankton groups (chlorophytes, diatoms, chrysophytes and euglenophytes). Diatoms generally dominated during spring and a during mixing episodes in other seasons.

In 2007 and 2008, identification and counts of cyanobacteria enabled estimations to be made of the relative biovolumes of the different taxa, and to combine these estimates with cyanobacterial biomass calculated from pigment analysis (Figure 7). In 2007, *Aphanizomenon* developed in May – June. From July to September, *Planktothrix agardhii*, probably favoured by cooler weather and mixing of the water column, became the dominant cyanobacterial species. Other taxa were present in the samples: *Microcystis aeruginosa*, *Anabaena* sp., *Limnothrix* sp., with *Gomphosphaeri*a sp. and *Aphanocapsa* sp. more sporadically observed. In 2008, the monitoring of blooms was carried out in Falemprise from April to mid-October: from mid-May onwards only blooms of *Aphanizomenon* and *Anabaena* sp. were observed, and a few *Microcystis* colonies were recorded.

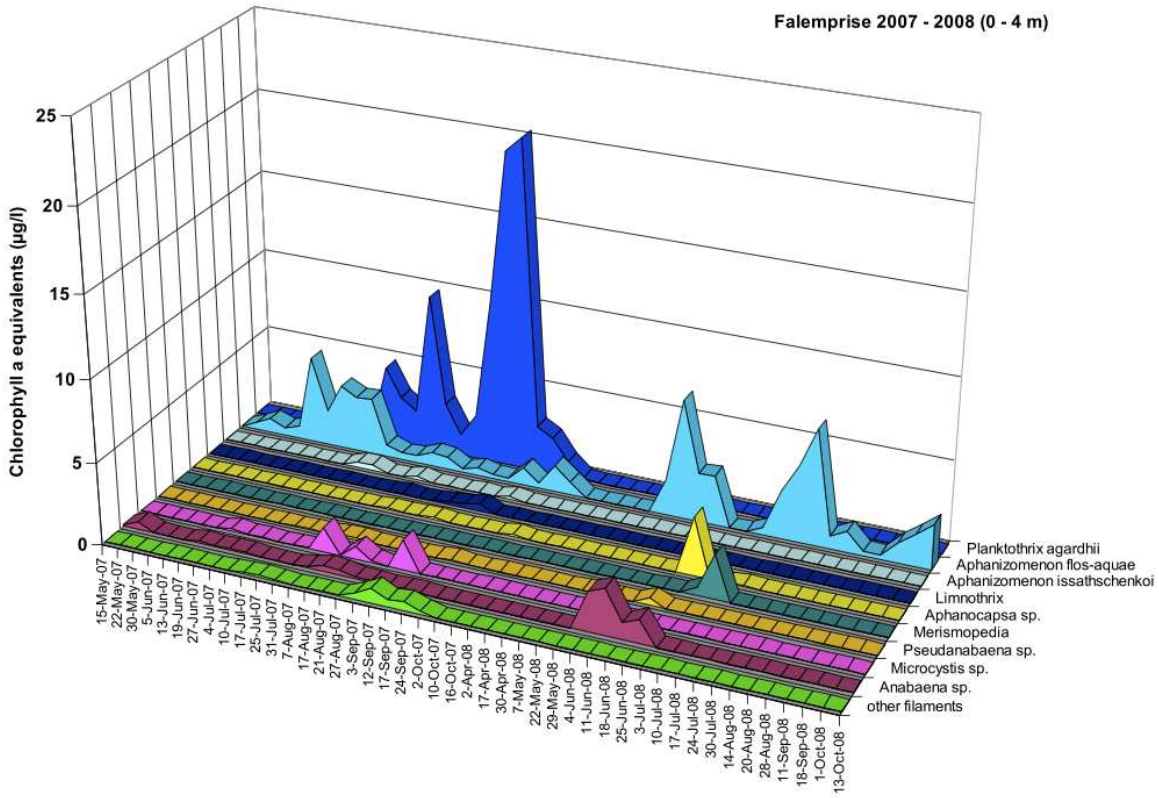


Figure 7: Biomass of the different cyanobacteria taxa in Falemprise (2007 and 2008), averaged for the 0 – 4 m samples, expressed as chlorophyll a equivalents.

A multivariate analysis of the Falemprise data, using ACP and CCA, was carried out. These analyses showed a strong seasonality in the environmental variables, but with some variability in phytoplankton structure, which may be due the unstable physical structure of the lake. However, the CCA clearly identified zooplankton as one of the factors determining composition and biomass of the phytoplankton: in particular, the development of the filamentous cyanobacteria dominant in this lake was related to the development of large cladocerans and calanoid copepods.

BLOOMNET in Wallonia

In 2007, only one significant *Microcystis* bloom was recorded: on 28 August in Lake Chérapont near Gouvy. In 2008, an important *Anabaena* bloom occurred in Lake Bambois and was sampled on July 30th. A similar bloom, dominated by *Anabaena* cf. *circinalis* was sampled in Lake Virelles in August 2008. Another sample was taken from the quarry of d'Ecaussines, used for drinking water water storage by VIVAQUA (ex CIBE): there, *Planktothrix rubescens* was well developed at depth (between 8 and 11 m). Sampling was performed on 20th June 2007 just before a test of water

aeration, as a measure to disrupt stratification. Microcystins were found by HPLC analysis of biomass extracts.

In 2009, several blooms were collected in summer. Again, only one bloom was reported by ISSeP in Lake Chérapont (Gouvy). This was a toxic *Microcystis* bloom, with 16.6 µg L^{-1} microcystin-LR equivalents, predominantly particulate. Lake Bambois presented short-lived *Anabaena* blooms at the end of August and at the beginning of September, and microcystins were detected in the water at low concentrations (max 2.6 µg L^{-1} microcystin-LR equivalents). The situation was similar in Lake Virelles, which was surveyed throughout the 2009 bloom season, but only discrete *Anabaena* blooms occurred, accompanied by few *Microcystis* colonies. These *Microcystis* were however microcsytin-producing strains, and low microcystin concentrations levels were measured. One sample from Renipont also presented a low microcystin concentration. More serious cyanobacterial blooms were observed in two lakes of the Eau d'Heure complex: Lakes Féronval and Ry Jaune, where bathing and other recreational activities were important in summer.. The situation was particularly serious in Lake Ry Jaune in 2009, where toxic *Microcystis* blooms developed from the end of July to mid-September, with total microcystins reaching a maximum 57.5 µg microcsytin-LR equivalents L^{-1}; the toxins was most of the time being mainly particulate.

In 2010, when the monitoring of phytoplankton was reinforced through the survey of all bathing waters in Wallonia, cyanobacterial blooms occurred with much lower frequency and intensity than in 2009, probabaly due to bad weather conditions in mid-summer. Nevertheless, a few eutrophic water bodies were added to the list of bathing waters presenting a high risk of developing toxic cyanobacterial blooms (Descy et al., 2010b).

2.2.2 Isolation of strains and characterization of their potential toxin production

A total of 520 strains were isolated and characterised on the basis of their morphology (Table III). Two strategies were used by UG and ULg to characterise intra-genus genotypic diversity and overall cyanobacterial diversity (see 3, Materials and Methods section). The ULg strategy was very effective to recover picocyanobacteria or small filaments (but not nitrogen-fixers), whereas the UG strategy was efficient to isolate dominant bloom-formers, e.g. *Microcystis* and *Planktothrix*.

At ULg, the cultures were tested for presence of *mcy* genes by PCR and/or sent to Dundee for microcystin analysis. Twenty one cultures were identified as potential microcystin-producers (table IV). The *mcyA* and *mcyE* genes were simultaneously detected in only one strain of *Microcystis* and one strain of *Cyanobium*. To date, this is the first report of a *Cyanobium* carrying *mcy* genes. The *mcyE* gene was also found in two other *Cyanobium* strains that were isolated from Lake Rénipont. A significant concentration of MC-LR equivalents (48.7 µg/L) was found in another *Cyanobium* strain from Lake Féronval.

Significant amounts of MC-LR equivalents were also detected in Oscillatoriales, including *Leptolyngbya*-like, *Phormidium*-like, *Pseudanabaena*-like and an unknown filamentous cyanobacterium. NRPS-like sequences were detected for the first time in a strain of the genus *Snowella*. This result shows that secondary metabolites, such as toxic coumpounds, can be produced by this genus.

A total of 39 *Cyanobium* strains were isolated. Phylogenetic analysis of 16S rRNA partial sequences clustered the strains into four groups (Figure 8). Interestingly, the three strains that were identified as potential microcystin producers are clustering in the cluster I.

Nine Oscillatoriales strains were not affiliated to any species on the basis of the BLAST analysis of their sequences and remain unidentified.

During this project, we were able to cultivate 'classical' bloom-forming cyanobacteria, including *Planktothrix* and *Microcystis,* and non-bloom forming cyanobacteria including *Cyanobium, Leptolyngbya, Pseudanabaena* and *Snowella*. Therefore, it will now be possible to focus on the features and role of non bloom-forming cyanobacteria in Belgium, such as the picocyanobacterial genus *Cyanobium*.

Table III. Number of strains obtained by UG and ULG for each sampling.

Waterbody	**Donkmeer**	**Donkvijver Oudenaarde**	**Driekoningenvijver**	**Falemprise**	**Féronval**
UG	29 (*P. agardhii*)	24 (*Microcystis*)	52 (*P. rubescens*)		
ULG				28 (*Cyanobium, Microcystis, Snowella*, Oscillatoriacae)	42 (*Cyanobium, Microcystis, Leptolyngbya, Snowella, Phormidium, Pseudanabaena*)
Waterbody	**Fort Bornem**	**Ixelles pond I**	**Ixelles pond II**	**Leeuwenhofvijver**	**Neerpede 4**
UG	24 (*Microcystis*)			95 (*Microcystis*)	
ULG		9 (*Cyanobium, Leptolyngbya*)	3 (*Wilmottia murray*)		1 (*Cyanobium*)
Waterbody	**Rénipont**	**Ri Jaune**	**Schulensmeer**	**Tiens Broek:**	**Vijvers Zonhoven**
UG			48 (*Microcystis*)	34 (*Microcystis*)	72 (*Microcystis*)
ULG	27 (*Cyanobium*, *Microcystis*, *Leptolyngbya*, *Snowella*, *Phormidium*, *Pseudanabaena*)	11 (*Cyanobium*, *Pseudanabaena*-like)	8 (*Cyanobium, Microcystis*)		11 (*Cyanobium, Pseudanabaena,* unknown, *Pseudanabaena*)
Waterbody	**Westveldparkvijver**				
UG	54 (*Microcystis*)				
ULG					

Table IV . Summary of strains of ULg that potentially produce secondary metabolites. - :absence, + : presence ; nd: not determined

Strain	Origin	Order	Most similar 16S rRNA (analysis by BLAST)	PCR detection			MC eq (µg/L)
				mcyA	*mcyE*	NRPS	
FW001	Féronval	Chroococcales	100% *Microcystis* PCC7820 (AF139300)	-	-	+	2.106
FW003	Féronval	Oscillatoriales	99.5% *Pseudanabaena* sp 1tu24s9 (AM259267)	-	-	+	nd
FW009	Féronval	Chroococcales	100% *Microcystis* LMECYA 82 (EU078494)	-	-	nd	3.42
FW027	Féronval	Oscillatoriales	99.7% *Pseudanabaena* sp 1tu24s9 (AM259267)	-	-	+	nd
FW028	Féronval	Oscillatoriales	99.3% *Phormidium tenue* NIES-611 (AB042842)	-	-	+	9.64
FW032	Féronval	Oscillatoriales	99.8% Uncultured bacterium clone 2\SC\37(EU340177)	-	-	+	
FW038	Féronval	Oscillatoriales	99.5% Uncultured bacterium clone 2\SC\37(EU340177)	-	-	-	6.83
FW057	Féronval	Chroococcales	99.9% *Snowella litoralis* 1LT47S05 (AJ781041)	-	-	+	nd
FW069	Féronval	Chroococcales	99.8% *Cyanobium* PCC7009 (AF216945)	-	-	nd	48.7
FW101	Féronval	Oscillatoriales	96.5% *Leptolyngbya* sp. LGE 06121 (HQ832945)	-	-	+	nd
FW118a	Féronval		Mixture	-	-	+	6.96
FW015	Rénipont	Chroococcales	100% *Cyanobium* sp. Sai002 (GU935363)	+	+	nd	nd
FW017	Rénipont	Chroococcales	100% *Cyanobium* sp. Sai002 (GU935363)	-	+	nd	nd
FW018	Rénipont	Chroococcales	100% *Cyanobium* sp. Sai002 (GU935363)	-	+	nd	nd
FW082	Rénipont	Oscillatoriales	99.0% *Leptolyngbya* sp. 0BB19S12 (AJ639895)	-	-	nd	2.217
FW088	Rénipont	Oscillatoriales	99.8% *Pseudanabaena* sp. 1tu24s9 (AM259269)	-	-	nd	9.61
FW039	Ri Jaune	Oscillatoriales	98.6% Uncultured bacterium (GU935361)	-	-	nd	3.803
FW071	Ri Jaune		Mixture	-	+	nd	nd
FW077	Ri Jaune	Oscillatoriales	99.9% *Pseudanabaena* sp. ABRG5-3 (AB527076)	-	-	nd	8.598
FW084	Ri Jaune	Oscillatoriales	99.9% *Pseudanabaena* sp. ABRG5-3 (AB527076)	-	-	nd	7.037
FW007	Schulensmeer	Chroococcales	99.9% *Microcystis aeruginosa* LMECYA 59 (EU078492)	+	+	nd	nd

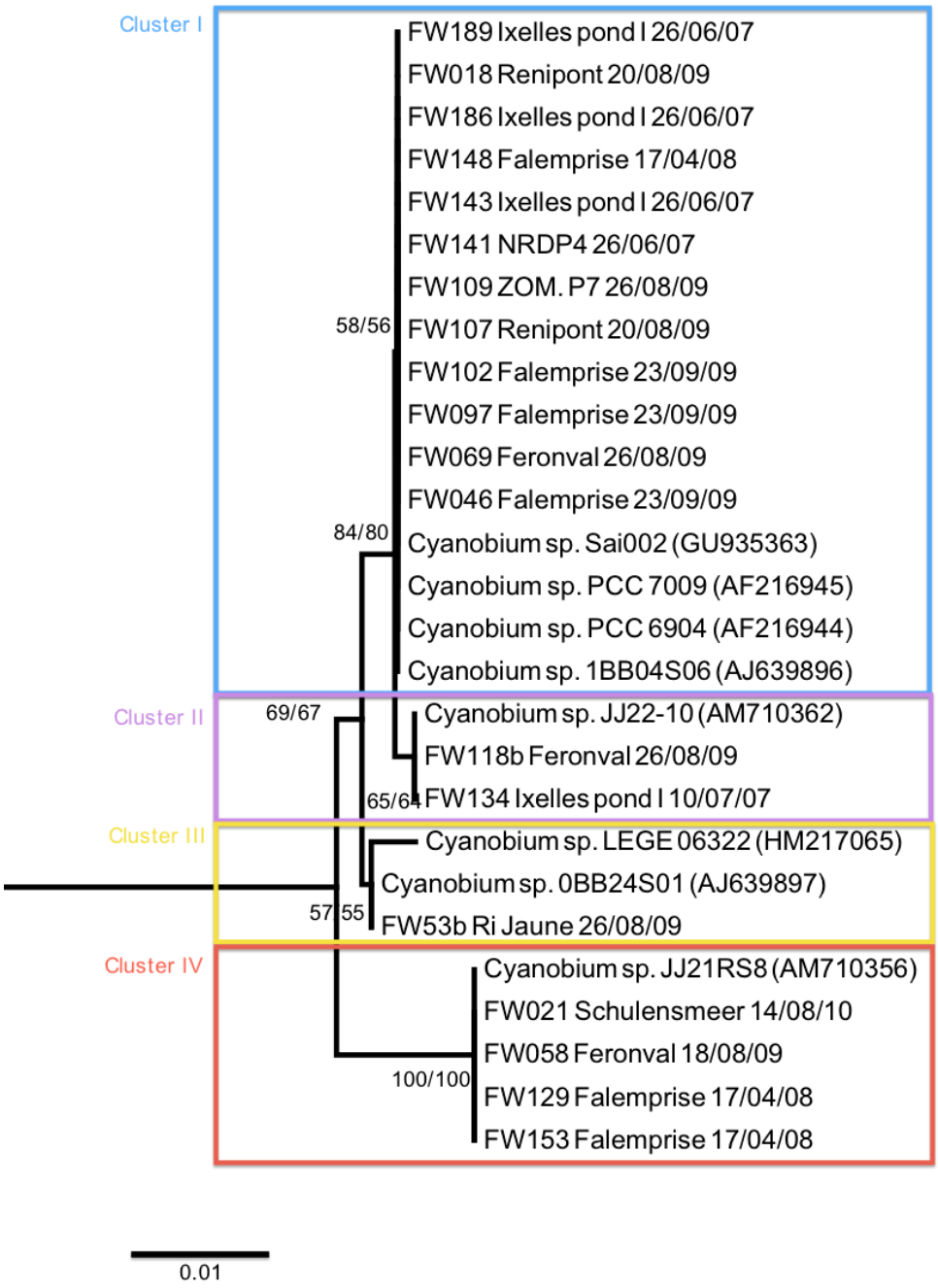


Figure 8. The 16S rRNA (523 nt) distance tree of *Cyanobium* strains. Distances were computed using the Jukes and Cantor correction and a Neighbor-joining tree was built (Saitou & Nei, 1987) with the software MEGA4 (Tamura et al., 2007). Minimum-evolution and neighbour-joining bootstrap replicates (1000 replicates) are indicated at the nodes as percentages. *Anabaena variabilis* ATCC 29413 was the outgroup. Clusters are framed in colour.

