SSD

SCIENCE FOR A SUSTAINABLE DEVELOPMENT

# CYANOBACTERIAL BLOOMS : TOXICITY, DIVERSITY, MODELLING AND MANAGEMENT

## «B-BLOOMS 2»

J.-P. DESCY, S. PIRLOT, G. VERNIERS, Y. LARA, A. WILMOTTE,
W. VYVERMAN, P. VANORMELINGEN, J. VAN WICHELEN,
I. VAN GREMBERGHE, L. TRIEST, A. PERETYATKO , E.EVERBECQ, G. A. CODD.

ENERGY

TRANSPORT AND MOBILITY

AGRO-FOOD

HEALTH AND ENVIRONMENT

CLIMATE

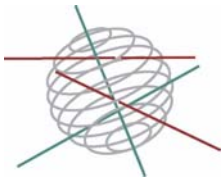
BIODIVERSITY

ATMOSPHERE AND TERRESTRIAL AND MARINE ECOSYSTEMS

TRANSVERSAL ACTIONS

.be

SCIENCE FOR A SUSTAINABLE DEVELOPMENT
(SSD)



*Terrestrial Ecosystems*

FINAL REPORT PHASE 1

# CYANOBACTERIAL BLOOMS : TOXICITY, DIVERSITY, MODELLING AND MANAGEMENT

## "B-BLOOMS 2"

## SD/TE/01A


**Promotors**

**Jean Pierre Descy**
Facultés Universitaires Notre-Dame de la Paix (FUNDP)

**Annick Wilmotte**
Université de Liège (ULg)

**Wim Vyverman**
Universiteit Gent (UGent)

**LudwigTriest**
Vrije Universiteit Brussel (VUB)

**GEOFFREY A. Codd**
**University of Dundee, Scotland**

**Authors**

JEAN-PIERRE DESCY, SAMUEL PIRLOT, GISELE VERNIERS, YANNICK LARA, ANNICK WILMOTTE, WIM VYVERMAN, PIETER VANORMELINGEN, JEROEN VAN WICHELEN, INEKE VAN GREMBERGHE, LUDWIG TRIEST, ANATOLY PERETYATKO, ETIENNE EVERBECQ, GEOFFREY A. CODD



BELGIAN SCIENCE POLICY


Rue de la Science 8
Wetenschapsstraat 8
B-1000 Brussels
Belgium
Tel: +32 (0)2 238 34 11 – Fax: +32 (0)2 230 59 12
http://www.belspo.be

Contact person: Sophie Veheyden
+32 (0)2 238 36 12



Jean-pierre Descy, Samuel Pirlot, Gisèle Verniers, Yannick Lara, Annick Wilmotte, Wim Vyverman, Pieter Vanormelingen, Jeroen Van Wichelen, Ineke Van Gremberghe, Ludwig Triest, Anatoly Peretyatko , Etienne Everbecq, Geoffrey A. Codd. ***Cyanobacterial blooms : toxicity, diversity, modelling and management "B-Blooms 2"***. Final Report Phase 1. Brussels : Belgian Science Policy 2009 – 59 p. (Research Programme Science for a Sustainable Development)

# Table of content

# ABSTRACT

The B-BLOOMS2 project consists of a study of cyanobacteria blooms in Belgium, involving sampling of a set of reference lakes in Flanders, Brussels and Wallonia, and the use of different approaches to assess environmental conditions, cyanobacterial diversity, potential toxicity through detection of the *mcy* genes, and toxin measurement using different techniques. The program was run for the years 2007 and 2008. Five reference lakes were selected: two lakes in Flanders (Westveld and Donkmeer), two in Brussels (Ixelles ponds 1 and 2) and one lake in Wallonia (Falemprise). Sampling was done on a regular basis (once a week or every fortnight from March to October). It comprised: water samples with measurements of nutrients and limnological variables, phytoplankton and zooplankton collection, subsampling for molecular analyses, isolation of strains in cultures and toxin analyses, collection of daily weather data. Phytoplankton was assessed at least at the class level, and cyanobacteria were counted and identified at least at genus level. A common protocol (see www.bblooms.be) was defined by the coordinator, and followed by the different teams. Samples were also collected through BLOOMNET, a network involving water users and managers in the different regions. In parallel, simulation models were developed for testing measures for bloom reduction in one of the lakes, and a probabilistic model was developed for predicting cyanobacterial bloom occurrence in ponds of the Brussels region.

Here we report (i) observations of phytoplankton composition with an emphasis on cyanobacteria in the reference lakes, and on environmental conditions associated with blooms development, (ii) genetic diversity assessed by DGGE and 16 S rRNA sequencing, (iii) genotoxicity assessed by detection of *mcy* genes, (iv) actual toxicity by measurement of cyanotoxins in water and in bloom material, (v) the results of Lake Falemprise simulation, and (vi) the results of the predictive modelling applied to the cyanobacterial blooms in the Brussels's ponds.

Phytoplankton and cyanobacteria dynamics were described in detail for the different lakes. Among the main results of the study already available, it can be stressed that most blooms consisted of potentially toxic belonging to the genera *Aphanizomenon*, *Microcystis*, *Planktothrix* and *Anabaena*. Observations and statistical analyses showed that, besides clearly depending on weather conditions and nutrient loading, cyanobacterial blooms were influenced to a varying degree by biotic interactions, such as nature and size of the planktonic grazers (Brussels ponds and Falemprise) or by parasites (amoebae and chytrids, in Donkmeer and Westveld). The assessment of bloom diversity was greatly improved by the molecular approaches, and change in strain dominance was shown in some lakes. The majority of DGGE bands amplified and sequenced showed high similarity with potentially toxic *Microcystis* and *Plankthotrix*.
A database on bloom molecular diversity in Belgian freshwaters (the ARB database) has been developed.

Several genes of the *mcy* cluster were regularly detected in the bloom samples: a total of 102 samples were extracted and tested for presence of mcy genes in the Brussels and Walloon regions in 2007 and 2008. While in 2007, only a part of the samples were positive, in 2008, the *mcyA*/*B*/*E* genes were detected in most samples, as in Falemprise and in Ixelles ponds, showing the presence of potentially toxic *Microcystis* in the majority of samples. A RFLP analysis showed succession of different *mcyE* genotypes in the same freshwater body.

Analysis has been carried out at University of Dundee using HPLC analysis of bloom samples and immunoassays, for determining particulate and dissolved toxins in the environment. Microcystins were also measured in single colonies or filaments isolated from environmental samples, using quantitative immunoassays. Toxin analyses showed presence of microcystins in all samples tested, the concentration of which exceeded the WHO guideline value (1 $\mu$g $L^{-1}$)

quite frequently if not all the time. Wide variations versus sample date were found in the compartmentation of microcystins between soluble and particulate phases.120 individual colonies and filaments have been analysed: the quotas measured ranged from 45.4 to 1620 picograms per colony or filament.

A modified simulation model, derived from the PEGASE model as applied to the Eau d'Heure watershed, has shown its ability to simulate cyanobacterial blooms in Lake Falemprise, and it can be used as predictive tool for future sewage treatment scenarios. However, improvements are still needed in order to fully adapt the model to lakes deep enough to develop summer stratification.
The predictive models developed for Brussels ponds allowed to calculate the probability of bloom occurrence in these ponds, with a considerable seasonal and interannual variation in the probability. The highest probability, based on the data from all the ponds studied, was shown by the combination of all the variables measured. This approach permits to identify the ponds prone to cyanobacterial bloom development and thus can help managers to focus their monitoring program on the most problematic ponds.

All these results will help understanding the mechanisms behind cyanobacterial bloom development and toxin production, and, from a management point of view, call for measures for blooms reduction for preventing public exposure to harmful blooms.

# ACRONYMS, ABBREVIATIONS AND UNITS

| | |
|---|---|
| LCL | large Cladocerans length |
| LCD | large Cladocerans density |
| SV | % submerged vegetation cover |
| T | temperature |
| SRP | soluble reactive phosphorus |
| TP | total phosphorus |
| MD | maximum depth |
| RT | hydraulic retention time |
| DIN | dissolved inorganic nitrogen |
| ITS | Internal Transcribed Spacer region |
| MC | microcystins |
| PCR | Polymerase Chain Reaction |
| rRNA | ribosomal RNA |
| DGGE | Denaturating Gradient Gel Electrophoresis |

# LIST OF THE PARTNERS

**FUNDP (coordination)**

Jean-Pierre Descy
Gisèle Verniers
Samuel Pirlot
Bruno Leporcq

**UGent**

Wim Vyverman
Pieter Vanormelingen
Ineke van Gremberghe
Jeroen Van Wichelen
Katleen Van der Gucht

**VUB**

Ludwig Triest
Anatoly Peretyatko

**ULg**

Annick Wilmotte
Yannick Lara (FRIA fellow)
Alexandre Lambion

**University of Dundee, Scotland (International Cooperation)**

Geoffrey A. Codd
Mariann Reilly
Diana Menzel

**AQUAPÔLE (Sub-Contractor)**

Etienne Everbecq

# 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 potential hazards 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 70% of the blooms are toxic (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 cyanobacterial occurrence, recurring associated health incidents, national guidelines or legislation, in several countries and international agency guidelines (World Health Organisation).

In view of the lack of knowledge about the situation in Belgium, three of the present partners initiated, 5 years ago, the BELSPO project, B-BLOOMS. Thanks to this work, it has been shown that many surface waters in Belgium are also plagued by cyanobacterial blooms, particularly in summer and autumn. Fifty-four % of the blooms 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 aims 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 propose strategies to reduce the impact.

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

- measurement of the major toxins present in the blooms and water samples by analytical methods, ELISA and Mass Spectrometry methods,
- collection of physical, chemical, biological and meteorological data on a 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 are:

- implement a network of samplers based on existing monitoring programmes of surface waters or on collaboration with health authorities or environmental organisations (BLOOMNET),
- transfer the knowledge about methods of monitoring and analysis of blooms that we will develop 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 population, to raise public awareness, contribute to future guidelines and risk assessment procedures, and improve monitoring and management.

# MATERIAL AND METHODS

## 1. BLOOMNET

BLOOMNET is the network of samplers already constituted under the B-BLOOMS1 project, and reactivated and extended under B-BLOOMS2. It is based on existing monitoring programmes of surface waters and on collaboration with health authorities or environmental organisations. As these may differ among regions, regions have been distinguished below. Contrary to the procedure initiated in B-BLOOMS1, in B-BLOOMS2, the BLOOMNET members, whenever a significant bloom is detected in a water body, have to call the respective partner in each region, , and the sampling and sample treatment is carried out by the project partners.

### 1.1. In Flanders

In Flanders, in 2007, phytoplankton blooms in 7 lakes were sampled and reported by the BLOOMNET partners, of which 6 contained a mass development of cyanobacteria. In 2008, an additional 13 blooms were sampled, of which 10 contained a mass development of cyanobacteria. Before the onset of this project, between June and October 2006, 11 additional blooms were sampled as well before the onset of this project of which 10 contained a mass development of cyanobacteria. The blooms were reported and sampled by the VMM (Vlaamse Milieu Maatschappij, n = 15), UGent (n = 13), and VVHV (Vlaamse Vereniging voor Hengelsport Verbonden, n = 3). The water samples were brought immediately after sampling to the PAE laboratory 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), and genetic (WP7) and toxin analyses (WP8).

On several occasions, the technical staff of VMM (Vlaamse Milieu Maatschappij) was trained by UGent in techniques for bloom sampling. At the moment the PAE team is also in contact with Frederik Thoelen from the "Natuurhulpcentrum Opglabbeek" who by profession deals with injured or sick waterfowl. Occasionally, private persons did contact UGent in case of problematic blooms, for instance in private diving lakes (Lake Dongelberg, Carlo Joseph).

### 1.2. In Brussels

The methodology of the three other projects carried out by the VUB dealing with nutrient fluxes and restoration potential of biomanipulation was adapted to that of the B-BLOOMS2 project so that the data collected were compatible. Thus, in addition to the 2 B-BLOOMS2 ponds, 17 other Brussels ponds were sampled according to the same methodology.

### 1.3. In Wallonia

The network was essentially based on the follow-up of swimming areas authorized in Wallonia and surveyed by the Walloon Region (DGRNE – 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 swimming areas once a week, and included counting of cyanobacteria in the regular survey. For the year 2007, only one significant *Microcystis* bloom was recorded on 28 August in the Lake Chérapont near Gouvy. In 2008, an important *Anabaena* bloom occurred in Lake Bambois: it was sampled on July 30$^{th}$. 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, exploited as drinking water water storage by VIVAQUA (ex CIBE): there, *Plankthothrix 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.

## 2. Sampling of reference lakes

A common protocol (see www.bblooms.be) was defined by the coordinator, and followed by the different teams, in order to ensure, as far as possible, acquisition of comparable results to be entered in the data base. Briefly, field and laboratory measurements are 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 toxin analysis and genetic / genotoxicity analysis, collection of samples for cultures of cyanobacteria.

All data on the reference lakes from the different teams, including genotoxicity and toxicity data, are being entered in an Excel file using the same format. This file uses the same format as the B-BLOOMS1 database, in order to make it possible to disseminate the data (WP12), and to carry out preliminary multivariate analyses, a step towards the design of predictive models of cyanobacterial blooms and of their potential and actual toxicity.

### 2.1. In 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 are known to suffer from recurrent cyanobacterial blooms. Westveldpark is a small parkland pond (± 2000 m²) while Donkmeer is a relatively large (± 86 ha) but shallow lake originated by peat-digging and 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 a bloom and twice a week in case a bloom was present. By the end of November, 52 samplings had been performed in the Westveldpark 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 Westveldpark and 23 for Donkmeer. In both lakes, an additional winter sample was gathered 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 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).

### 2.2. In Brussels

#### Sampling sites and frequency

Two Brussels ponds, IxP1 and IxP2 (étangs d'Ixelles, Place Flagey), selected as reference ponds, were sampled on 88 occasions between March 2007 and December 2008, approximately once a week from June to August, and twice or once a month during the colder season.

Twenty other Brussels ponds were sampled monthly from May to September 2007 (nine selected ponds that showed high phytoplankton biomass and/or elevated concentrations of cyanobacteria in September were also sampled in October 2007). In 2008, beside the regular sampling of 2 reference ponds, 31 Brussels ponds were sampled 3 times (May, July, August). An additional 2 ponds that developed conspicuous cyanobacterial blooms were sampled in August 2008.

The composition and surface cover of aquatic vegetation, wherever present, were assessed during each field visit.

Morphometric variables of the ponds were measured in the field (depth), or using topographic maps (area). Hydraulic retention time was estimated on the basis of the outlet discharge and the corresponding pond volume.

To quantify nutrient fluxes and their relationship with phytoplankton in general and cyanobacteria in particular, water samples for nutrient analyses were taken at the inlets and outlets of a number of the ponds studied as well as from ground water seepages whenever it was possible. Within the framework of another project (Teissier, 2008), benthic chambers were set in a number of the Brussels ponds in order to study nutrient exchange between the sediment and the water column.