2.2.3. Molecular ecology of cyanobacteria

Molecular diversity of cyanobacteria in reference lakes

ARB database: Belgian freshwater cyanobacteria

The ARB database was created to record the richness of cyanobacteria in Belgian waterbodies. The majority of sequences (62.5%) from the database cluster with *Aphanizomenon/Anabaena* (36.4%) and *Anabaena/Aphanizomenon issaatchenkoi* (26.1%). During the B-BLOOMS1 project, clone libraries of bloom samples showed the presence of only one to three cyanobacterial dominant genera.

About 20 sequences in the database belong to the genus *Microcystis*. These sequences were found in 9 different Belgian lakes, which suggests a wide distribution of *Microcystis,* as previously observed by Willame et al. (2005). About 23 sequences belonged to *Planktothrix agardhii/rubescens*. *Snowella* and *Woronichinia* sequences (25) were recovered from 3 different lakes and were present in two reference lakes (Lake Falemprise and Ixelles pond II). Nevertheless, blooms of *Woronichinia* were quite frequently observed in Belgium and can be associated with concentrations of microcystins at least 4-fold higher than in some *Microcystis* blooms (Willame et al. 2005). Picocyanobacteria, with *Cyanobium/Synechococcus*-like sequences, were recovered from the quarry of Ecaussines, Lake Falemprise, Lake Ry Jaune, and Lake Blaarmeersen. Other sequences belonging to clusters of the genera *Nostoc*, *Phormidium* and *Pseudanabaena* were added to the database. These genera are mostly known to belong to benthic environments, but can be associated with hepatotoxin and neurotoxin production (Codd et al., 2005a,b; Jungblut et al., 2006; Cadel-Six et al., 2007).

16S rRNA DGGE analysis

DGGE analysis carried out on Lake Falemprise samples from 2007 showed the presence of *Microcystis*, *Planktothrix*, *Cyanobium*, *Synechococcus* and *Snowella* spp.. The majority of the bands amplified and sequenced showed high similarity with potentially toxic *Microcystis* and *Plankthotrix*, already observed in 2007 samples from Ixelles pond II, Lake Chérapont and the Ecaussinnes quarry. All of these sequences were uploaded into the ARB database as summarized in Table V.

Table V. Affiliation of cyanobacteria based on DGGE band sequences.

Lakes	Week sample number	Similarity	First relatives strains	AN
Falemprise				
	22, 23, 24, 25, 26, 27, 28, 29*, 30*, 34, 36, 40, 41, 42	100%	*Aphanizomenon flos-aquae* strain 1TU26S2	AJ630443
	35	99,7%	*Aphanizomenon flos-aquae* strain 1TU26S2	AJ630443
	35	99,48%	*Aphanizomenon flos-aquae* LMECYA 88	EU078540
	29, 30, 31, 32, 33, 36, 37, 40	99,7 - 100%	*Planktothrix agardhii* LMECYA 153F	EU078516
	28, 30	100%	*Microcystis aeruginosa* LMECYA 157	EU078503
	31, 41	100%	*Microcystis aeruginosa* LMECYA 59	EU078492
	27, 30, 31, 40, 41, 42	99,7-100%	*Microcystis aeruginosa* PCC 7806	AM778951
	25, 32	99,5 - 100%	*Cyanobium sp.* JJ19B5	AM710354
	28	100	*Cyanobium sp.* JJ22K	AM710364
	35	99,20%	*Synechococcus* sp. 0BB26S03	AJ639899
	26, 35	99,5-99,7%	*Synechococcus* sp. 0TU30S01	AM259220
	35*,36, 37, 38*, 39*, 40, 41, 42*	100%	*Snowella litoralis* 1LT47S05	AJ781041
Ixelles pond II				
	23, 32*, 34*	100%	*Planktothrix agardhii* LMECYA 153F	EU078516
	32	100%	*Aphanizomenon issatschenkoi* 473	EU157982
	23, 26*, 28*	100%	*Microcystis aeruginosa* PCC 7806	AM778951
	23, 26*, 28*, 32	99,70%	*Woronichinia naegeliana* 0LE35S01	AJ781043
	26, 28*, 32*	100%	Uncultured alga isolate WL8-6	AF497901
Lake Chérapont				
	35	100%	*Aphanizomenon issatschenkoi* 473	EU157982
	35	100%	*Microcystis aeruginosa* PCC 7806	AM778951
Carriere Ecaussinnes				
	25	100%	*Planktothrix agardhii* NIVA-CYA 29	AB045931
	25	100%	*Synechococcus sp* . MW6C6	AY151243

Genotypic analyses of microcystin-producing and non-producing cyanobacteria

A total of 162 samples were extracted and tested for the presence of *mcy* genes in the Brussels and Walloon regions between 2007 and 2009.

In 2007, *mcyB* detection by nested PCR was the most efficient procedure to detect potential microcystin-producing *Microcystis* genotypes. All samples from Brussels-Wallonia were positive, except two (Falemprise 24/09/07 and Ixelles Pond II 01/08/07). The *mcyE* detection results showed the presence of *mcyE* in 9 Falemprise samples from 27th June to 27th August 2007, and then, *mcyE* became undetectable until 2nd October. It was detected again from 2nd October to 16th October. In the quarry of Ecaussines (20th June 2007), *mcyE* detection was positive.

In 2008, all samples were positive for the presence of at least one of the *mcy* gene markers used in this project.

In Lake Falemprise, all samples were positive for *mcyA/B/E* except one (17th April 2008). Nonetheless, *mcyB* showed the presence of potentially toxic *Microcystis* in this sample.

In IxP2, *mcyA* analysis showed 17/20 undoubtedly positive results, while the samples of 26th March and 24th April did not show clear bands and the sample of 25th June showed no band at all. The *mcyE* gene was detected in 15/20 samples. The *mcyB* 2-step amplification was positive for all samples. Results of the detection of the three markers in Ixelles pond II suggest that the microcystin-producing cyanobacteria included *Microcystis* plus other genera .

In IxP1, *mcyA* amplifications were observed in18/19 samples. 19/19 were positive after the 2-step *mcyB* PCR. A *mcyE* amplification was obtained with 17/19 samples.

In 2009**,** *mcyA* and *mcy*E genes were detected in the 12 samples of Lake Ri Jaune from 28/07/09 to 21/10/9. In Lake Féronval, *mcyA* was detected in all samples from 28/07/09 to 21/10/9 and *mcyE* was detected in all samples, except that of 18/08/09. In IxP2, *mcyA/E* were detected in 3 samples of 08/04/09, 13/08/09 and 24/08/09. In Gouvy, Bambois, and Renipont, *mcyA* and *mcyE* were always detected.
In general, the *mcy* gene analysis showed the presence of potential microcystin-producing *Microcystis* in the majority of the Walloon samples.

We have also considered the link between *mcy* detection and microcystin concentrations measured in the soluble and particulate fractions. The *mcyA* (Hisbergues et *al*., 2003) and *mcyE* (Rantala et *al.,* 2004) gene detections were performed for all extracted samples In parallel, the controls PCR for the presence of cyanobacteria, using the 359F and 23S30R primers (Taton et al., 2006) were always positive. Eighty-two percent of the samples that were extracted were positive for the presence of the gene *mcyA,* and 89% were positive for the presence of the *mcyE* gene. The *mcyE* detection seems to be more efficient to detect potential microcystin-producing genotypes but there were cases where the *mcyA* gene was detected whereas *mcyE* was absent, as in Lake Féronval in 2009 (Figure 9). When the particulate toxin fraction represented at least 50% of the total toxin concentration (ELISA), both genes were always found in the samples. Negative detections of one or both markers were observed when the total microcystin pool was below 2.5 µg/L

microcystin-LR equivalents and the soluble fraction was higher than 60%. In the case of the Virelles samples, it is possible that the low biomass of cyanobacteria may explain the lack of detection of microcystin gene markers.

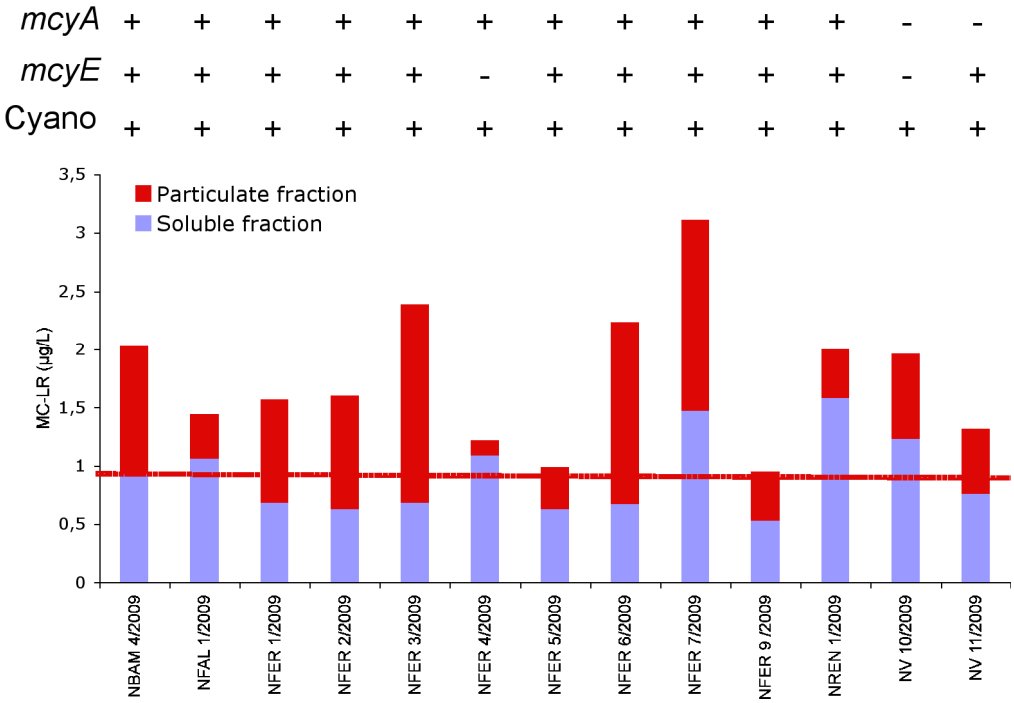


Figure 9: Relation of total, soluble, and particulate MC-LR equivalents concentrations measured by ELISA, with PCR detection of microcystin synthetase genes *(mcyA, mcyE)* and cyanobacterial presence (Cyano) from Lakes Bambois (NBAM), Falemprise (NFAL), Féronval (NFER), Virelles (NV)

RFLP analysis of *mcyE* was carried out on samples from 9 filtered samples from IxP2 from June to September 2007. This suggested the coexistence of two different *mcyE* genotypes of *Microcystis*. The same pattern was observed in IxP1 2008 and in Chérapont 2007 samples. In addition, a potential microcystin-producing *Planktothrix* was clearly identified in the quarry of Ecaussines (2007-2008).

In Falemprise, RFLP analysis of 2007 and 2008 samples showed a succession of the two different *mcyE* genotypes of *Microcystis* (Figure 10).

Thus, two *mcyE* genotypes of *Microcystis* were present in the majority of the Walloon samples.

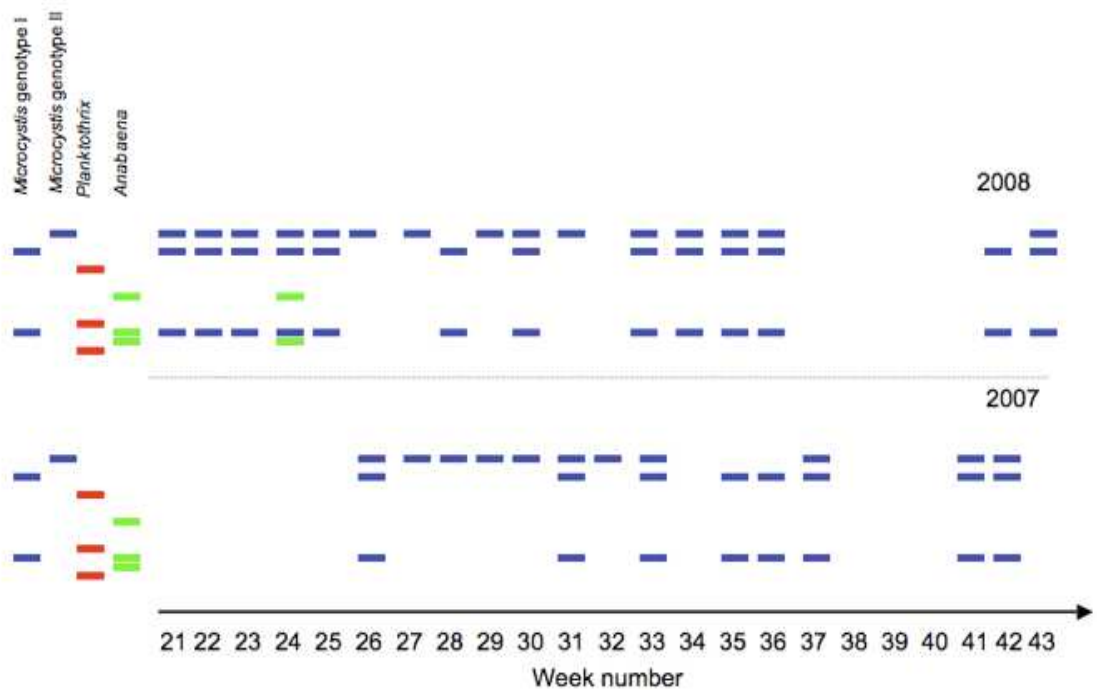


Figure 10: RFLP of *mcyE* in Lake Falemprise (2007-2008).

ITS genotype analysis

The ITS diversity of 62 BLOOMNET samples from the Flanders region (*Microcystis* blooms: 32, *Planktothrix* blooms: 24, mixed blooms: 6, obtained from B-BLOOMS1 together with the strains obtained by B-BLOOMS2 was studied by ITS DGGE.

For *Planktothrix*, DGGE analysis of 29 Donkmeer strains isolated on 7/09/2007 showed the presence of two to four ITS bands per strain, indicative of the presence of multiple copies of ITS, as shown by Janse *et al.* (2003). As a result, it is difficult to determine the number of different ITS genotypes present in natural bloom samples. However, the rather low number of bands encountered in *Planktothrix* bloom samples (on average 2.75 ± 0.88 bands), and the low spatial (the same bands were seen in most blooms) and temporal (no differences in banding pattern in Donkmeer during the entire sampling period) turn-over between blooms suggest a very low local and regional genetic diversity in *Planktothrix* blooms. This agrees well with the fact that the Donkmeer strains belonged to only one or two different ITS genotypes. Also, all *Planktothrix rubescens* strains isolated from Driekoningenvijver had identical ITS sequences based on DGGE analysis and sequencing of the DGGE bands.

For *Microcystis*, the DGGE profiles revealed a high ITS richness within blooms, since most bloom samples contained several to many ITS types (mean: 3.6 stdev: 1.8). This was also the case for the ponds sampled in B-BLOOMS2. In Westveldparkvijver, 10 *Microcystis* ITS types were detected during the whole sampling period, although only 3 types were dominant. A rapid and dramatic shift in genetic structure occurred at the same moment as the shift from *M. aeruginosa* to *M. viridis*, suggesting that the different morphotypes correspond to different ITS types. This was clarified with the isolates of 2008 for which the original colony morphology was noted. At Fort Bornem, 7 *Microcystis* ITS types were present, of which only 2 were dominant and always present during the sampling period. In one sample of Tavervriendenvijver (Humbeek) from 18/08/2010, 7 different *Microcystis* ITS types were detected.

The studied Flemish *Microcystis* blooms were, next to having a high ITS richness, also functionally diverse. *Microcystis* strains isolated from the same bloom (Tiens Broek or Leeuwenhofvijver) were shown to differ in growth rate, colony formation, microcystin production (van Gremberghe et al. 2009a), and, as a result, competitive ability and sensitivity to zooplankton grazing (van Gremberghe et al. 2009b,c). This is also evident from the different sensitivity of *Microcystis* strains from Westveld pond to amoebae-grazing (see above). This suggests that these blooms have the potential to respond to changing environmental conditions through a rapid genotype turn-over.

For Leeuwenhofvijver, 2 of the 4 different *Microcystis* ITS genotypes detected were positive for *mcyA/E* gene sequences. In Tiens Broek, this was true for 3 of the 7 *Microcystis* ITS genotypes. For Driekoningenvijver, the single *Planktothrix* ITS genotype present was also positive. The 3 main *Microcystis* ITS types present in Westveldparkvijver were all identified as potential microcystin-producers. Of the 29 strains isolated from Donkmeer in 2007, 10 contained *mcyA* genes.

The ‚single-colony' approach for Microcystis and Woronichinia colonies

For the first time, we were able to amplify and sequence several genetic loci of the genome of 8 single colonies of *Microcystis* from 3 different ponds (Figure 11).