### 2.3. In Wallonia

Lake Falemprise is one of the Eau d'Heure Lakes (surface area 47 ha), and is an official swimming area in the Walloon Region. Therefore, this lake is monitored for microbiological quality by the Walloon authorities. Sampling of Falemprise Lake has taken place near the dam where the depth is maximal (5m). It 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 are being measured according to the analysis protocol; weather data are acquired in situ with a Davis Vantage Pro (mod 6150 C) meteorological station, and irradiance is recorded with a surface LICOR sensor LI-190 SB connected to a data logger. Limnological profiles are acquired using a YSI 6600 or Hydrolab DS5 multiprobe.

## 3. Isolation of strains

At UGent, individual colonies of *Microcystis* were picked out using sterile glass Pasteur pipettes under a binocular microscope to establish monoclonal cultures from Leeuwenhofvijver (date: 07/09/04, number of strains: 34), Tiens Broek (10/08/05, 34) and Westveldparkvijver (26/07/07, 54, in 2008, isolations were made on five time points and establishment of pure cultures is still in progress:

22/04/08: 18 *Microcystis viridis* and 4 *M. aeruginosa* isolated, 29/04/08: 11 *M. viridis* and 1 *M. aeruginosa* isolated, 13/05/08: 15 *M. viridis* and 9 *M. aeruginosa* isolated, 03/06/08: 24 *M. aeruginosa* isolated, 17/06/08: 48 *M. aeruginosa* isolated). The same was done with individual filaments of *Planktothrix* from Driekoningenvijver (31/03/05, 52) and Donkmeer (7/09/07, 29). The strains were grown in WC-medium in a culture room. Colony morphology of the *Microcystis* strains was examined and pictures were taken. As morphology might change in culture (especially with regard to colony formation), we noted the morphology of individual colonies in the recent isolations from Westveld was noted to more easily link the morphotypes observed in nature with the established strains and their ITS rDNA sequence. Strains from Leeuwenhofvijver were identified as *Microcystis aeruginosa*, strains from Tiens Broek as *Microcystis flos-aquae*, strains from Driekoningen as *Planktothrix rubescens*, and strains from Westveldparkvijver as *Microcystis aeruginosa* or *M. viridis*. Strains from Donkmeer belonged to *Planktothrix agardhii*.

Isolation of cyanobacteria from Falemprise and Ixelles samples was carried out at ULg. Fresh samples were inoculated on agar plate and in liquid medium containing 500 $\mu$g /mL of cycloheximide to avoid growth of eukaryotic algae. BG11, B110, Z8, and WC media were used to increase the diversity of cyanobacteria and the number of strains of toxic and non-toxic genotypes. Cultures were transferred several times from liquid to solid media until mono-cyanobacterial stage is reached. They were checked under epifluorescence microscopy to investigate contamination by picocyanobacteria. At ULg, a total of 20 fresh samples were inoculated into culture media. Nine fresh field samples from the reference lakes Ixelles Ponds II and the Lake Falemprise (Ixelles Pond II 18/06/07, 26/06/07, 10/07/07, 26/07/07, 01/08/07, 10/08/07, 17/08/07, 24/08/07; Lake of Falemprise 07/08/07) were taken in 2007. Eleven fresh field samples from additional lakes (Ixelles Pond I 26/06/07, 10/07/07, 26/07/07, 01/08/07, 10/08/07, 17/08/07, 24/08/07, Quarry of Ecaussines 21/06/07; NRDP1 26/07/07; NRDP4

26/07/07; MIKI 26/07/07) were taken during occasional samplings or blooms in 2007 by the VUB and FUNDP partners, and sent to ULg. To date, 2 *Pseudanabaena*-like strains were obtained from Lake Falemprise (07/08/07). 12 *Microcystis* strains were obtained (5 from NRDP4 26/07/07, 7 from Ixelles pond II 18/06/07) and 15 *Coelosphaerium*-like strains from Ixelles pond II (26/07/07). Samples of the 2007 survey are still kept on agar plates and in liquid media. 15 strains are not identified yet. Isolation of cyanobacteria strains continued in 2008 following the protocol described above.

## 4. Environmental conditions for reference and BLOOMNET samples

Limnological variables and chemical parameters were analysed as described in the 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). At VUB, 10 sub-samples of 1 L taken by a plastic tube sampler were combined in the field, filtered through a 64 µm mesh net and preserved in 4% formaldehyde final concentration 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. Individual size of large zooplankton taxa was also measured. . The length of large cladocerans was also measured and used as proxy for grazing intensity and size-selective predation (Pinel-Alloul, 1995; Carpenter et al., 2001).

## 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 on 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 sonications 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 standard 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. 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 program, 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 chlorophyll 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, in addition samples from the Ixelles ponds (VUB) were analysed with this technique, as well as those from most BLOOMNET samples.

## 6. Molecular ecology of cyanobacteria

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

### Sample storage for molecular biology and DNA extraction

Brussels and Walloon reference lakes and additional BLOOMNET samples were filtered on 0.2 $\mu$m pore size membrane 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 $\mu$m pore size GSWP (Millipore) filter for DNA extraction and frozen at -80°C.

### 6.1. Molecular diversity of cyanobacteria in reference lakes

During the last decades, intensive efforts have been devoted to the identification and characterisation of freshwater cyanobacteria. Analysis of the 16S rRNA gene sequences has been shown to be an efficient phylogenetic marker for prokaryotic classification (Rosselo-Mora and Amann, 2001). It is now widely used for prokaryotic identification (Ouellette and Wilhlem 2003) and 16S rRNA genes amplification from freshwater samples is now used in routine 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 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 has been created. The aims of this database are 1) to make an inventory of potentially toxic and non-toxic cyanobacteria that are present in freshwater reservoirs; 2) to observe diversity changes in reference ponds and lakes or in sites that were repeatedly sampled during several years; 3) to detect potential invasive species such as the tropical *Cylindrospermopsis raciborskii* in Belgian freshwater bodies.

### ARB database: Belgian freshwater cyanobacteria

We collected 272 cyanobacterial sequences that were previously isolated from Belgian freshwater samples in order to create a 16S rRNA database. Cyanobacterial and chloroplast sequences from the SILVA rRNA database (SILVA 98 release) were kept into an ARB database and other sequences were deleted. Then, each Belgian sequence was imported into ARB, aligned and inserted in the phylogenetic tree by the ARB parsimony application. Their number is still quite limited, and the B-BLOOMS1 project has been the major source till now Origins of the Belgian sequences are listed in Table 1.

**Table 1.** Belgian cyanobacterial 16S rRNA database composition

| Sequence origin | Methodology | Number of samples | Number of sequences |
|---|---|---|---|
| Willame et al., 2006 | Cultivation approach | 22 | 26 |
| B-BLOOMS (2003-2005) | Clone library analysis | 5 | 201 |
| B-BLOOMS2 (2007- present) | DGGE analysis | 32 | 45 |

### The 16S rRNA-DGGE analysis

For the Brussels and Walloon samples, 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) and were used as template 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 for nested PCR. A second PCR was performed for DGGE analysis as described by Boutte et al. (2006). Bands were excised from gels and amplified using CYA 359F/CYA781R. PCR products were purified with GE Healthcare GFX PCR product purification kit and sent for sequencing.

## 6.2. Genotypic analyses of toxic and non-toxic cyanobacteria

The *mcy* gene cluster encodes for genes involved in microcystin biosynthesis. They belong to the families of non-ribosomal peptide synthesis proteins (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 Oullette and Whilhem 2003 for review). Recent findings showed that deletions and insertions could occur in these genes. For example, deletion of the *mcyT* region in *Planktothrix mcy* operon gave a non-toxin producing strain (Christiansen et al., 2008). In such case, positive PCR detection is not concomitant with toxicity. On the other hand, deletion of other regions might have no consequences on toxin 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 occurrence is responsible for the production of a different variant of microcystin. Nevertheless, it was previously shown that the presence of *mcyABDEF* and *mcyH* genes was 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/B* and *mcyE* with primers described in the literature.

Cyanobacterial species composition in lakes is relevant for the microcystin concentrations and the microcystin variants in the environment (Kardinaal and Visser, 2005). A combination of PCR and Restriction Fragment Length Polymorphism (RFLP) analysis gives the opportunity to differentiate potentially toxic genera present in a sample (Hisbergues et al., 2003; Dittmann and Borner, 2005). To quickly identify the potential microcystin producers present in our samples, we have 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 on morphological criteria and 16S rRNA sequences. Therefore, a genotypic analysis is required on the basis of different DNA sequences.

Previous studies of the Microcystis population dynamics based on ITS-DGGE have shown a succession of different genotypes during a bloom. This succession suggested a relation with the concentration of microcystins (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.

Toxin and/or mcy gene detections in single colony/filament previously showed that populations were composed of both toxic and non-toxic genotypes (Janse et al., 2004; Via Ordorika et al., 2004). It also suggested that analysis of *Microcystis* morphotypes was an indication of microcystin production. Nevertheless, it was not possible to have data on toxin concentrations. However, Young et al. (2008) from the laboratory of Dundee demonstrated that it is now possible to quantify MC-LR in single colonies from a cultivated strain. We would like to

combine, for the first time, toxins concentrations and genetic analysis from a single environmental *Microcystis* colony. Genotypic analysis of single colonies/filaments requires a large amount of DNA. So far, in the literature, the genotypes of environmental single colonies of *Microcystis* were characterized on the basis of one or two PCR products. In the framework of this project, we have developed a new approach that allows to perform Multi Locus Sequence Analysis (MLSA) on a single colony or filament.

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

Three different PCR protocols were used to detect *mcyA* (Hisbergues et al., 2003), *mcyB* (Nonneman and Zimba, 2001) and *mcyE* genes (Rantala et al., 2006). These primer pairs have different targets that are necessary for microcystin production. The *mcyA* (mcyA-CdF/ mcyA-CdR) and *mcyE* (mcyEF2/ mcyER4) primers were respectively based on *Anabaena*, *Microcystis*, *Planktothrix, Anabaena* and *Nostoc* microcystin synthetase sequences. In this way, we wanted to detect the presence of potential toxic genotypes from several toxin-producing genera in a single reaction. In addition, *Microcystis*-specific primers *mcyB* (mcyF1/ mcyR1/ mcyR2) were used in two successive PCRs (nested strategy) to detect potential microcystin-producing *Microcystis* genotypes in our samples. Additionally, *mcyE* RFLP analysis was performed on lake Falemprise samples 2007/2008 and additional samples from Ixelles pond II 2007/2008, lake Chérapont 2007, quarry Ecaussines 2007/2008, Virelles pond 2008, Lake Féronval 2008, Lake Bambois 2008, and Ixelles pond I 2008. The *mcyE* PCR products obtained according to Rantala et al. (2006) were digested with the restriction enzyme AluI. PCR programs were performed as described in Table 2.

**Table 2.** PCR program description

| Target gene | Primers | Progam | | | | |
|---|---|---|---|---|---|---|
| | | cycle 1 (1X) | cycle 2 (35X) | | | cycle 3 (1X) |
| *mcyA* | mcyA-CdF/ mcyA-CdR | 94°C-5min | 94°C-1min | 54°C-1min | 72°C-1min | 72°C-7min |
| | | cycle 1 (1X) | cycle 2 (28X) | | | cycle 3 (1X) |
| *mcyB* | mcyF1/mcyR1 | 94°C-3min | 94°C-30sec | 57°C-45sec | 72°C-1min | 72°C-7min |
| | | cycle 1 (1X) | cycle 2 (35X) | | | cycle 3 (1X) |
| *mcyB* | mcyF1/mcyR2 | 94°C-3min | 94°C-30sec | 57°C-45sec | 72°C-1min | 72°C-7min |
| | | cycle 1 (1X) | cycle 2 (35X) | | | cycle 3 (1X) |
| *mcyE* | mcyEF2/mcyER4 | 95°C-3min | 94°C-30sec | 60°C-30sec | 68°C-1min | 68°C-10min |

**The ITS-DGGE analysis**

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

In Flanders, from each sampling of the reference lakes and each BLOOMNET sample, filters were used for DGGE of PCR-amplified 16S-23S rDNA intergenic spacer sequences to investigate the genetic structure of the populations of the dominant cyanobacteria *Microcystis* and *Planktothrix* (Janse et al., 2003; 2004). 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* described by 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). 500 colonies were isolated from Ixelles pond II, rinsed, and pooled in the same tube under a dissecting microscope. DNA was extracted using a DNA extraction kit for soil (MoBio). 16S-23S rRNA intergenic spacer was first amplified using the

cyanobacteria-specific primers CYA359F/ 23S30R. In the mean time, *mcyB/E* detection was performed as described previously.

**Single filaments/colonies approach**

Colonies and filaments were isolated and washed under a dissecting microscope, starting from fresh samples sent by FUNDP, UGent and VUB. Single colonies/filaments were observed and photographed under the microscope with 400X magnitude. They were stored at -20°C and underwent a heat shock (1 min at 99°C) to be split into two portions. Then, 0.5 $\mu$l of mixture was used as template for genetic analysis. We used the Repli-g mini kit (QiaGen) to amplify the whole genome of cells in the mixture by Multiple strand Displacement Amplification (MDA) with the phy29 polymerase. MDA products were used as template for cyanobacteria or *Microcystis* specific PCR reactions.

Eleven environmental *Microcystis* and 5 environmental *Woronichinia* were amplified with MDA. Housekeeping genes *ftsZ*, *gyrB*, *recA* were detected following protocols described in Tanabe et al. (2007). The *rpoC1* gene was detected as described in Yoshida et al. (2008). The *rbcLX* gene was amplified following Rudi et al. (1998)'s protocol. The ITS amplicons for DGGE were obtained as described in the previous section. The 16S rRNA gene was amplified with the ITS region using primers 359F/23S30R or CH/23S30R depending on taxa or colonies. The *mcyA/E* were amplified as described in the previous section.

The second portion of 8 *Microcystis* were sent to the University of Dundee for quantitative ELISA assays for microcystins.

In 2007, a total of 88 *Microcystis* colonies were isolated from Ixelles Ponds or cultures. 43 *Planktothrix* filaments were isolated from PCC cultures. 48 *Woronichinia* colonies were isolated from the Ixelles and Tervuren Ponds. All were stored at -20°C.

## 7. Determination of toxin contents

Partners have been collecting and processing environmental samples throughout the 2007 and 2008 bloom season for the analysis of microcystins by HPLC. This has involved sample filtration to provide a particulate (cyanobacteria-containing) fraction and recovery of soluble (extracellular phase) microcystins by solid phase extraction. All fractions for analysis are being stored by partners for shipment to University of Dundee at the end of November. Analysis has been carried out at University of Dundee using HPLC and immunoassays as described below. Toxin analysis was carried out mainly in 2008 and 2009.

### 7.1. HPLC detection of cyanotoxins

Measurements of intracellular and extracellular microcystin concentrations by HPLC with photodiode array detection (HPLC-DAD) is being carried out from selected blooms samples, collected from the reference lakes and from water bodies sampled thanks to BLOOMNET. Samples are 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 5$\mu$m) 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 toxins, only bloom samples having the two microcystin synthase genes (*mcyE* and *mcyB*) were processed. Toxin concentrations will be expressed in $\mu$g toxin / g dried weight and, if sampling volume has been recorded, as $\mu$g 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 for implementing detection of other toxins in Belgian blooms, in particular anatoxin.