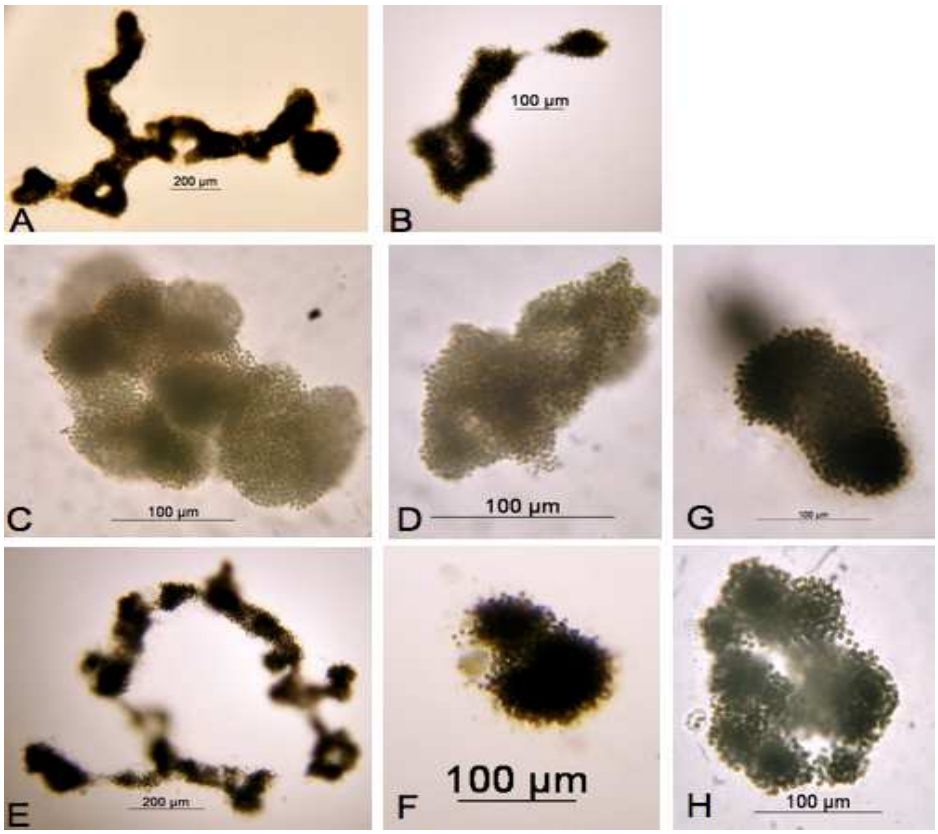


Figure 11: Photomicrograph of *Microcystis* environmental colonies. (A) Colony X5, (B) Colony X38, (C) Colony X8, (D) Colony X11, (E) Colony X39, (F) Colony X40, (G) Colony T1, (H) Colony F13. All colonies were manually isolated under the dissecting microscope and transferred onto a slide with wells for photography with 400X magnification. X: Ixelles Pond, T: Tervuren Pond, F: Falemprise pond.

Up to now, the WGA approach was efficient for 70% of the tested colonies. Positive amplifications were obtained for rRNA-ITS, 16S rRNA, *rpoC1*, *recA*, *gyrB*, *rbcLX*, and *gvpA*. Detection of the microcystin synthetase operon was possible with the PCR reactions for *mcyA*, *mcyB*, and *mcyE*. The concentrations of MC-LR equivalents ranged from 0.01 to 0.9 ng colony^{-1} in 5 *Microcystis* colonies, in coincidence with the presence of *mcy* genes (Lara et al., submitted). The ITS sequences were grouped into two clusters, though with low bootstrap values. Colonies that were positive for at least two genes and ELISA were grouped into cluster I, whereas negative colonies were grouped into cluster II (Figure 12)

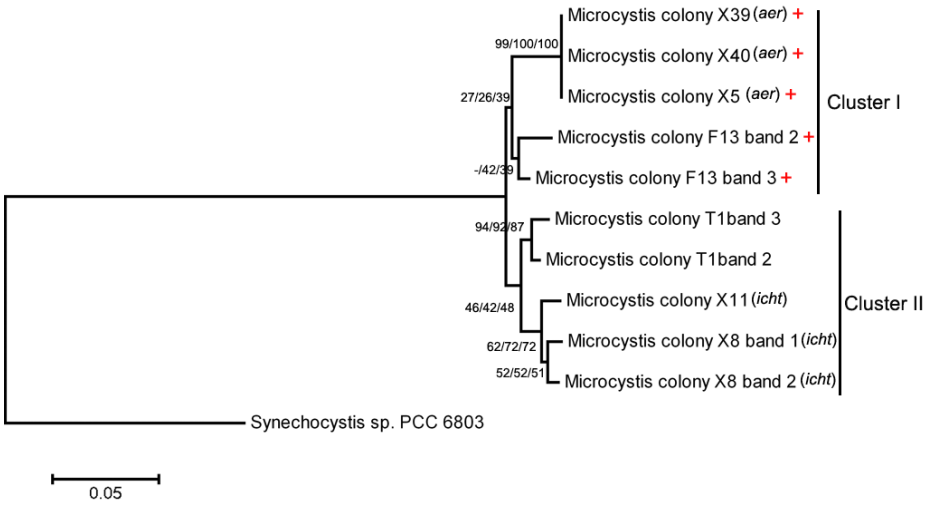


Figure. 12. Distance tree of ITS sequences (460 nt). Distances were computed using the Jukes and Cantor correction and a Neighbor-joining tree was built (Saitou & Nei,1987) with the software MEGA4 (Tamura et al., 2007). Maximum-parsimony, minimum-evolution and neighbour-joining bootstrap percentages (1000 replicates) are indicated at the nodes. *Synechocystis sp.* PCC 6803 sequence was used as outgroup. (*aer: aeruginosa* 'like'; *iht: ichthyoblabe* 'like'; the '+' means that MC measured was over the minimum detection limit and at least two *mcy* genes were detected). Distance scale is expressed as the number of substitutions per site.

The housekeeping genes *ftsZ, gltX* and *recA* were successfully amplified and sequenced for 7 *Microcystis* colonies. It seems that each colony is a genetically homogeneous group of cells that share the same sequence type (ST). Indeed, we determined that the 3 (*ftsZ, gltX, recA*) gene loci were present in only one copy in the genome of *Microcystis* NIES-843 (Kaneko *et al.*, 2007). Moreover in all chromatograms, neither ambiguities, nor mixtures of sequences, were observed after careful examination. The alignment of concatenated sequences (1196 bp) revealed 6 Sequence Types (ST) for 7 colonies but no distinct clusters of MC-producing and non-MC-producing colonies, in contrast to the ITS. The comparison of the ST acquired during this study with sequences of Asian *Microcystis* (Tanabe *et al.*, 2007) indicates that the Belgian ST are unique (Lara et al., submitted).

In addition, we have obtained the first sequences of *rpoC1, rbcLX* and rRNA-ITS from 6 individual colonies of the genus *Woronichinia* (identified by microscopy, Figure 13). This information is particularly significant as blooms of *Woronichinia* are quite frequently encountered in European waterbodies. As it is very difficult to isolate and maintain this genus in culture, few data are available in Genbank and the question of its toxicity is yet unresolved.

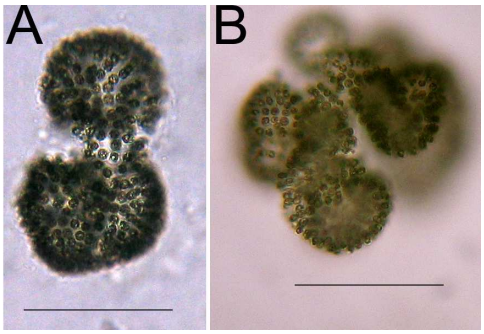


Figure. 13. Photomicrographs of two of the *Woronichinia* environmental colonies. *Woronichinia* individual colonies were isolated as described for *Microcystis* colonies. Distance bars represent 50 µm. (A): colony T2; (B) colony T5.

We performed *mcyE* gene PCR detections (Rantala et al. 2004) which showed the presence of a 750 nt DNA fragment in one *Woronichinia* colony. This fragment was cloned and sequenced. BLASTp analysis of the translated sequence showed 50% identity with a beta-ketoacyl carrier protein synthase from *Microcystis* NIES-843 (Kaneko et al., 2007). Beta-ketoacyl-ACP synthases are important enzymes involved in polyketide and fatty acid synthesis. However, the ELISA result of this colony was below the minimum detection limit of 17 pg per colony (Young et al., 2008).

Moreover, we have determined the presence of operons involved in secondary metabolite production in *Woronichinia*. As microcystins and other secondary metabolites are encoded by a combination of NRPS and PKS, we have used cyanobacterial specific primers to amplify the A-domain (adenylation domain) of NRPS and the KS (ketide synthase) region of PKS, respectively, which are 2 regions characteristic of these synthases. Clone libraries for both PCR products were constructed for 6 *Woronichinia* colonies. As an example, the results for one colony are presented. For the A-domain clone library, the analysis of the translated sequences (Figure 14a) showed high identities with cyanopeptolin gene clusters (71.3% to 98.5%). Other sequences were clustered with undefined cyanobacterial or putative A-domain sequences. The sequence analyses of the KS clone library (Figure 14b) showed highest identities with a KS region of the microginin operon (96.5 to 97.4%). Other sequences were related, with lower identity values, to cyanobacterial putative PKS domains. Recently, it was shown by Gademann et al. (2010) that cyanopeptolins can have a toxicity comparable to that of some microcystins. Here, we report the presence of cyanopeptolin-like genes in a *Woronichinia* colony and we

suggest that this genus should be considered to include potential cyanopeptolin-producing species. More effort should be made to isolate *Woronichinia* strains to confirm the production of secondary metabolites.

In conclusion, this ‚single colony' approach allows to work with a small amount of DNA, and represents a potentially valuable solution to the lack of data on non- or hardly-culturable cyanobacteria and a novel approach to the investigation of the diversity of toxigenic cyanobacteria. . In the future, it would be interesting to couple this approach with ELISA analysis and chemotyping by MS (MALDI TOF) to characterize *Microcystis* populations.

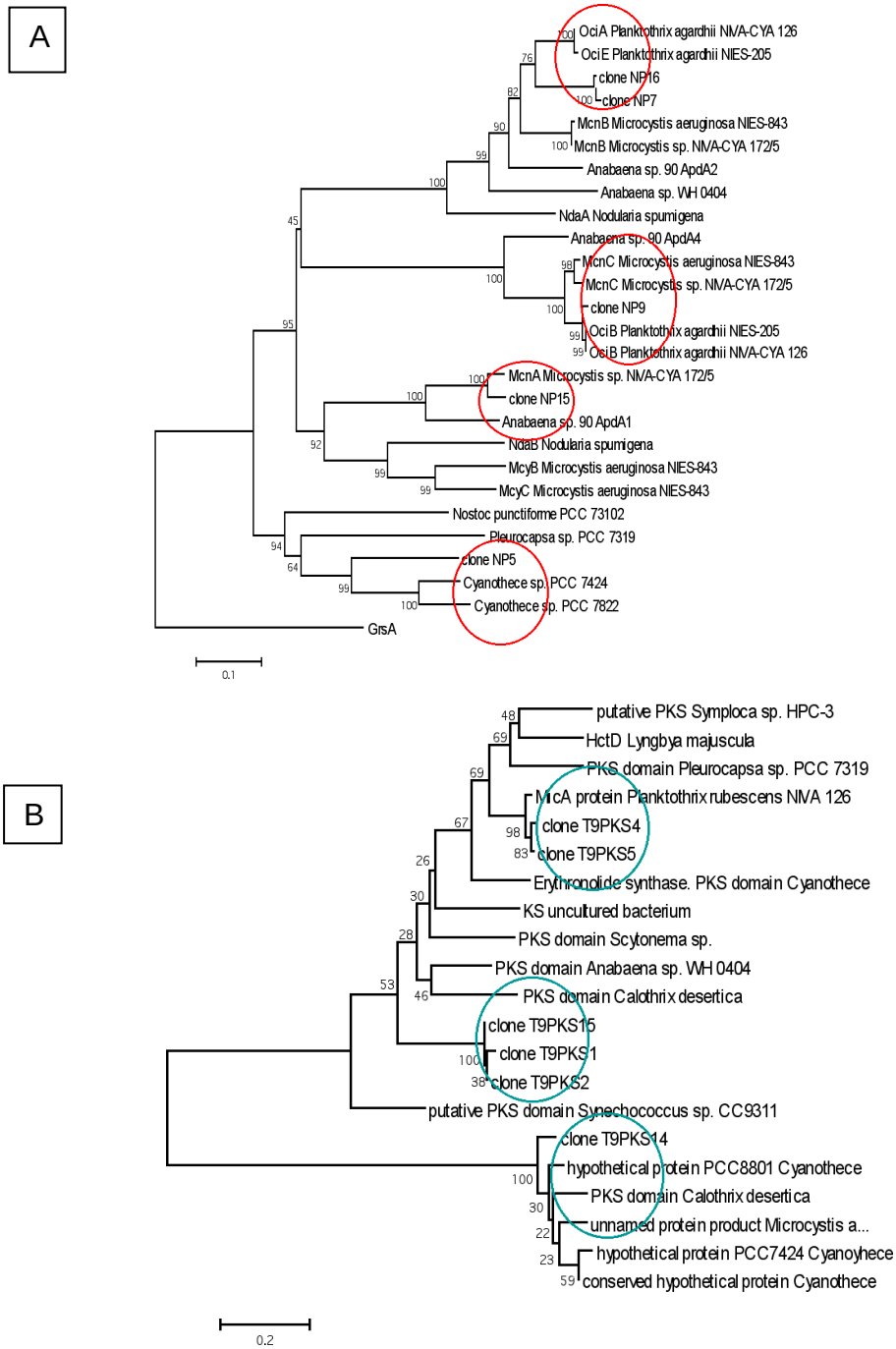


Figure 14: A) Neighbour-joining A-domain distance tree. (Satou and Nei, 1987). Distances were computed using the Poisson model on a 405 AA length alignment; - B) Neighbor-joining KS domain distance tree (Satou and Nei, 1987). Distances were computed using the Poisson model on a 235 AA length alignment. Colored circles indicate the position of our clones.

2.2.4. Determination of toxin content

178 phytoplankton samples for microcystin analysis were received by U. Dundee in 2007-2010.

All samples were analysed by ELISA in triplicate to determine total microcystin concentration and the % distribution of the toxins in the soluble phase (dissolved microcystins) and in the particulate (intracellular) phase. All samples were positive for microcystins although wide variations occurred in concentration and compartmentation. The ranges of microcystin concentrations (µg L^{-1} total MC-LR equivalents) were:

- Wallonia: Lake Falemprise (samples from 2007 and 2008): 0.120 – 6.110 µg L^{1};

  Lake Chérapont: 0.524 µg L^{-1}; Ecaussines quarry: 7.892 µg L^{-1}
- Brussels: Ixelles Ponds, IxP1 and IxP2 (samples from from 2007 and 2008): 0.210 - 1106 µg L^{-1}
- Flanders: Westveldparkvijver (samples from 2007 and 2008): 0.77 - 250.7 µg L^{-1}; Donkmeer (samples from 2007 and 2008): 0.260 – 5.740 µg L^{-1}; results from additional Flemish lakes (2007-2010) are summarized in Table VI.

Table VI: Microcystin concentrations measured in BLOOMNET samples of surface blooms (2007-2010) and analysed (ELISA immuno-essays) by Dundee University. Toxicity is expressed as µg MC-LR equivalents L^{-1}.