### 7.2. Quantitative immunoassay of microcystins in environmental and other water samples

Water samples collected from Belgian water bodies were aliquoted 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 have been 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 microcystin. The resulting analyte was therefore the sum of the intra- and extracellular toxin pools; (b) extracellular microcystin, by centrifuging 1ml 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).

### 7.3. 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).

### 7.4. 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, aliquoted in glass vials and lyophilized for use by all partners as required throughout the project (Fastner et al. 2002).

### 7.5. Measurements of anatoxins 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).

## 8. Deterministic watershed simulation modelling of Eau d'Heure Lakes

The existing model of the Eau d'Heure basin has been 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.

The model 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 on soil and in 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 include specifically the cyanobacteria. The model allowed running simulations of various management scenarios. In the framework of the B-BLOOMS 2 project, the model was improved thanks to the data on lake monitoring (including those from B-BLOOMS 1).

## 9. Statistical predictive models for Brussels ponds

The data from the 22 Brussels ponds sampled monthly from May to September 2007 were combined with the data from the same or other Brussels ponds acquired between 2003 – 2006, according to the same methodology, into a single data matrix. The resulting data matrix comprises 42 ponds sampled on 381 occasions. The combined dataset was analysed using

multivariate and univariate statistical techniques and incorporated into a probabilistic model based on conditional probability calculation.

# RESULTS AND DISCUSSION

## 1. Ecology

### 1.1. Flanders

Fifteen percent of the BLOOMNET cyanobacterial blooms from 2006 - 2008 were dominated by *Aphanizomenon*, 11% by *Anabaena*, 7 % by *Planktothrix (rubescens)* and 48 % by *Microcystis*. Three blooms were particularly important because of their socio-economic consequences. In spring 2007, a massive bloom of *Aphanizomenon* in the Gavers in Harelbeke coincided with the mortality of big carp and some waterfowl. However no anatoxin-a or saxitoxins (analysis done at Institute Pasteur, Paris) could be detected in the cyanobacteria and the reasons for the high mortalities remain unknown. Also in Schulensmeer in Lummen a massive bloom of *Woronichinia* and *Microcystis* developed in autumn 2006, 2007 and 2008. In both cases, the local governments closed these lakes to the public for several weeks for public 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 $\mu$g $L^{-1}$. Due to this problem, this lake couldn't be opened by the owners for recreation (diving).

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 and 2008 (Figure 1). 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 coccal green algae.

In Westveld pond, in 2007 at the beginning of May, a bloom of *Euglena* was present which was replaced by *Microcystis* after two weeks. In 2008, the bloom of *Microcystis* came up later (Figure 2). 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.

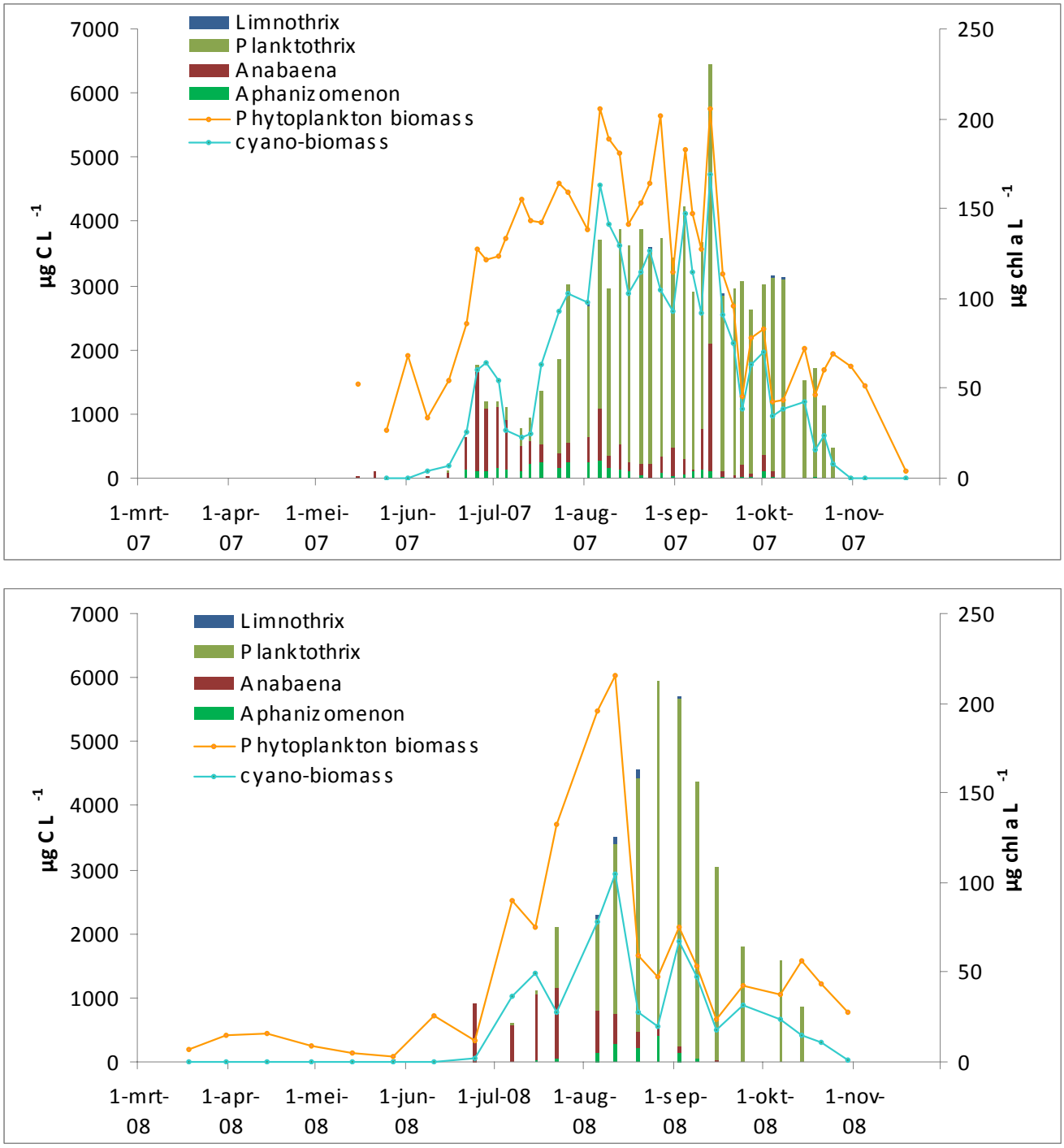



**Figure 1**: Cyanobacterial bloom dynamics in the reference pond Donkmeer in 2007 (above) and 2008 (below), as revealed by microscopical countings, and a comparison with estimates of total and cyanobacterial biomass based on HPLC pigment analysis (performed by FUNDP, Namur).