Locality	Collector	Sampling date	Bloom-forming taxon	Toxicity
Westveldvijver (Sint-Amandsberg)	UG	21/05/2007	*Microcystis aeruginosa*	629
Westveldvijver (Sint-Amandsberg)*	UG	25/05/2007	*Microcystis aeruginosa*	7.7
Westveldvijver (Sint-Amandsberg)	UG	25/05/2007	*Microcystis aeruginosa*	1576
Donkmeer (Overmere)	UG	21/05/2007	*Anabaena subcylindrica*	22
Donkmeer (Overmere)	UG	21/06/2007	*Anabaena* spp.	3.14

Donkmeer (Overmere)	UG	25/06/2007	*Aphanizomenon flos-aquae, Anabaena* spp.	3.64
Parkvijver (Kraainem)	UG	15/08/2007	*Aphanizomenon flos-aquae/issatschenkoi*	0.5
Grindplas (Kessenich)	UG	24/08/2007	*Microcystis* spp.	75
Spaarbekken AWW (Ranst)	VMM	28/08/2007	*Microcystis flos-aquae*	49
Schulensmeer (Lummen)	VMM	14/09/2007	*Woronichinia naegliana, M. flos-aquae*	633
Gewad (Meerhout)	VMM	17/09/2007	*Microcystis wesenberghii*	7.46
Ringsloot (Oostakker)	UG	17/01/2008	heterotrophic purple bacterium	1.24
Dongelberg (Jodoigne)	Carlo Joseph	24/02/2008	*Planktothrix rubescens*	69
Visvijver Indiana (Eke)	VVHV	13/05/2008	*Planktothrix rubescens*	37500
Dikkebussevijver (Dikkebus)	VMM	3/06/2008	*Microcystis aeruginosa, Aph. flos-aquae*	91
Visvijver (Diest)	VVHV	24/06/2008	*Microcystis aeruginosa*	31010
vijver VVHV	VVHV	3/07/2008	*Microcystis flos-aquae*	205
Schulensmeer (Lummen)	VVM	28/07/2008	*Woronichinia naegliana, Microcystis* spp.	0.75
Walburgvijver (Sint-Niklaas)	UG	17/08/2008	*Microcystis aeruginosa*	72
Verloren Broodvijver (Merelbeke)	UG	23/08/2008	*Anabaena flos-aquae*	0.49
Damslootmeer (Destelbergen)	VMM	8/09/2008	*Anabaena planctonica*	256
Blokkersdijk (Antwerpen)	UG	19/09/2008	*Aphanizomenon flos-aquae*	1.03
Vijver Tavervrienden (Humbeek)	VVHV	18/08/2010	*Microcystis flos-aquae*	472
Fort (Bornem)	VVHV	18/08/2010	*Microcystis aeruginosa, M. flos aquae*	7730
Fort (Bornem)	UG	24/08/2010	*Microcystis aeruginosa, M. flos aquae*	18060
Fort (Bornem)*	UG	24/08/2010	*Microcystis aeruginosa, M. flos aquae*	1289
Fort (Bornem)	UG	31/08/2010	*Microcystis aeruginosa, M. flos aquae*	14280
Fort (Bornem)	UG	6/09/2010	*Microcystis aeruginosa, M. flos aquae*	12720
Fort (Bornem)	UG	13/09/2010	*Microcystis aeruginosa, M. flos aquae*	13290
Fort (Bornem)	UG	20/09/2010	*Microcystis aeruginosa, M. flos aquae*	14250
Fort (Bornem)	UG	27/09/2010	*Microcystis aeruginosa, M. flos aquae*	13880

Fort (Bornem)	UG	4/10/2010	*Microcystis aeruginosa, M. flos aquae*	20450
Fort (Bornem)	UG	13/10/2010	*Microcystis aeruginosa, M. flos aquae*	21460
Fort (Bornem)	UG	22/10/2010	*Microcystis aeruginosa, M. flos aquae*	15800
Donkmeer (Overmere)*	UG	19/08/2010	*Planktothix agardhii*	4.31
Donkmeer (Overmere)	UG	19/08/2010	*Microcystis* spp.	2357
Donkmeer (Overmere)	UG	19/08/2010	*Microcystis* spp.	2088
Donkvijver (Oudenaarde)	VMM	3/09/2010	*Microcystis flos-aquae, M. aeruginosa*	206.8

*integrated sample of the whole water column. All microcystin concentrations: means of triplicate determinations.

Table VII: Microcystin concentrations measured in BLOOMNET samples from official recreational lakes (2007-2010) taken by VMM and analysed (ELISA immuno-essays) by Chemiphar NV. Toxicity is expressed as µg MC-LR L^{-1}. n.d.: not determined.

Location	Sampling day	Bloom-forming taxon	Toxicity
De Gavers (Harelbeke)	30/03/2007	*Aphanizomenon flos-aquae*	< 0.1
De Gavers (Harelbeke)	20/04/2007	n.d.	< 0.1
Schulensmeer (Lummen)	21/09/2007	*Woronichinia naegliana, Microcystis* spp.	> 80
Schulensmeer (Lummen)	01/10/2007	n.d.	> 80
Zwartwater (Linkhout)	08/10/2007	n.d.	710
Schulensmeer (Lummen)	08/10/2007	n.d.	8600
Schulensmeer (Lummen)	08/10/2007	n.d.	1000
Zwartwater (Linkhout)	22/10/2007	n.d.	< 10
Schulensmeer (Lummen)	22/10/2007	n.d.	14
Schulensmeer (Lummen)	22/10/2007	n.d.	370
Zwartwater (Linkhout)	29/10/2007	n.d.	< 10
Schulensmeer (Lummen)	29/10/2007	n.d.	< 10
Schulensmeer (Lummen)	29/10/2007	n.d.	< 10
Dikkebussevijver (Dikkebus)	16/06/2008	*M. aeruginosa, Aph. flos-aquae*	4
Schulensmeer (Lummen)	23/07/2008	*Woronichinia naegliana, Microcystis* spp.	> 80
Schulensmeer (Lummen)	23/07/2008	*Woronichinia naegliana, Microcystis* spp.	42
Schulensmeer (Lummen)	28/07/2008	n.d.	15
Schulensmeer (Lummen)	31/07/2008	n.d.	< 5
Schulensmeer (Lummen)	31/07/2008	n.d.	< 5
Schulensmeer (Lummen)	31/07/2008	n.d.	< 5
BLOSO-vijver (Hofstade)	04/08/2008	n.d.	< 5
Schulensmeer (Lummen)	25/09/2008	n.d.	22
Dikkebussevijver (Dikkebus)	01/07/2009	*Aphanizomenon flos-aquae*	< 5
Ter Heide vijver (Rotselaar)	29/07/2009	*Aphanothece stagnina, Anabaena* sp.	< 5
Ter Heide vijver (Rotselaar)	30/07/2009	*Aphanothece stagnina, Anabaena* sp.	< 5

Schulensmeer (Lummen)	31/07/2009	*Microcystis-flos-aquae, W. naegliana*	8
Schulensmeer (Lummen)	03/08/2009	n.d.	5.4
De Vijvers (Averbode)	31/08/2009	*M. aeruginosa, Cylindrospermopsis raciborskii*	27
De Vijvers (Averbode)	08/09/2009	*Aphanizomenon flos-aquae*	20
Keienven (Wuustwezel)	09/09/2009	n.d.	< 5
Schulensmeer (Lummen)	09/09/2009	*Microcystis aeruginosa, M. flos-aquae*	38
Schulensmeer (Lummen)	29/10/2009	*Aphanizomenon flos-aquae*	< 5
De Gavers (Harelbeke)	12/05/2010	n.d.	< 5
De Gavers (Harelbeke)	31/05/2010	n.d.	< 5
De Gavers (Harelbeke)	14/06/2010	n.d.	< 5
Dikkebussevijver (Dikkebus)	28/06/2010	n.d.	5
Familiestrand Postel (Mol)	29/06/2010	n.d.	< 5
Paalse Plas (Beringen)	12/07/2010	*Anabaena flos-aquae*	> 80
Paalse Plas (Beringen)	15/07/2010	n.d.	< 5
Paalse Plas (Beringen)	15/07/2010	n.d.	< 5
Dikkebussevijver (Dikkebus)	26/07/2010	n.d.	< 5
Dikkebussevijver (Dikkebus)	09/08/2010	n.d.	13
Heidestrand (Zonhoven)	10/08/2010	*Spirogyra* sp., *Oscillatoria* sp.	< 5
Donkvijver (Oudenaarde)	03/09/2010	*Microcystis flos-aquae, M. aeruginosa*	207
Schulensmeer (Lummen)	13/09/2010	*Microcystis flos-aquae*	< 5
Boudewijnpark vijver (Brugge)*	16/12/2010	*Planktothrix rubescens*	21
Boudewijnpark vijver (Brugge)*	30/03/2011	*Planktothrix rubescens*	32 - 2954
Boudewijnpark vijver (Brugge)*	11/04/2011	*Planktothrix rubescens*	217 - 273
Boudewijnpark vijver (Brugge)*	24/05/2011	*Planktothrix rubescens*	<5 - 12
Schulensmeer (Lummen)	06/06/2011	*Microcystis flos-aquae, M. aeruginosa*	5

*sample taken and analysed (Chemiphar NV) by Stad Brugge. All microcystin concentrations: means of triplicate determinations.

Since 2006, the recreational waters in Flanders have also been monitored for the presence of cyanobacteria and microcystins by the VMM (Table VII). Every year, blooms are detected, samples taken and sent to Ghent University (microscopical analysis) and Chemiphar NV (microcystin analysis). When the presence of cyanotoxins is confirmed, the waterbodies are closed for recreation until the concentrations have decreased under the recreational water threshold level of 20 µg MC-LR L^{-1}. Several waterbodies have been closed on occasions since 2007 (see earlier). The toxin analyses carried out at U. Dundee also included samples from private waterbodies that are mainly used for line fishing (Table VI). These small and shallow ponds generally contain very high fish populations and are all hypertrophic. The analyses reveal that these waterbodies are especially prone to very toxic surface blooms of mainly *Microcystis* spp. that can prevail for long periods, as for example at Fort Bornem in 2010. Also in deeper and less nutrient-enriched waterbodies, sometimes a dense bloom of *Planktothrix rubescens* can migrate upwards to form a surface layer, sometimes very toxic, as observed in a lake in Eke with a surface area of 10 ha and a maximal depth of 16 m that is used for trout stocking. Moreover, urban lakes constitute a potential health threat to people and pets as demonstrated by the presence of toxic *Microcystis* blooms in Westveldparkvijver (Sint-Amandsberg) and in Walburgvijver (Sint-Niklaas).

All samples received for immunoassay for total microcystin content were examined for the possibility to isolate single cyanobacterial colonies and filaments for further immunoassay. The aim was to quantify the microcystin content (quota) per colony and filament. It was possible to isolate single *Microcystis* colonies from: Donkmeer, Westveld, Falemprise, IXP1 and IXP2. Single filaments of *Planktothrix* were isolated from Donkmeer and Falemprise. One series of *Anabaena* coiled filaments was isolated from Donkmeer.

Over 120 individual colonies and filaments were analysed. In some cases where samples were not received within a few days of sample collection at the lakes and microscopic evidence of colony and filament lysis was evident. It was not possible to isolate single colonies and filaments from these samples.

Microcystin quotas were determined for over 50 of the single colony/filament samples. *Microcystis* colony areas were also measured to permit estimation of microcystin quota per cell. *Planktothrix* filament length and width measurements also permitted determinations of microcystin concentration per unit biovolume. Attempts to measure microcystin quotas in the remaining samples have proved to be difficult and due to possible changes in the suppliers of immunoassay plasticware.

The quotas measured ranged from 45 to 1620 picograms per colony or filament. This range indicates a wide diversity in microcystin quota, probably extending to per unit biovolume and is consistent with the notion of a single cyanobacterial cell quota in the femtogram range (Orr and Jones, 1998; Akcaalan et al. 2006).

Wide variations versus sample date were found in the compartmentation of microcystin between soluble and particulate phases. For example, at Donkmeer, this ranged from 26% to 100% soluble microcystin (Figure 15). The total microcystin pool was predominantly in the soluble phase (80 to ca. 100%) from the beginning of sampling in 2007 until late June (Figure 15). This was the period when the Donkmeer cyanobacterial bloom was largely dominated by *Anabaena sp.*. From early July until late October, when the Donkmeer bloom was dominated by *Planktothrix* sp., the proportion of total microcystin in the soluble phase was low (e.g. 30 to 50%), although total microcystin concentrations were greatest in this period (Figure 15). The results emphasise the need to determine microcystin concentrations in both dissolved and particulate material in lake sampling to obtain an overall understanding of toxin pools. Comparisons of the microcystin concentrations and compartmentation alongside phytoplankton composition and biomass and the *mcy* DNA profiling were presented in Section 4.

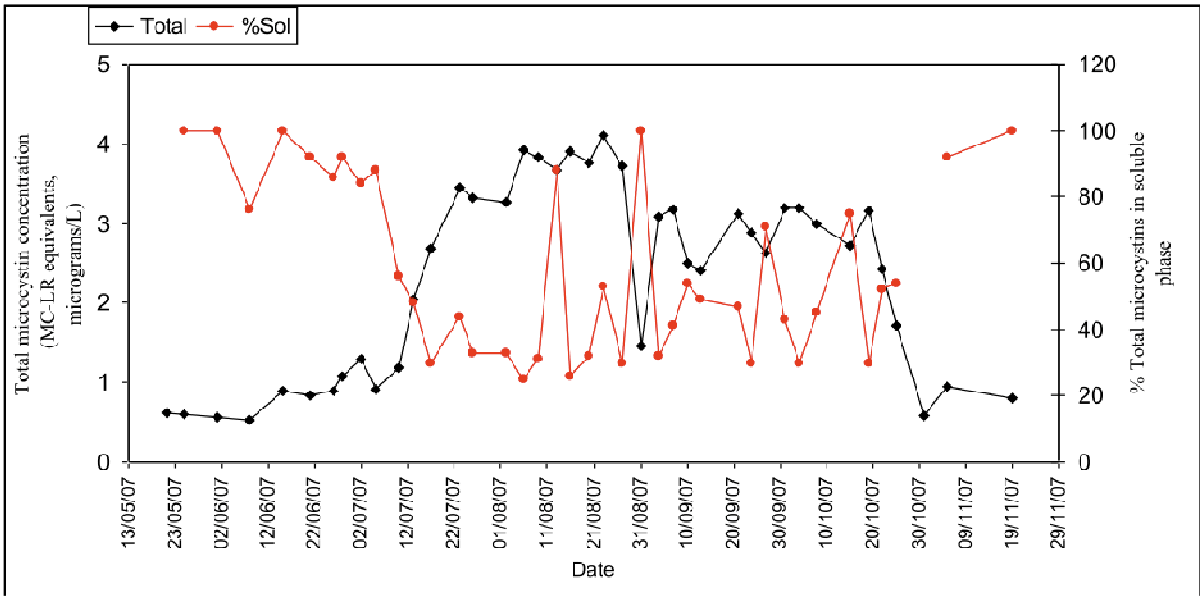


Figure 15: Total microcystin concentrations (determined by ELISA) and % total microcystin pool in soluble (dissolved) phase, for Donkmeer, 2007. Each point is the mean of triplicate determinations.

Many results have been obtained in this study which cannot be detailed here. Given the importance of those data for water management, we report here a summary, focusing of total microcystin concentration in water of the B-BLOOMS2 reference lakes where samples were analysed throughout 2007 and 2008 (Figure 16) i.e. Falemprise in Wallonia and Donkmeer and Westveldparkvijver in Flanders. Microcystin was detected in all samples, and many samples were well above the WHO drinking water Guideline Value of 1 µg microcsytin-LR L^{-1}. This occurred in a few summer samples in Falemprise, and in more samples from Donkmeer. These two lakes presented the same range of microcystin concentration, whereas Westveldparkvijver had much higher microcystin concentration. In the latter pond, all measurements were above the WHO drinking water Guideline Value. The highest concentration was measured in the Ixelles pond I during summer 2007 when the concentration in the Ixelles pond II was also high. However, in 2008, Brussels reference pond concentrations never reached the WHO drinking water Guideline Value. All of these results concern samples integrating the whole water column. However since cells can become concentrated at the surface and in the litoral zone, microcystin concentrations in these scum layers can be orders of magnitude higher. This was demonstrated for Westveldparkvijver where a surface bloom on 25/05/2007 contained more than 1500 µg MC-LR l^{-1} (based on ELISA immunoassay), while an integrated sample contained less than 10 µg (Table VI). This was also the case for Donkmeer where the litoral surface blooms show much higher concentrations than

that present in the water column, especially when these were dominated by *Microcystis* spp. as was the case on 19/08/2010 when more than 2300 µg MC-LR l-1 was measured in surface blooms in comparison with 4.3 µg measured in a sample from the integrated *Planktothrix agardhii* bloom present at the same time (Table VII).

Beside these results based on ELISA, HPLC analysis of microcystins from filters (i.e. in the bloom material) and SPE cartridges (concentrated water samples) was done. These analyses confirmed the results obtained with ELISA, and allowed quantification of various microcystins in the Belgian samples. U. Dundee also produced and quantified purified microcystin-LR and anatoxin-a standards and provided analytical protocols for FUNDP for analytical methods development. HPLC analysis of anatoxin-a was developed at FUNDP. Several methods and HPLC columns were tested and applied to several bloom samples but anatoxins have not been detected so far.

In addition to the cyanobacterial studies, specimens from various groups of other aquatic organisms sampled in Donkmeer (40 samples) and Westveldparkvijver (112 samples) were analysed for microcystin content (Tables VIII and IX). In 40 % of the samples from Donkmeer, a microcystin content above the detection limit was measured while this was the case for 63 % of the samples from Westveldparkvijver. The positive results yielded a range of microcystin-LR equivalents from 17.5 to 118.4 picograms per animal for Donkmeer and from 7.0 to 157.5 picograms per animal for Westveldparkvijver. These are the first findings of microcystins (and/or microcystin detoxication products) in individual freshwater zooplankton species organisms reported. They open new possibilities to investigate the passage of cyanotoxins through trophic levels in zooplankton at both population and single organism level. Since affected organisms belong to all main heterotrophic compartments of the aquatic foodweb (from amoebae and ciliates to zooplankton and fish larvae) the risk of transfer of microcystins in the lakes' foodweb is rather high.

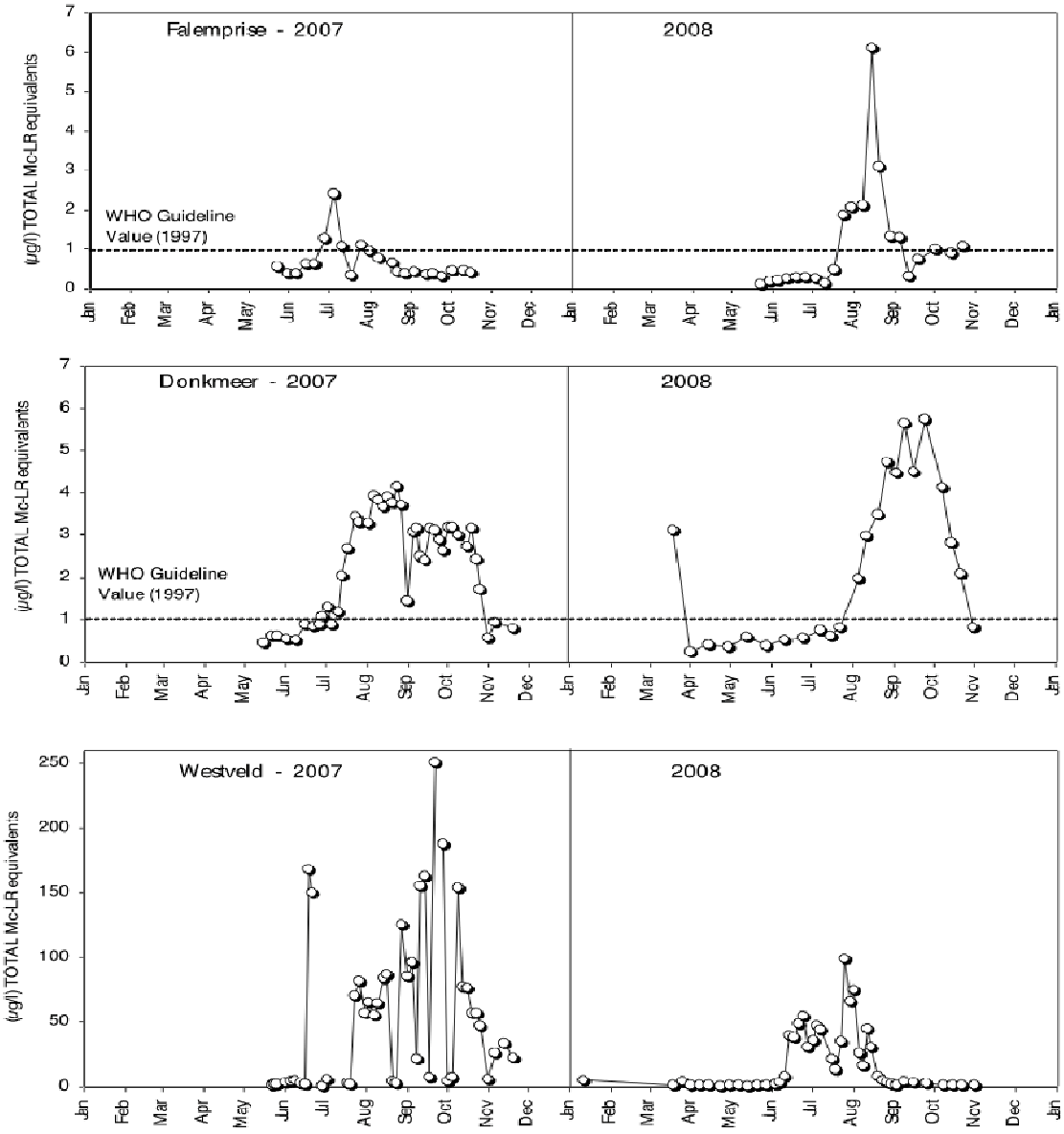


Figure 16: Total microcystin concentrations (determined by ELISA for Falemprise, Donkmeer and Westveld in 2007 and 2008.

Table VIII: Microcystin concentrations (pg MC-LR equivalents ind.$^{-1;}$) of isolated specimens of aquatic organisms sampled in Westveldparkvijver during *Microcystis* dominance (2007-2008).

organism group	Taxon	% positive	maximal toxicity	average toxicity
cyanobacteria	*Microcystis aeruginosa* (10)*	100	898	421
ciliates	ciliate sp. (5)	40	45	20
amoebae	*Arcella gibbosa* (2)	50	53	26
rotifers	*Asplanchna priodonta* (1)	0	-	-
	Brachionus angularis (14)	50	133	37
	Brachionus calyciflorus (20)	75	158	49
	Brachionus diversicornis (4)	75	84	35
	Brachionus dorcas spinosa (1)	100	47	47
	Brachionus sp. (2)	100	58	53
	Filinia longiseta (1)	100	65	65
	Keratella cochlearis (28)	64	156	41
	Keratella quadrata (2)	0	-	-
	Lecane lunaris (1)	100	46	46
	Polyarthra remata (4)	50	89	41
	Trichocerca cf. *rattus* (3)	100	72	52
copepods	Nauplius cyclopoid copepod (7)	100	65	43
	Cyclopoid copepod (12)	50	61	19
cladocerans	*Daphnia* sp. (2)	50	75	38
mites	*Hydrozetes lacustris* (1)	0	-	-
insects	Insect larvae (1)	0	-	-
fish	Fish larvae (1)	100	65	65

()* Amount of specimens tested for each taxon.

Table IX: Microcystin concentrations (pg MC-LR equivalents ind.$^{-1}$) of isolated specimens of aquatic organisms sampled in Donkmeer during *Planktothrix* dominance (2007-2010).

organism group	**Taxon**	**% positive**	**maximal toxicity**	**average toxicity**
cyanobacteria	*Anabaena subcylindrica* (10)	100	211	159
	Microcystis sp. (4)	100	135	127
	Planktothrix agardhii (6)	100	248	122
rotifers	*Brachionus angularis* (3)	100	52	42

	Keratella cochlearis (1)	100	58	58
	Nauplius cyclopoid copepod			
copepods	(1)	100	23	23
	Cyclopoid copepod (2)	0	-	-
cladocerans	*Bosmina longirostris* (5)	80	118	
	Daphnia sp. (23)	17	46	6,3
	Daphnia sp. (55)*			0,74
	Leptodora kindtii (21)*			3,24

*results from bulk analysis.

2.2.5. Deterministic watershed simulation modelling of Eau d'Heure Lakes

Simulations

Simulations of phytoplankton (and more specifically cyanobacteria) were made on the "Eau d'Heure lakes" Féronval and Falemprise (Figure 17) between 2001 and 2003. The model was able to represent cyanobacteria for this type of lake (Figure 18).

In the B-BLOOMS2 project, it was planned to use the model applied to the Eau d'Heure lakes (including Falemprise)to :

- test and validate the model for the year(s) 2007 and 2008
- improve the description and understanding of the cyanobacteria
- develop and test scenarios for controlling cyanobacterial blooms (P reduction etc).

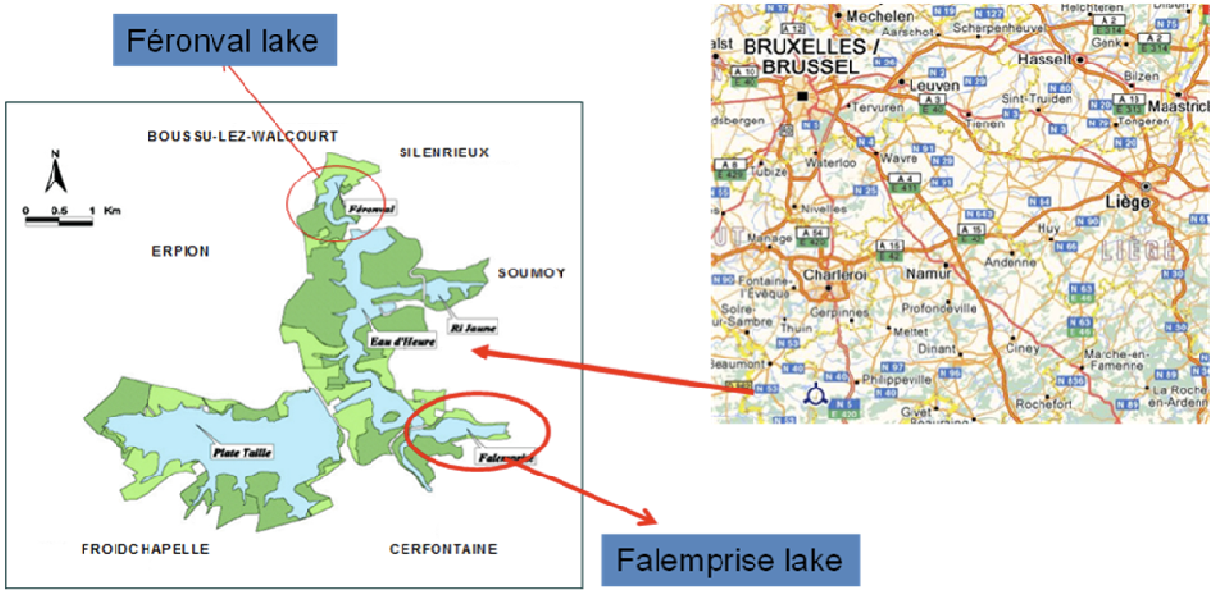


Figure 17: The Eau d'Heure Lakes.

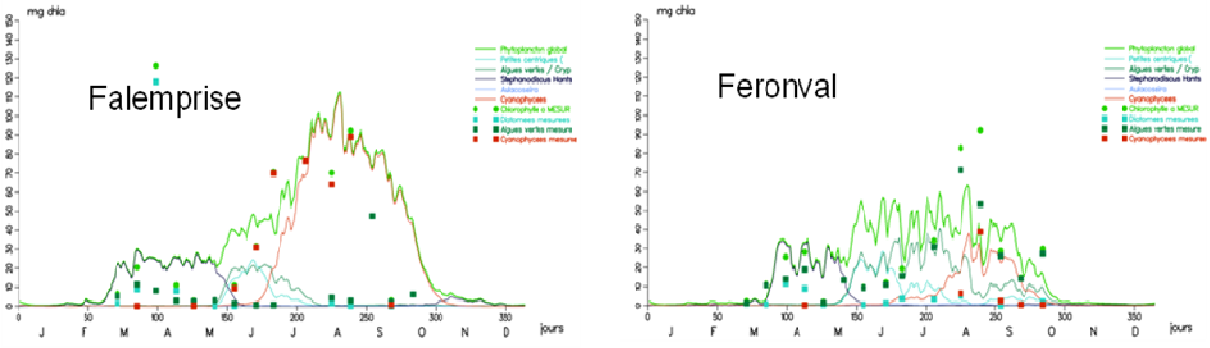


Figure 18: Annual simulations of cyanobacteria in the Falemprise and Feronval lakes for 2002 (Eau d'Heure Project).

The first step was to collect and format data necessary for a 2007 simulation. (It was impossible to do 2008 simulations because meteorological data were not available):

- Water discharges (daily values)
- Collection of meteorological data from 6 stations (MET)
- Reconstruction of two « virtual » measurement stations
- Pre-treatment for incorporation into PEGASE
- Collection of water temperature data (daily values)
- Collection and treatment (interpolation) of data collected during the study
- Collection of daily total radiation data (J/m^2.day) at the IRM Dourbes Station
- Calculation of semi-hourly values for each day

Determination of pollution inputs (point and diffuse loads): urban releases : 2 sewage treatment plants plus sewer discharge systems; diffuse loads : use of semi-statistic input functions.

In the watershed of Lake Falemprise, two sewage treatment plants are operating (Figure 19) :

- At Soumoy since 1989 (170 I.E., collect rate = 67%, with phosphorus removal)
- At Senzeille since 2003 (960 I.E., collect rate = 67%, with phosphorus removal)

Collect rates for the year 2015 are planned to be 90% at Soumoy and 82% at Senzeille.

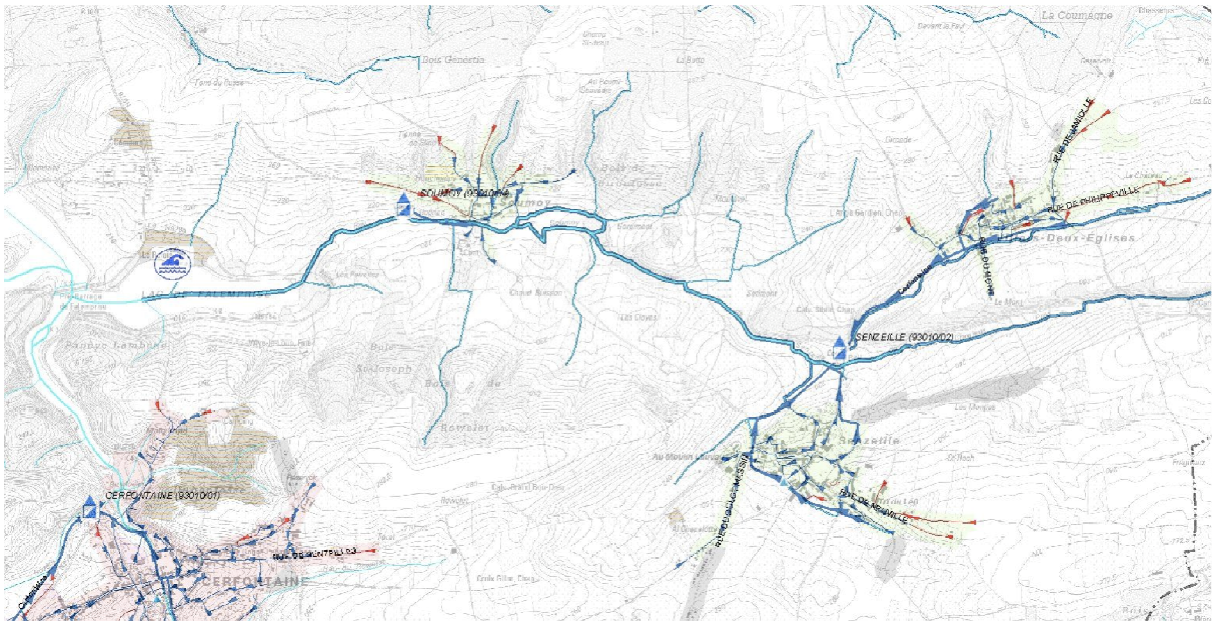


Figure 19 : Sewage treatment plants and sewage networks in the Falemprise Lake basin.

Figure 20 shows the results of a simulation made for the year 2007, with the parametrizations used for the 2002 simulations. It can be seen that cyanobacteria simulated in Lake Falemprise (red lines) were much greater that the measured concentrations (red markers). This may have resulted from an inadequate calibration of the phosphorus-requirement of cyanobacteria, not detected in the 2002 simulations, as P inputs were greater at that time, before the Senzeille plant was working. Therefore, P kinetics of cyanobacteria were modified and new simulations were run with a revised calibration (Figure 21).

The simulations show that validation for 2007 is much better, while that for 2002 is still consistent. Additional simulations were run, based on the P inputs expected for 2015, assuming the same weather conditions as in 2002 and 2007 (in particular, for irradiance and temperature).

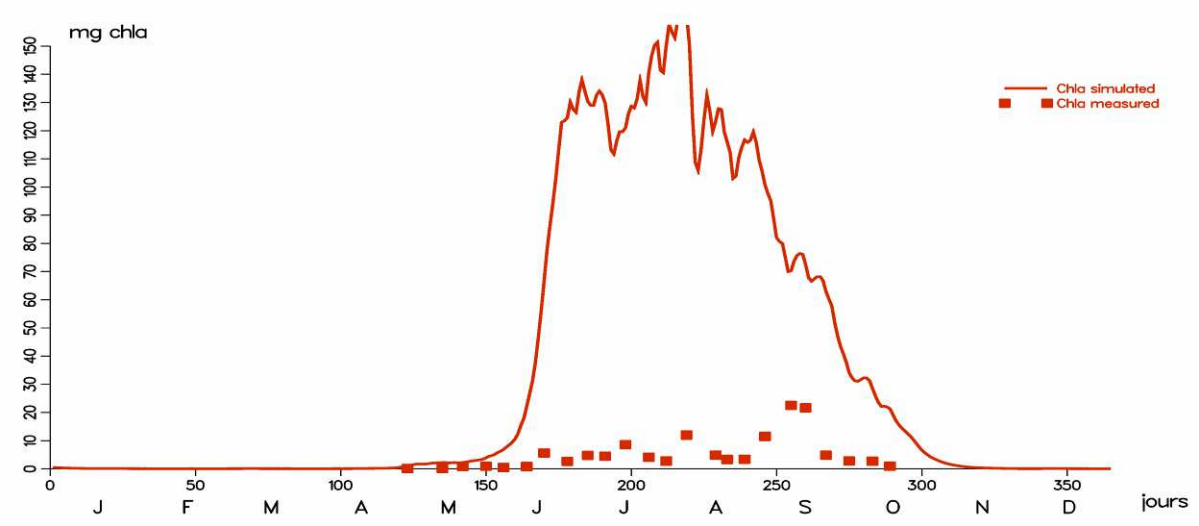


Figure 20: 2007 simulations of cyanobacteria in Lake Falemprise with 2002 calibration.