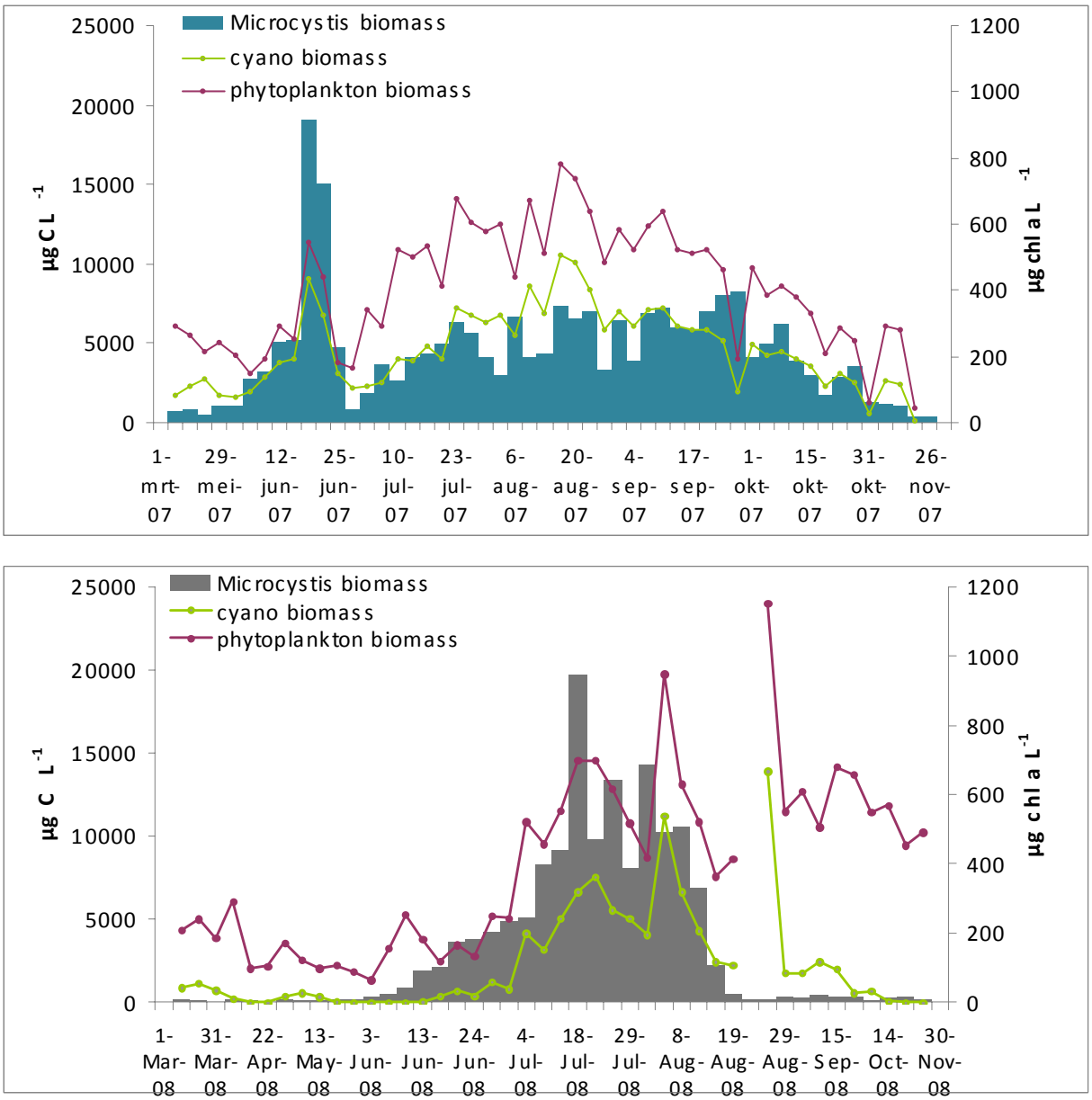



**Figure 2**: Cyanobacterial bloom dynamics in the reference pond Westveld in 2007 and 2008, as revealed by microscopical countings, and a comparison with estimates of total and cyanobacterial biomass based on HPLC pigment analysis (performed by FUNDP, Namur).

Microscopical examination of the samples form Westveld pond 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, 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. This suggests an important role for the amoeba in causing a major and extremely rapid shift in *Microcystis* population structure and a temporal biomass decline. This contrasts with the zooplankton, which consisted almost exclusively of rotifers, which had an abundance peak before the bloom maximum and were present only in low densities throughout the bloom period. A chytrid fungus, *Chytridium microcystidis*, also reached high infection

frequencies (up to 75% infected colonies) in both morphotypes but was apparently incapable of controlling *Microcystis* biomass and remained present throughout the bloom period. In 2008, the dominance of *M. aeruginosa* in summer and a peak of amoebae was detected again (although later in summer), but the dominance of *M. viridis* after the peak of amoebae was not present. The DGGE patterns seem to correspond with the microscopical determination.

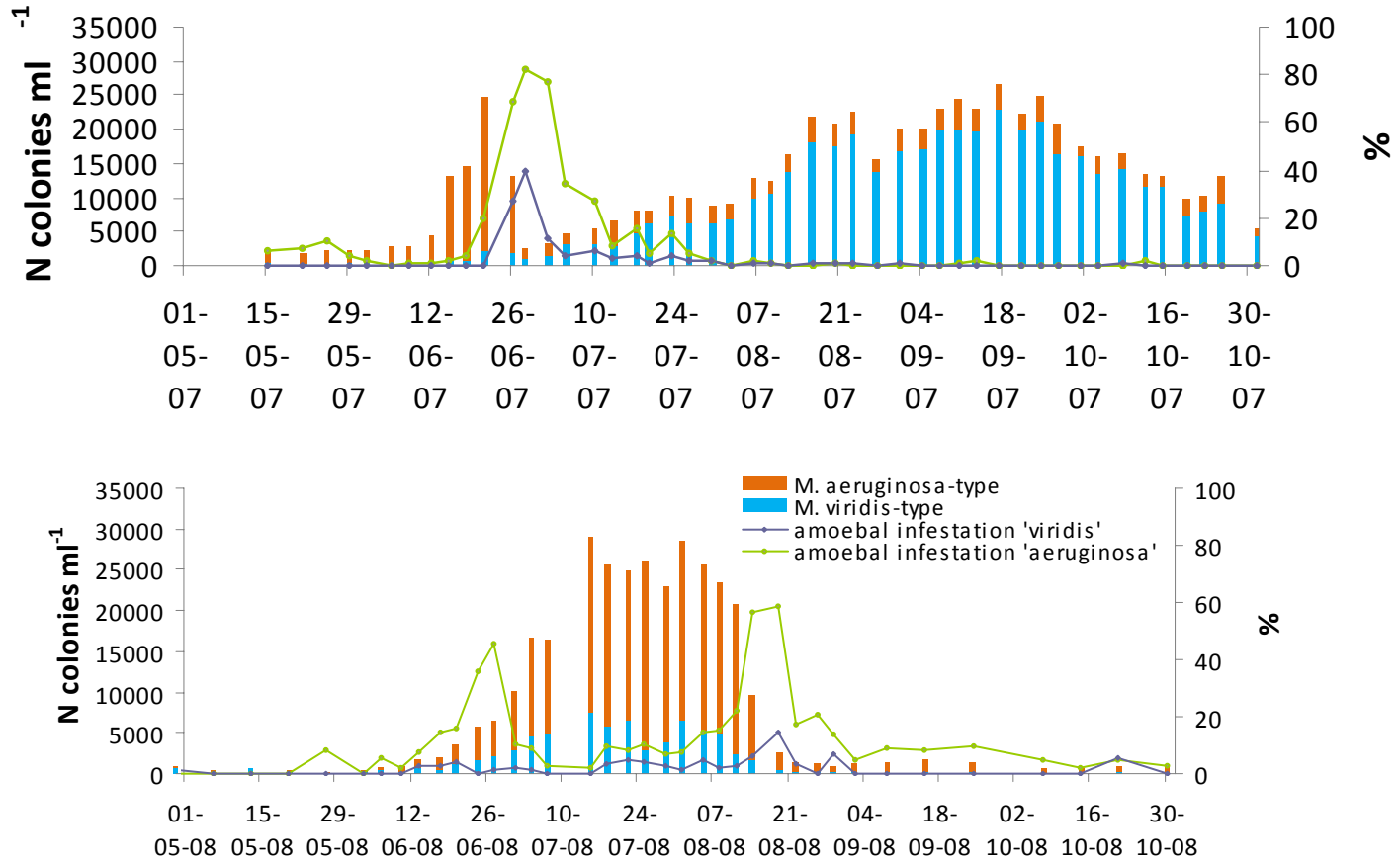



**Figure 3**: *Microcystis aeruginosa* and *M. viridis* biomass in Westveld in 2007 and 2008 and the percentage of amoebal infestation during the same period.