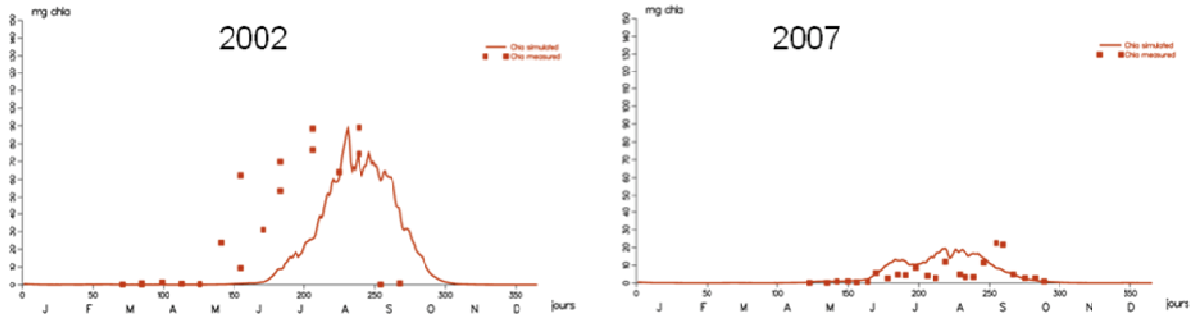
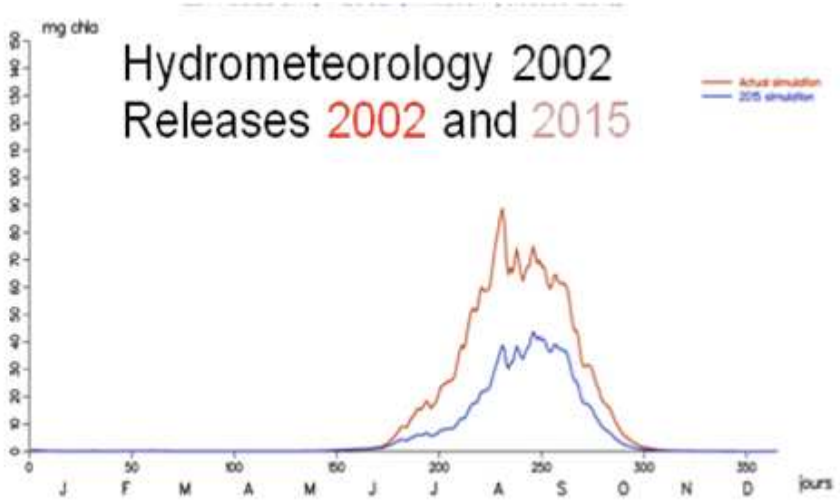
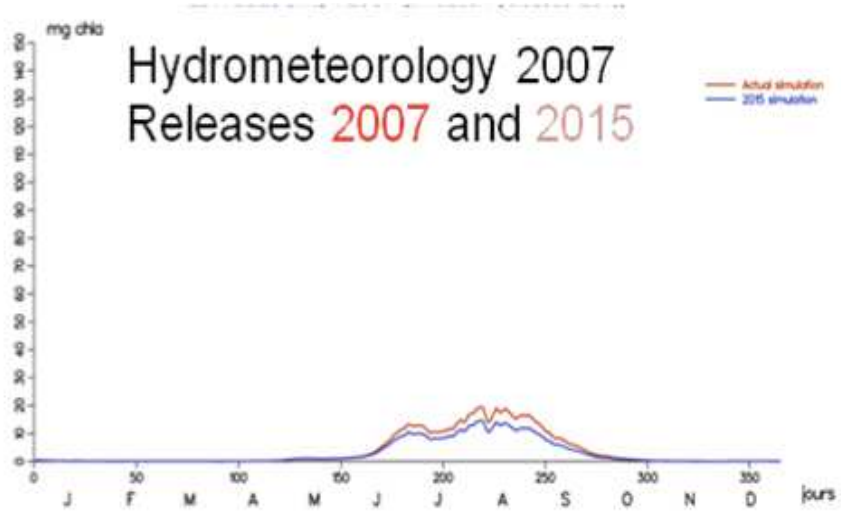


Figure 21: 2002 and 2007 simulations of cyanobacteria in Lake Falemprise with new parametrization of phosphorus kinetics.

The simulations with this scenario allowed prediction of a decrease in cyanobacterial concentrations (Figure 23), mainly for the « 2002 » situation for which the expected P reduction in 2015 is comparatively much higher than for 2007.





Figures 22: 2002 and 2007 simulations of cyanobacteria in the Falemprise Lake with estimated phosphorus releases for the year 2015.

In conclusion, the modified simulation model has shown its ability to simulate cyanobacterial blooms in Lake Falemprise. The upgraded model can be used as a predictive tool for future sewage treatment scenarios. However, further improvements are still needed in order to fully adapt the model to lakes deep enough to develop summer stratification. Obviously, a complete representation of vertical structure and depth profile of paparameters for the lake to be modelled should be achieved, with at least two layers; a vertical migration sub-model for cyanobacteria should be developed, and the sedimentation-resuspension processes should be represented.

2.2.6. Statistical predictive models for Brussels ponds

The data from 22 Brussels ponds sampled monthly from May to September in 2007 and 32 ponds sampled in May, July and August in 2008 and 2009 (Peretyatko et al.,2009) were combined with the data from the same or other Brussels ponds acquired between 2003 – 2006, according to the same methodology, into a single matrix. The resulting data matrix comprises 48 ponds sampled on more than 500 occasions. The combined dataset was analysed using multivariate and univariate statistical techniques and incorporated into a probabilistic model based on conditional probability calculation.

First, linear relationships between different phytoplankton groups (divisions) and environmental variables were investigated by the multivariate statistical method, Redundancy analysis. RDA results showed that pH, submerged vegetation cover, length and density of large cladocerans and SRP and DIN had significant relationships with phytoplankton biomass and explained the greater part of the variation in the phytoplankton data. Significant negative relationships of dissolved nutrient concentrations (SRP, DIN) with the phytoplankton data indicate phytoplankton control of nutrients rather than the reverse. The strength of nutrient to phytoplankton relationship in these ponds are detailed in Teissier et al. (2011). Ponds with high phytoplankton biomass showed SRP and DIN concentrations near detection limit, whereas in the clearwater ponds they could exceed 0.5 ml PL^{-1} and 2.5 ml NL^{-1}.

Linear relationships between cyanobacteria and environmental variables measured were also investigated by means of regression analysis that showed their poor predictive capacity. Only pH and total phytoplankton biovolume explained 9 % each of the variation in the cyanobacteria data. The other variables explained less than 1% of the variation. The relationships between cyanobacteria and a number of environmental variables seem to be of a threshold, rather than a linear nature (Figure 23).

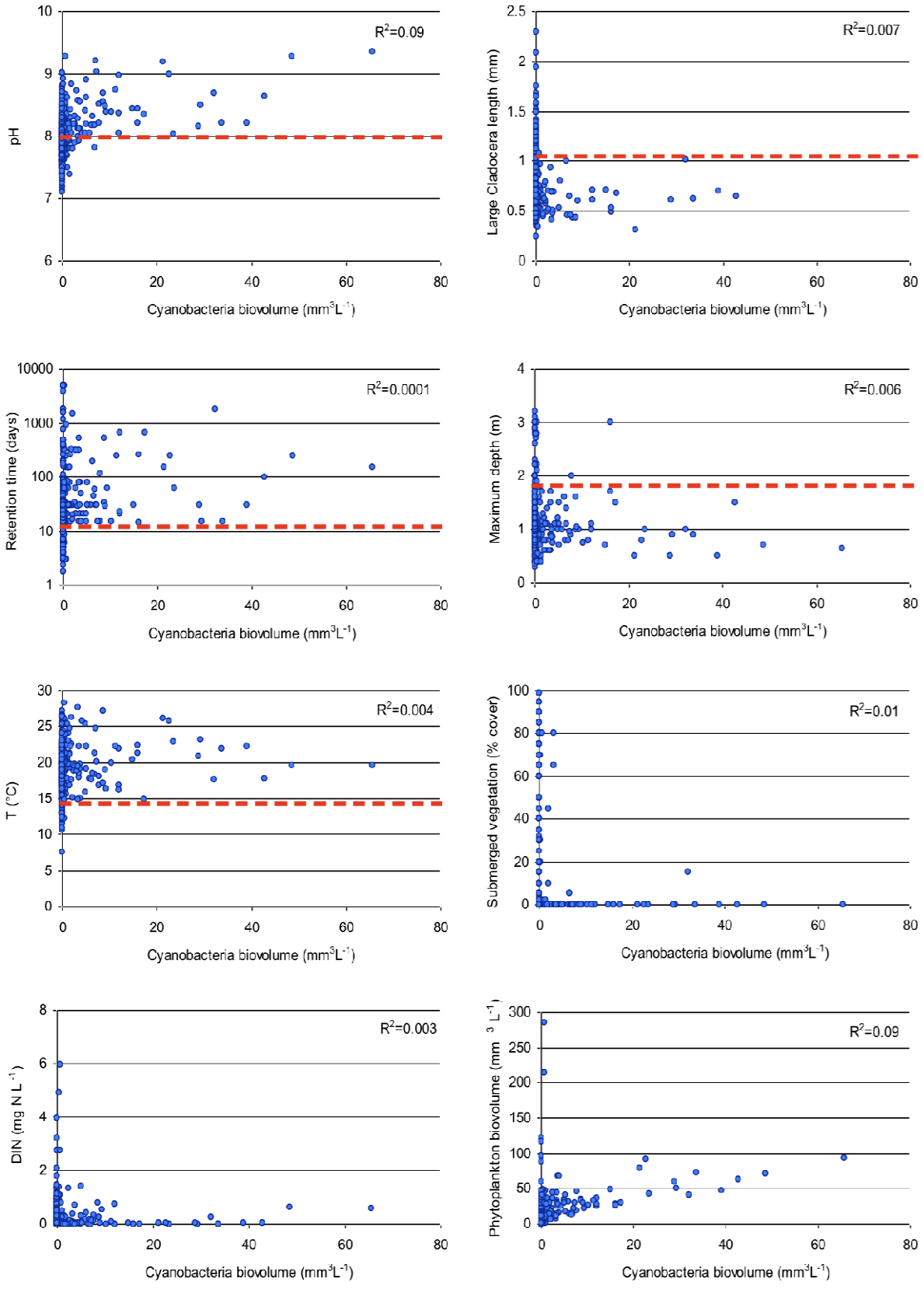


Figure 23 Scatter plots based on 2003-2008 data from 48 Brussels ponds showing relationships between cyanobacteria and environmental variables. Dashed lines indicate thresholds in the distribution of cyanobacterial biomass along a given gradient.

Therefore, a probabilistic approach to the assessment of the risk of cyanobacterial bloom development was adopted (Peretyatko et al., 2010). The threshold relationships between cyanobacterial biomass and environmental variables indicate that some conditions favour/act against cyanobacteria more than others. This permits the reduction of the sample size corresponding to the conditions which are the most propitious for cyanobacterial bloom development by means of conditional probability calculation (Figure 24).

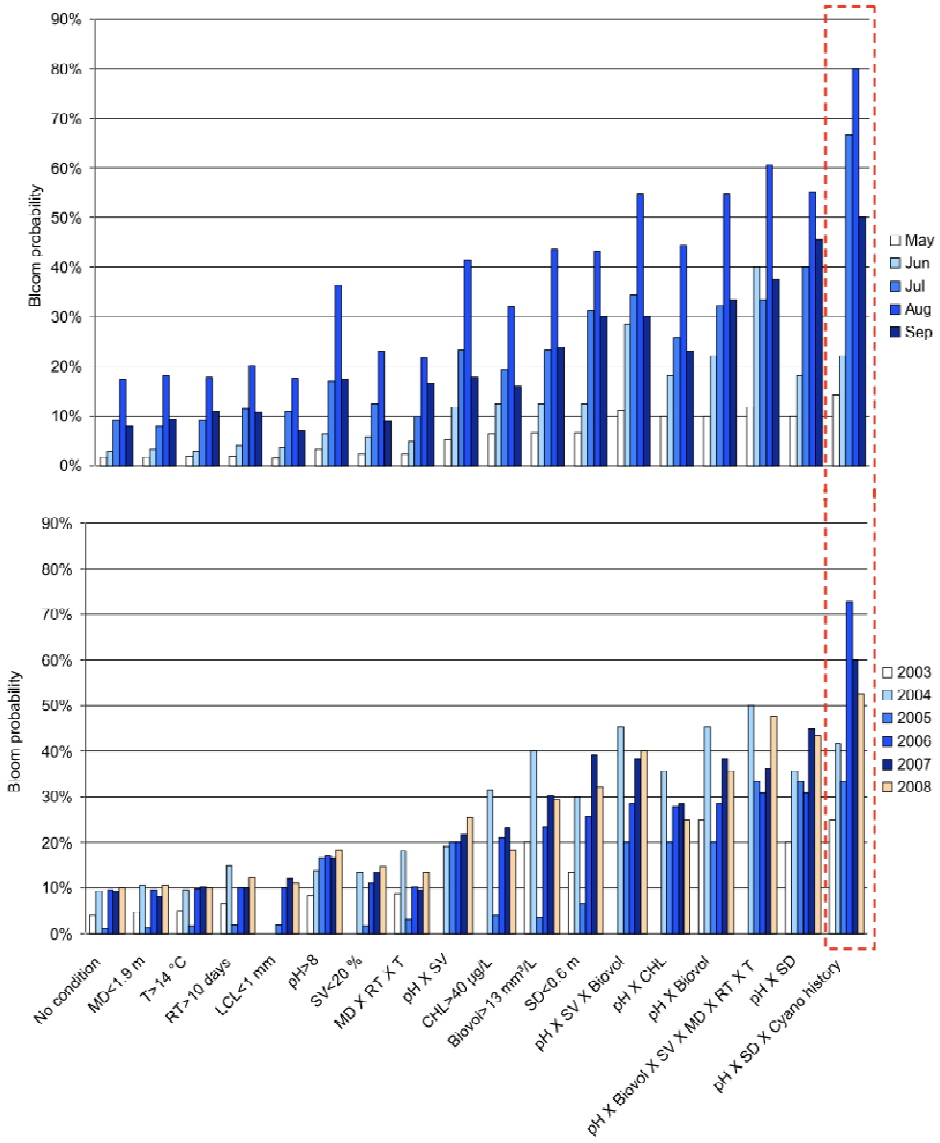


Figure 24: Seasonal and inter-annual variations in the unconditional and conditional probabilities of cyanobacterial bloom (5 mm3 L-1 of cyanobacteria) occurrence in the ponds studied. Higher probability values indicate higher predictive capacity of a given condition. Red rectangle indicates the probability of bloom occurrence in ponds with a history of cyanobacterial blooms given the conditions that pH>8 and SD<0.6.

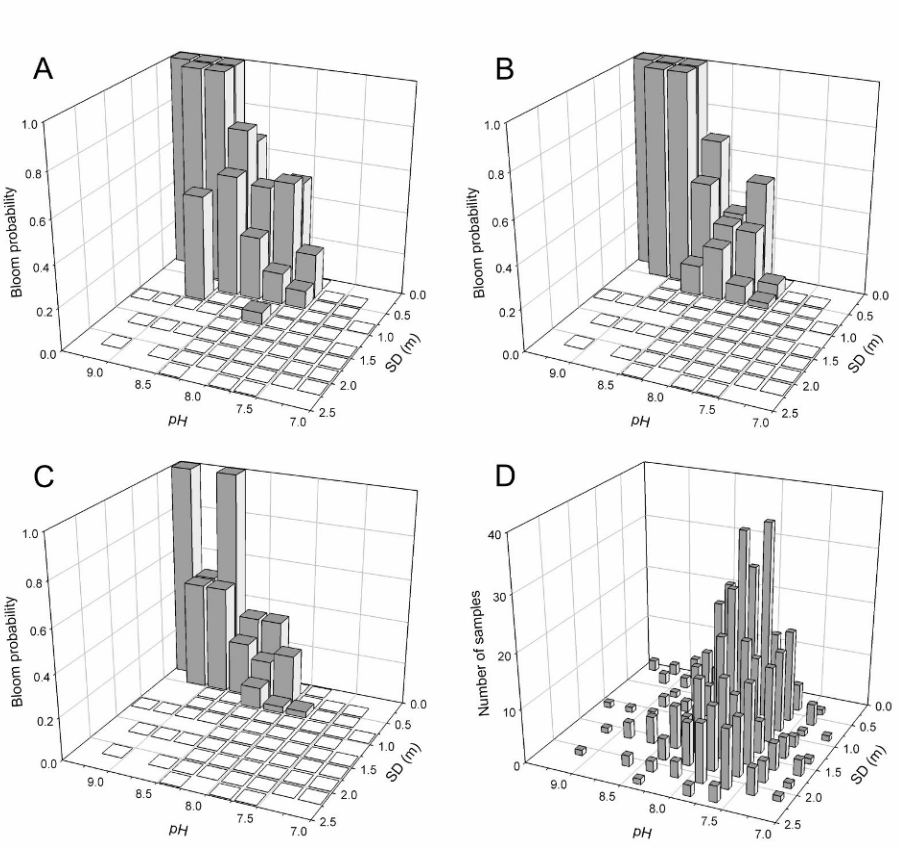


Figure 25: A-C: Risk of cyanobacterial bloom occurrence corresponding to different ranges of pH and SD calculated stepwise (step length for SD=0.25, for pH=0.5) for different levels of cyanobacterial biomass (A: ≥2 mm3 L-1, B: ≥5 mm3 L-1, C: ≥10 mm3 L-1). D: number of samples corresponding to each region used in probability calculation.

The probability value was considered as a measure of the predictive capacity of a given condition based on one or several variables (e.g. pH>8 or pH>8 and LCL < 1mm). The probability calculation was automatised in MS Excel by superposition of different data matrices (map algebra approach). The possibility to modify the magnitude of cyanobacterial bloom and condition (threshold) levels allows the risk of cyanobacterial bloom development of a given magnitude under given conditions to be assessed.

The results of probability calculation (Figure 24) showed a considerable seasonal and interannual variation in the probability of cyanobacterial bloom occurrence. The highest probability, based on the data from all the ponds studied, was shown by the combination of all the variables measured. The combinations of pH and phytoplankton biovolume, and of pH and Secchi depth used as conditions for probability calculation, rendered probability values that are not significantly different

from the latter (Wilcoxon matched pairs test). This implies that a large number of variables, which may be logistically difficult to measure, might be substituted by a few easily measured variables for the rapid assessment of cyanobacterial bloom risk.