In Donkmeer, no cyanobacterial parasites were observed and a more diverse zooplankton community was present with rotifers, *Bosmina*, copepods and large *Daphnia* sp. in autumn.

### 1.2. Brussels

Phytoplankton, zooplankton, and nutrient samples collected in 2007 and 2008 have been processed. All the data acquired were organized into respective MS Access databases.

Both reference ponds (IxP1 and IxP2) are hypereutrophic in terms of TP concentrations (mean TP > 0,15 mg P $L^{-1}$). The preliminary results show considerable interpond as well as interannual variation in the dynamics of phytoplankton biomass, large Cladocerans density and size (Figure 4, Figure 5) and cyanobacteria biomass and composition (Figure 6, Figure 7). The main factors controlling cyanobacteria in the reference ponds seem to be zooplankton grazing, pH, retention time, nitrogen availability and depth. Large Cladocerans density appeared to be less important for phytoplankton control than their size. Density might even have positive correlation with cyanobacteria (Figure 8), suggesting that large cladocerans, when small in size, can avoid predation and feed preferentially on other algae, which are more palatable than cyanobacteria. This can promote cyanobacterial bloom development as cyanobacterial losses to zooplankton

grazing are lower that those of other phytoplankters in such situations (Benndorf et al., 2002). When cladocerans are large enough (>1mm), they can considerably restrain phytoplankton growth, including bloom-forming cyanobacteria (see also section 7).