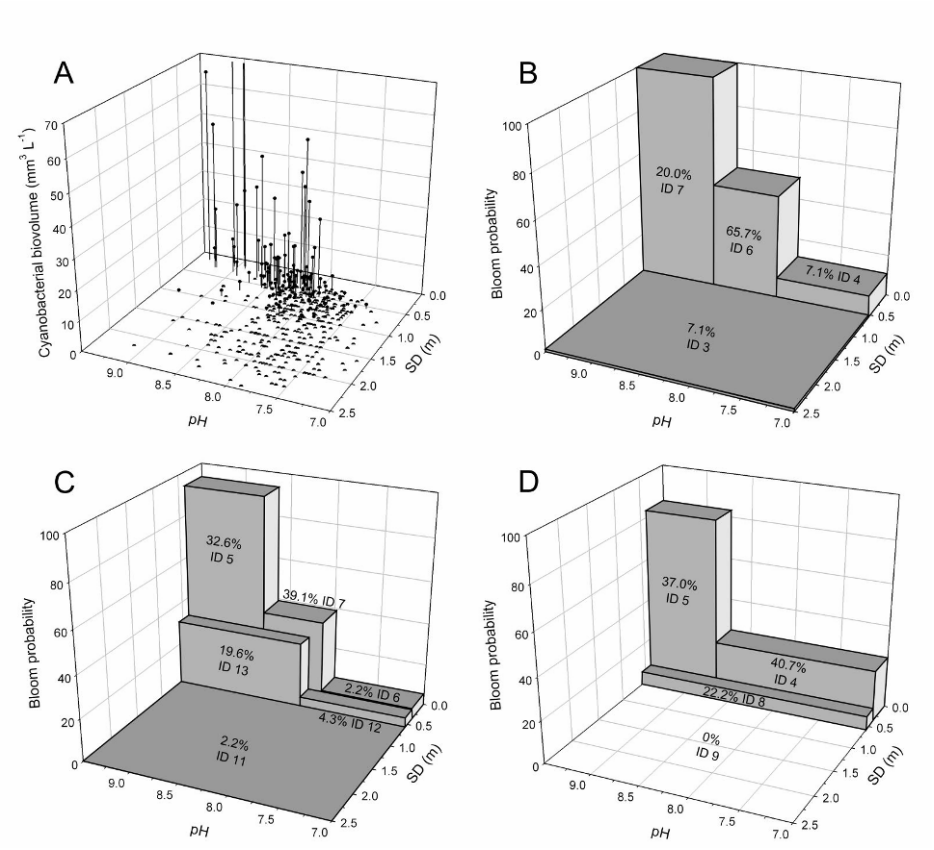


Figure 26: A: Relationship of cyanobacterial biovolume to SD and pH. B – D: Probability of cyanobacterial bloom occurrence corresponding to different ranges of SD and pH as predicted by the classification trees for different levels of cyanobacterial biomass (B: ≥2 mm^3 L^{-1}, C: ≥5 mm^3 L^{-1}, D: ≥10 mm^3 L^{-1}). Each bar shows bloom probability corresponding to the node of the respective classification tree with an indication of the ID as well as the percentage of all blooms accounted by the node (Peretyatko et al., 2011a).

The probability values and thus predictive capacity of the model markedly increased when only ponds with a history of cyanobacterial blooms were taken into account (dash-line frame; Figure 24. It shows that the probability of bloom occurrence in a Brussels pond previously affected by cyanobacteria is the highest in August (80%).

The probabilistic model also allows the stepwise calculation of bloom probability for different ranges of factors or their combinations and different levels of cyanobacterial biomass. The result of the model run for the relatively narrow ranges of the 2 best predictors of cyanobacterial blooms, pH (step=0.5) and Secchi depth (step=0.25), and 3 levels of cyanobacterial biomass, 2, 5 and 10 mm^3 L^{-1}, is shown on Figure 25 and 26.

This permits assessing the risk of occurrence of blooms of a given magnitude in particular environmental conditions, which further improves the predictive capacity of the model (see Peretyatko et al. 2010 for details).

The results of the probabilistic model were verified by classification trees analysis (Breiman et al., 1984), also designed for the treatment of data with non-linear relationships. The classification trees analysis rendered similar results to those obtained by the probabilistic model (Peretyatko et al., 2011b). The best predictors of cyanobacterial blooms detected by the classification trees are the same as those identified by the probabilistic approach (Secchi depth and pH). The consistent results obtained by the two independent methods prove that they are not an artifact of a specific methodological approach.

The probabilistic approach permits identification of the ponds prone to cyanobacterial bloom development and thus can help managers to focus their monitoring programmes on the most problematic ponds. Such ponds can be more frequently monitored during the time of elevated risk of bloom development in order to detect cyanobacterial bloom formation at early stages and, subsequently, take the appropriate preventive and mitigative measures as well as inform the users of the affected ponds about the risk of potential health effects. The fact that the occurrence of cyanobacterial blooms is mostly confined to the warmest months of the year suggests that climate warming may lead to the increase in the frequency, magnitude and duration of cyanobacterial blooms.

2.2.7. Data base

The B-BLOOMS2 data comprise Excel files gathering most data collected during the study, from the follow-up of the reference lakes (Phase 1, 2007-2008) and from BLOOMNET samples.

The processing of these data is still ongoing but some results are already available. Analysis of the Falemprise data (n= 100) using boosted regression trees (BRT) produced interesting results in terms of predictors of cyanobacterial blooms in this lake. The technique was used to identify the conditions in which *Aphanizomenon flos-aquae* or *Planktothrix agardhii* developed best. The BRT model (Figure 27) predicted *Planktothrix* development in Lake Falemprise when total phosphorus exceeded 100 µg L^{-1} and when the euphotic depth went below 2 m, with some influence of total nitrogen above 1 mg L^{-1} and of increased rainfall. By contrast, *Aphanizomenon*

development was favoured by photoperiods longer than 15 h, by total phosphorus in the range 50 – 100 µg L^{-1}, by low inorganic nitrogen, by epilimnion temperature above ~17°C, by low to medium irradiance associated with low rainfall, relatively high water clarity and by the presence of ammonium. In other words, in agreement with the ecological preferences of these two taxa (e.g. Reynolds, 2006), *Planktothrix* developed in bad weather conditions, favouring vertical mixing of the water column and allowing TN and TP to increase, thereby meeting requirements for growth of this taxon. Also, *Planktothrix* is known as a superior competitor for light, which is shown here by its success when euphotic depth was reduced. *Aphanozimenon* has quite different preferences: whereas its P requirements are relatively high, it needs stratification conditions (hence high temperature and calm weather) in order to be able to take advantage of its strong buoyancy capacities.

Also, stable stratification favours denitrification in the eutrophic Lake Falemprise, which

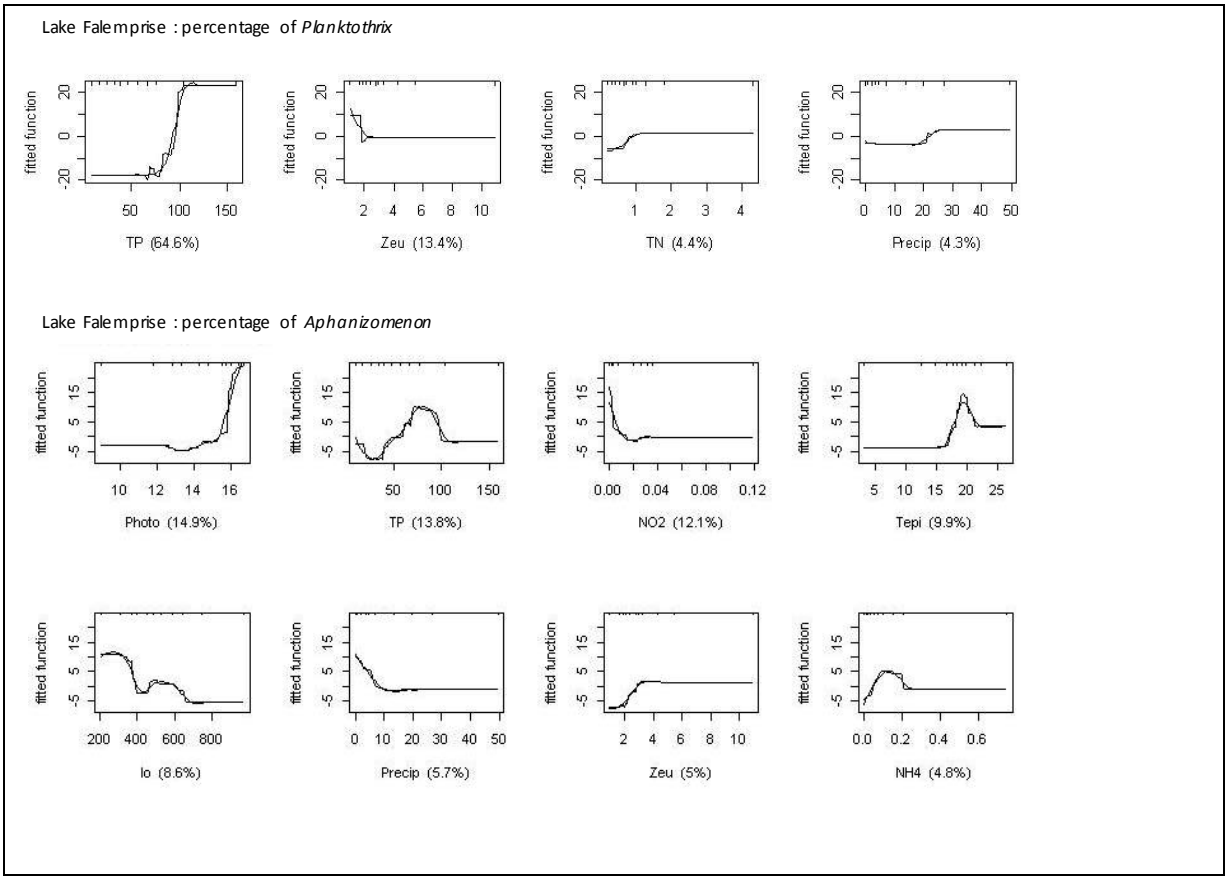


Figure 27: Fitted functions for the most influential variables in the BRT model relating relative biomass of *Planktothrix agardhii* and *Aphanizomenon flos-aquae* in Lake Falemprise. The data set comprises observations made from 2002 to 2008 (n=100). The percentages indicate the relative contribution of predictor variables to each model.

may allow *Aphanizomenon,* a di-nitrogen fixer, to outcompete other phytoplankton taxa. Therefore, the BRT application to the Falemprise data helps to confirm the major effect of weather conditions on the development of cyanobacteria in this relatively shallow eutrophic lake. This has implications on possible presence of cyanotoxins in the water of this bathing area, as *Planktothrix* is a potential microcystin producer, while *Aphanizomenon* spp. include populations with the potential to produce saxitoxins and cylindrospermopsins (Codd et al.2005a,b; Fastner *et al*., 2007).

Another BRT analysis was run on the data of the more eutrophic and shallow water bodies (Donkmeer, Westveldparkvijver and Ixelles ponds), with the aim of predicting total cyanobacterial biomass and the relative contribution of different taxa (Figure 28 a, b). Total phosphorus was by far the best predictor of total cyanobacterial biomass, followed by low euphotic depth (< 0.5 m) and very low dissolved inorganic nitrogen. The contribution of *Microcystis* to blooms was predicted by low water transparency (Zeu < 0.25 m), low dissolved inorganic nitrogen, low wind velocity in the preceding days, and total phosphorus above 600 µg L^{-1}. *Anabaena* spp. were best predicted by long photoperiod (> 15h), relatively low TP (<200 µg L^{-1}), epilimnion temperature above 16°C and relatively high euphotic depth (increase between 0.5 and 1 m).

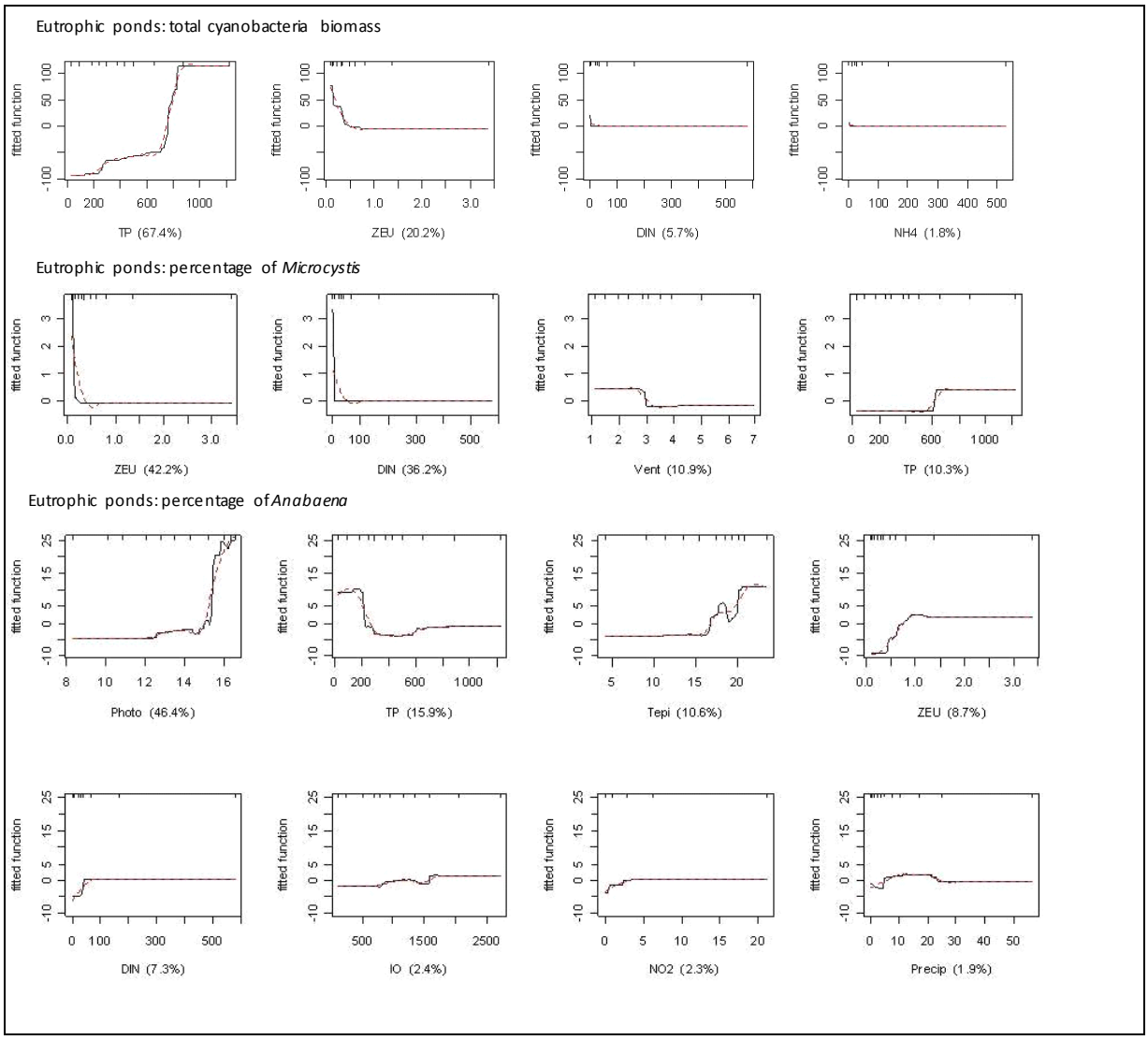


Figure 28 a: Fitted functions for the most influential variables in the BRT model relating total biomass of cyanobacteria, relative biomass of *Microcystis,* and relative biomass of *Anabaena* in shallow eutrophic waterbodies (Donkmeer, Westveldparkvijver and Ixelles ponds). The data set comprises observations made from 2007 to 2008 (n=130). The percentages indicate the relative contribution of predictor variables to each model.

In those waterbodies, *Aphanizomenon flos-aquae* development is best predicted, remarkably, by the same environmental variables as in Lake Falemprise: long photoperiod, epilimnion temperature above ~14°C, total phosphorus <200 µg L^{-1}, relatively high water clarity, and by low rainfall. The effect of inorganic nitrogen seems secondary for *Aphanizomenon* development in those water bodies. The BRT model for *Planktothrix agardhii* in the eutrophic ponds, as in Lake Falemprise, points to macronutrients and relatively short photoperiod as predictor variables, illustrating again the need of this taxon for high N and P concentration and its ability to develop in low-light conditions.

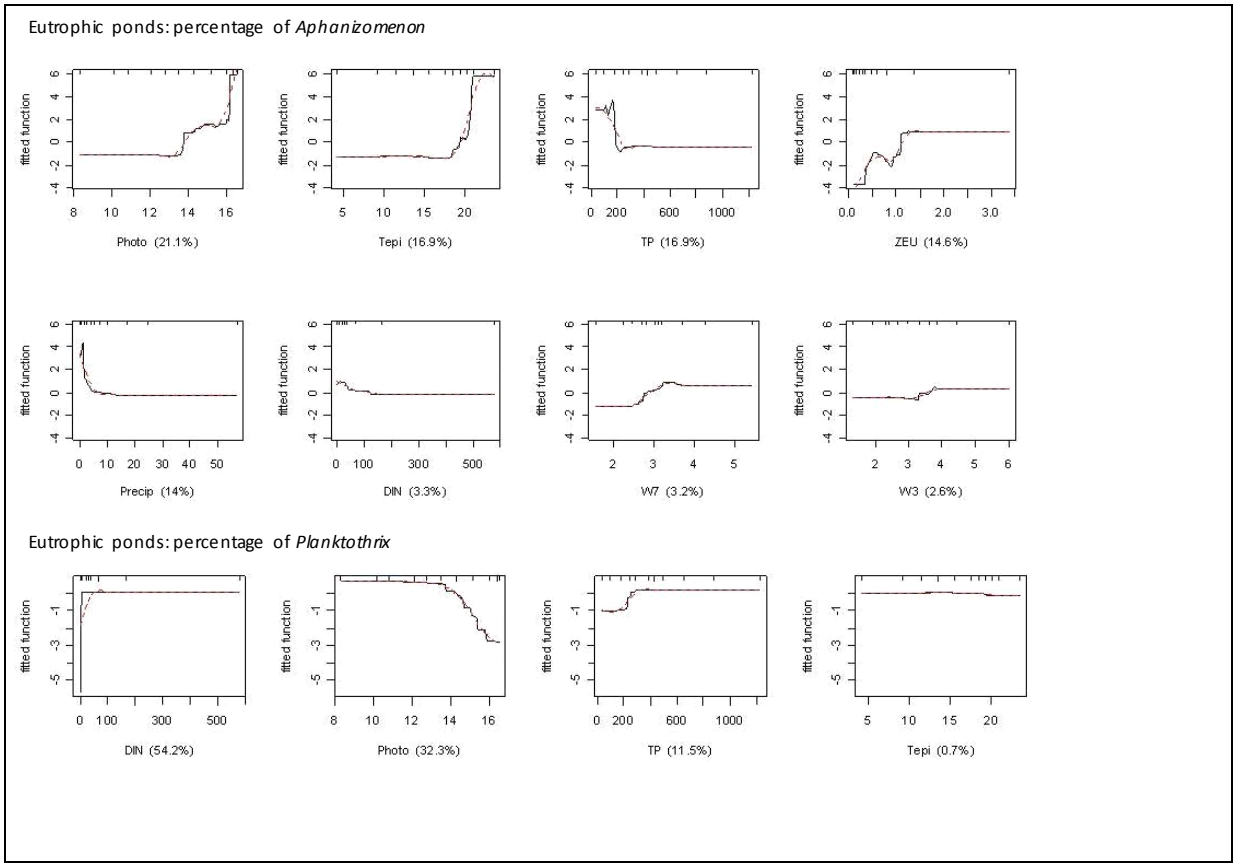


Figure 28 b: Fitted functions for the most influential variables in the BRT model relating relative biomass of *Aphanizomenon* and of *Planktothrix* in shallow eutrophic waterbodies (Donkmeer, Westveldparkvijver and Ixelles ponds). The data set comprises observations made from 2007 to 2008 (n=130). The percentages indicate the relative contribution of predictor variables to each model.

3. POLICY SUPPPORT

Regarding support for the monitoring and management of cyanobacterial blooms, several key results were obtained in the B-BLOOMS2 project. The three teams involved in regular field sampling (FUNDP, UGent and VUB) adopted common standard procedures for monitoring environmental data and plankton in a range of freshwater bodies of increasing degree of trophy, thereby covering a range of lakes in which cyanobacterial blooms develop to varying degrees. The active participation of BLOOMNET, the network of water-users and managers, further expanded the opportunities to detect and analyse blooms in high resource Belgian freshwaters for taxonomic composition, genetic diversity, genotoxicity and actual toxicity. From this survey, data have been collected at national scale in a standardised manner and entered into a common data base. The data processing so far was carried out separately by each team – which has already provided results, such as the identification of the main drivers of blooms – and a large amount of work has been done to exploit the data and correlate environmental, taxonomic, genetic and toxicity information. The B-BLOOMS database currently contains all data collected in 2007 and 2008 in the reference lakes, and additional data from BLOOMNET and parallel studies. The processing of the data, using non-linear modelling techniques, has yielded prediction models ot total cyanobacterial biomass and of the contribution of the main taxa found in the blooms observed in the reference lakes. The steps taken by the VUB team, which has data on a large number of ponds, already demonstrate that data-based modelling approaches can help identifying the key drivers of blooms in a series of high resource urban lakes. Such data-based modelling approaches are valid for the set of lakes monitored and can be useful tools for designing management measures for reducing cyanobacterial blooms. Hereafter we stress the main results obtained in each region and indicate how these results may contribute to monitoring and management of blooms.

In Flanders, both reference lakes suffered heavily from cyanobacterial blooms, but with contrasting bloom dynamics and species-dominance. The intensive sampling at both the cyanobacterial community and population level (both selectively neutral and toxin genes) allowed to study in extraordinary detail bloom dynamics at both levels (and possible interactions) and the major abiotic and biotic factors governing them. This is exemplified by the rapid and dramatic shift in genotype composition in Westveldparkvijver for which the first results suggest that it is caused by amoebae

and occurs so fast that it would easily be missed with a less frequent sampling regime.

In the Brussels ponds, cyanobacterial growth is inhibited mainly by submerged vegetation and large zooplankton grazing. Ponds with extensive cover of submerged vegetation generally showed very low cyanobacterial biomass despite their nutrient richness. Submerged vegetation also provides shelter to large cladocerans, the most efficient phytoplankton grazers. In ponds lacking submerged vegetation, large cladoceran populations are decimated by fish. In such ponds, phytoplankton growth is generally controlled only by nutrients and self-shading. Because of high nutrient availability, this leads to fast increase in phytoplankton biomass and, subsequently, to high pH. As pH increases, cyanobacteria get a competitive advantage over other phytoplankters and can develop massive blooms. In order to prevent mass developments of cyanobacteria in eutrophic ponds, submerged vegetation should be restored and conserved. This can be done through manipulation of fish community structure, as many Brussels ponds are overstocked with planktivorous and benthivorous fish. This has proven to promote submerged vegetation growth and large zooplankton grazing. In ponds excessively polluted with nutrients, biomanipulation should be accompanied by nutrient level reduction, because when nutrient loadings are too high, biomanipulation might not achieve the desired results.

In Wallonia, the survey of Lake Falemprise, which was followed by the FUNDP team since 2002 in other programmes, is of particular interest as it is an official bathing area located in the Eau d'Heure lakes area, where diverse recreational activities take place. This is why Lake Falemprise has been monitored by the Walloon Region authorities for compliance with the 2006 European Bathing Waters Directive, which includes the need for the monitoring of cyanobacterial proliferation, the identification of heath risks, and for taking adequate measures to prevent exposure of the public. According to our survey, cyanobacterial blooms regularly occurred in Lake Falemprise in the period 2002-2009. These blooms are dominated by *Aphanizomenon flos-aquae*, but other potentially toxic taxa occur, including *Microcystis aeruginosa* and *Planktothrix agardhii*. The presence and the potential toxicity of these taxa has been confirmed by genetic analyses, and microcystin toxins were detected at concentrations above the WHO Guideline Value in 2007, 2008 and 2009. This clearly calls for measures to be taken by the Walloon authorities to reduce the risk of exposure to cyanobacteria and their toxins, in compliance with the EC Directive. Even

more severe problems occurred in the lakes of the same area, as Lake Ry Jaune (another water body devoted to several recreative activities): the microcystin concentrations observed in its water in 2009 should have prompted risk assessment and possible interdiction of the recreational activities. Several other waterbodies at risk of developing cyanobacterial blooms were identified in Wallonia in 2010.

A major result of this project is the improved detection of dissolved and particulate microcystins, thanks to the analyses carried out using an ELISA test by the international partner, University of Dundee. These measurements made it possible to show that microcystins were present in all bloom samples provided by the Belgian partners throughout the project, at variable concentrations. This single fact demonstrates the relevance of the B-BLOOMS2 project, which aims, among other things, to assess toxicity from cyanobacteria blooms in eutrophic Belgian lakes. Additional data on microcystin content of various aquatic organisms, particularly zooplankton, pointed to significant risk of concentration of this toxin in the aquatic food web. The detection of *mcy* genes seemed to be a good proxy for the presence of microcystins in the samples tested. The molecular tools applied have shown that there was a genetic heterogeneity of the blooming taxa *Microcystis* and *Plantkothrix*, on the basis of the ITS sequences.

The B-BLOOMS2 project also, while pursuing investigations on genetic diversity and toxigenicity, focused on practical and science policy objectives: developing BLOOMNET, transferring knowledge to the official laboratories in charge of water quality control and protection, and improving information and awareness of the authorities and the general population. Understanding the processes driving cyanotoxin production and their dependence on environment and genetic diversity has also been a key component of the project. A first processing of the database has shown that it is possible to predict the occurrence of potentially toxic genera – *Microcystis, Planktothrix, Anabaena, Aphanizomenon* – in eutrophic lakes.

The molecular tools have filled some knowledge gaps and allowed to detect the potential toxicity of new genera in Belgium. For example, they indicated that the *Woronichinia* genus might produce toxic cyanopeptolins and other secondary metabolites, that *Snowella* and the picocyanobacterium *Cyanobium* might also produce secondary metabolite which may be harmful. More attention could be directed towards the isolation of Nostocales strains, as their toxicity in Belgium remains unknown.

Thanks to the maintenance of the B-BLOOMS website, the information gathered during the project will remain accessible to end-users, as well as the scientific results, through the presentations of the workshops and the publications of the teams. It is a wish of the project partners to maintain contact with the managers and the wider community, and to contribute to disseminate information on cyanobacterial blooms and their management.

4. DISSEMINATION AND VALORISATION

Most activities related to this task were carried out through BLOOMNET. In Wallonia, advice for sampling, identification of cyanobacteria and analysis of toxins was regularly provided by the University of Namur (FUNDP) to the ISSeP staff in charge of lake monitoring (related to the WFD implementation) and of the application of the EC Bathing Waters Directive. FUNDP, through two successive contracts with the Walloon authorities (DGARNE, Namur and DEMNA, Gembloux), has been involved in phytoplankton analysis at the Walloon reservoirs (Descy et al., 2010) for the determination of the ecological potential of these waterbodies. Also notable is the study conducted by FUNDP on the 18 bathing zones for PROTECTIS, with the production of a report containing the methods and results. Through that study, a protocol for estimating the risk of eutrophication and cyanobacterial bloom development has been proposed which will hopefully be used in the monitoring of the waterbodies presenting potential or actual health risks.

During the course of the project, the public and the government were informed in the form of interviews and press releases for newspapers,popular publications in regional magazines and presentations given on several occasions at local (City Council: Lummen-problems with toxic blooms in Schulensmeer), regional (presentation for the Flemish Agency for Care and Health, Brussels) and national/international levels (presentation at Belgian Wildlife Disease Society, Brussels).

4.1 WEB SITE

Two versions of the website were developed in succession. The present one, www.bblooms.be, was created specifically for the second phase of B-BLOOMS2 and designed for communication with end-users and BLOOMNET members. For instance, it was used for the announcement and registration of the Final Workshop, and for allowing access to the documents produced earlier, including the presentations of the Mid-term Workshop held after Phase1. Several peer-refereed scientific

publications by the team produced during the projects can also be accessed, and the site has web links to several documents of interest. The field, laboratory and data analysis procedures used during the project can also be viewed.

4.2 COMMUNICATION WITH THE END-USERS COMMITTEE AND WORKSHOPS

During Phase 1, two meetings were organised, one at FUNDP in April 2007 and the other in the afternoon session of the Mid-term Workshop held in Brussels at the end of October 2008. The reports of these two meetings can be accessed on the web site.

The final seminar was a valuable opportunity to communicate with end-users and managers, with 50 people attending the meeting held at BELSPO on December 2010. After the morning scientific session, a lively debate followed with afternoon presentations made by the persons in charge of the implementation of the 2006 Bathing Waters Directive in the three regions.

The first point for debate focused on the definition of the threshold concentrations for toxicity, as given by international norms and listed in European guidelines. For the representatives of the various managing authorities present, these guidelines remain vague, heterogeneous, and lack clarity, not to mention that they are sometimes considered as only provisional. The European Water Framework Directive does not necessarily help clarify the situation, as governments are largely left to their own devices when asked to define their own sets of thresholds. In some cases, e.g. Belgium, threshold values are set at the regional level. In neighbouring France, these thresholds are nationally defined and enforced, whereas in the Netherlands, despite a nationally-defined set of values, enforcement is conducted on a voluntary basis. The terminological vagueness of the EU WFD, and the reliance on member states to work out the best possible scenarios independently, leaves managing authorities somewhat disoriented. Professor Codd's intervention at one stage aimed at easing some of these concerns, by reminding the audience that the seemingly diverging sets of threshold values defined throughout EU member states remain well within the confidence interval of WHO guidelines, and as such they should be considered adequate at present, despite their disparity.

A second point focused on the apparent multiplication of events associated with the proliferation of cyanobacteria, and to a growing perception of their danger, as such events almost inevitably will happen more frequently and be more widespread in the

foreseeable future if current trends are confirmed. With this in mind, corollary questions naturally arise from these observations. Will economic interests become more and more threatened by the increasing incidence and frequency of such proliferation events? Is the burden of having to decide on restricting access to the public manageable for local authorities, given the economic consequences and the need for public understanding and compliance? How should authorities best communicate to the public about the motives underlying their decisions? What roles should scientists and the media fulfil in informing the public about these issues?

Two interventions are worth mentioning in connection with these questions. Professor Codd warned against excessive alarmism, by reminding participants that the problems caused by cyanotoxins are very different in nature from those caused by "classical" pathogens such as bacteria and viruses, in that no contagion is involved and that epidemics do not arise from exposure to cyanobacteria. Bearing this in mind, even if bloom proliferation events become more frequent, some of them leading to increased risks of waterborne toxicity, the potential health hazards should remain largely manageable, given good awareness among decision-makers and water-users with contingency plans in place.

As to the implication of scientists and journalists in informing the public, there is a general agreement among scientists themselves that they are ill-equipped to communicate with the general public, as they are trained to deal efficiently with specialized audiences. Scientific journalists, on the contrary, can do a much better job of framing the debates in general terms, but suffer from a lack of visibility in the printed press. They are also a species in decline. Potentially important news with a scientific twist is often confined to the back pages of newspapers. Consequently, to get their message across, journalists could be inclined to throw in a pinch of alarmism, use punch lines that grab the interest of the general readership. Between running the risk of quickly becoming yesterday's news, and unwillingly instilling fear, there is a delicate balance to strike.

The role of scientists is best confined to two fields: educating future generations, as everyone will agree, and adequately briefing authorities and managers by maintaining a level of quality in their applied research, in other words: telling it like it is…

A third important topic dealt with the difficulties of long-term management. In many cases, managers are faced with heavy legacies from past eutrophication, and where solutions exist to efficiently limit additional inputs of nutrients into water bodies, managing contaminated sediments is a totally different proposition. Technical, workable solutions exist but the dwindling availability of credit is often a constraint.. As far as perception is concerned, the long-term economic benefits of rehabilitation efforts are sometimes difficult to get across, too. An additional difficulty in raising the necessary funds is that, in the EU WFD, only lakes and impoundments are considered: ponds are not, that make the bulk of water bodies affected by cyanobacteria.

At the end of the debate, the possibility of testing "green technology" (for a blue-green problem) was briefly evoked, in direct relationship with this topic.

The session was concluded by a proposal to hold annual workshops at national or regional level on the subject of cyanobacterial blooms, their consequences and management, with the aim of sharing experience and strengthening co-operation between scientists and managers. The idea was welcomed.

4.3 DISSEMINATION TO A WIDE AUDIENCE

- Article in the Bulletin of the ASBL 'Science et Culture', June 2010
http://www.sci-cult.ulg.ac.be/Bulletins/Bulletin426.pdf

- Article on the outreach website of the University of Liège 'Reflexions' :
http://reflexions.ulg.ac.be/cms/c_16490/algues-bleu-vert-ou-rouges-couleurs-toxiques?cid=j_15783

http://www.demorgen.be/dm/nl/5397/Milieu/article/detail/1193788/2010/12/09/Alarmerende-concentraties-giftige-stof-in-Belgische-vijvers.dhtml

http://www.lavenir.net/article/detail.aspx?articleid=39301568

http://www.fundp.ac.be/universite/services/sevrex/sevrexp/librecours/2011/libre-cours-78/lc-78

http://www.lalibre.be/actu/gazette-de-liege/article/602772/baignade-interdite-pour-cause-de-cyanobacteries.html

http://www.lalibre.be/archives/divers/article/632748/les-algues-bleues-proliferent.html

5. PUBLICATIONS

Several scientific publications were produced during the project and communications to scientific audiences were made by all teams(see the web site).

In peer-reviewed journals

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van Wichelen Jeroen, Ineke van Gremberghe, Pieter Vanormelingen, Ann-Eline Debeer, Bruno Leporcq, Diana Menzel, Geoffrey A. Codd, Jean-Pierre Descy and Wim Vyverman (2010). Strong effects of amoebae grazing on the biomass and genetic structure of a *Microcystis* bloom (*Cyanobacteria*). Environmental Microbiology 12 (10): 2797-2813.

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2007

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Communication targeted to the physicians and veterinaries

- Article in « Health and Food », n°109, Hiver 2001; p.5-6. « Quand le lac est tout vert … », « Als de vijver groen kleurt ». www.healthandfood.be

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