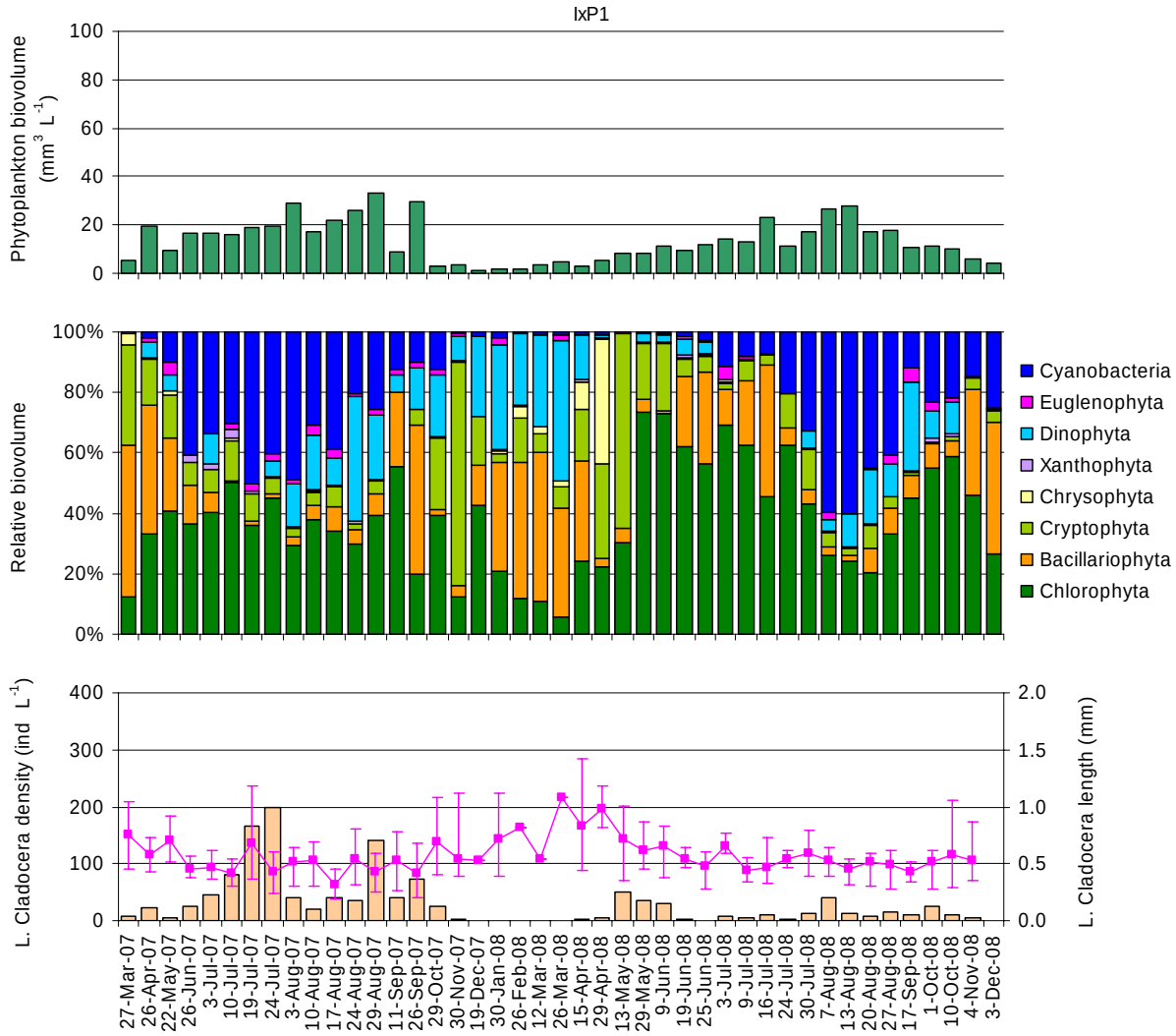



**Figure 4**: Temporal variation in phytoplankton biomass and composition and large Cladocerans density and size; IxP1

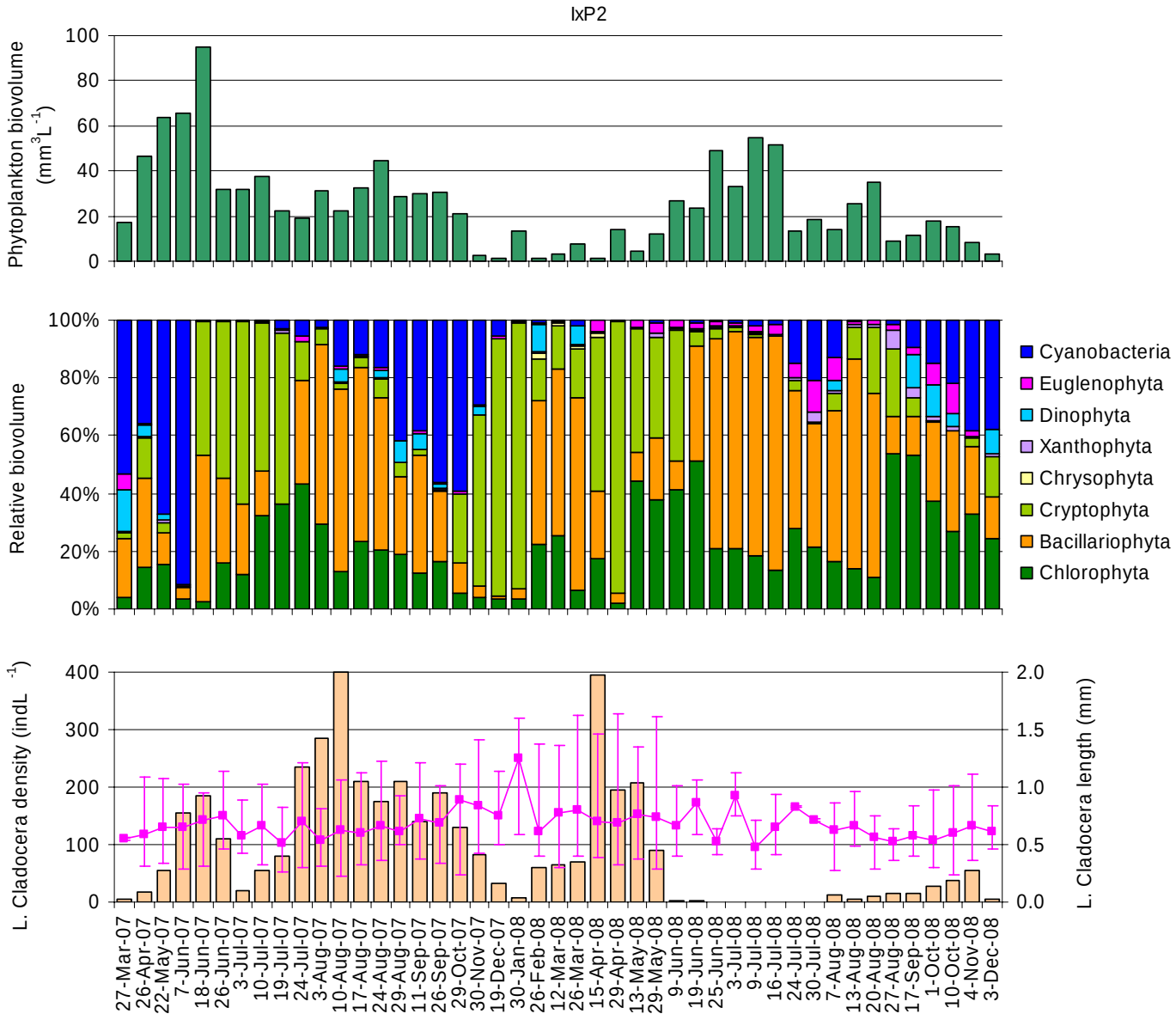



**Figure 5**: Temporal variation in phytoplankton biomass and composition and large Cladocerans density and size; IxP2

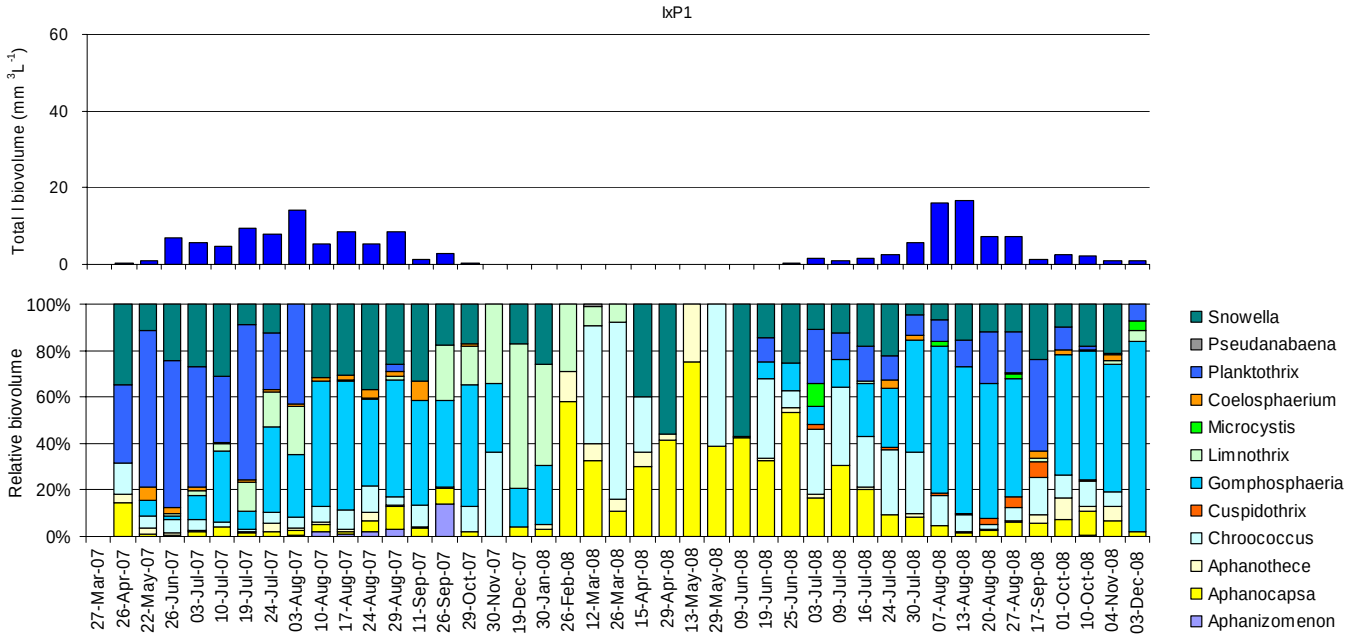



**Figure 6**: Temporal variation in biomass and composition of cyanobacteria; IxP1

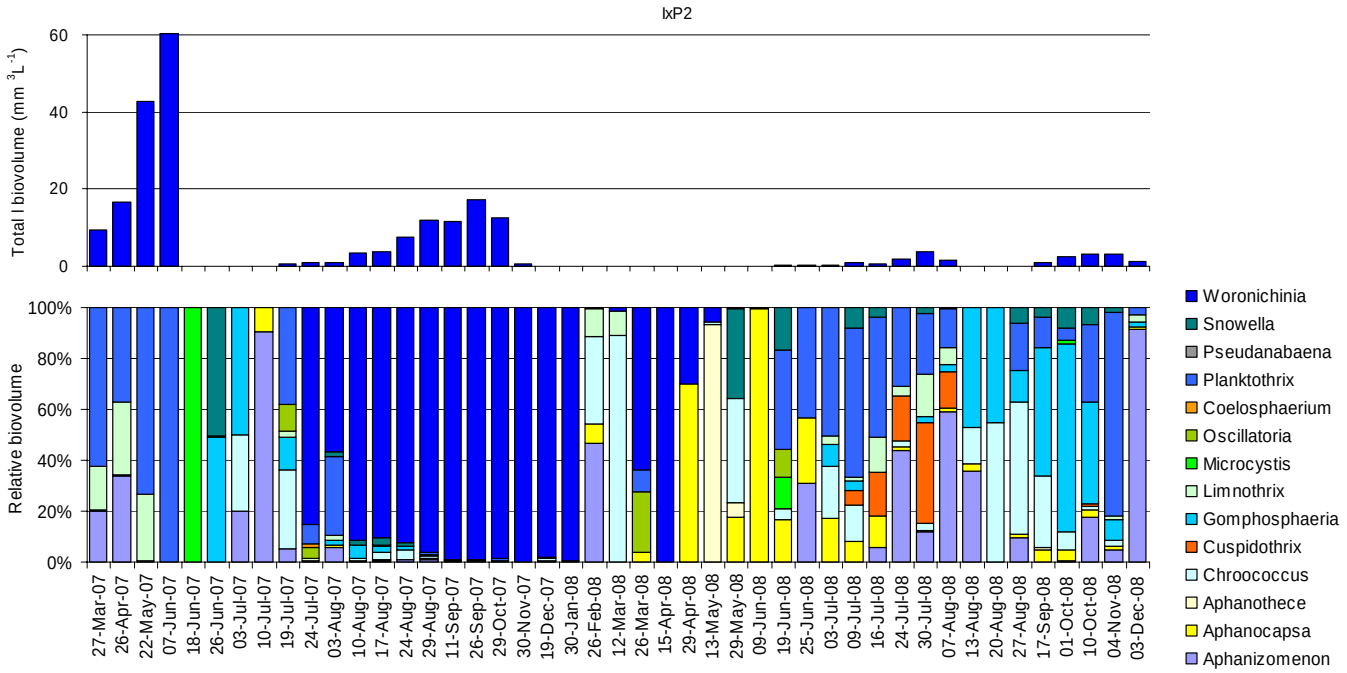



**Figure 7**: Temporal variation in biomass and composition of cyanobacteria; IxP2

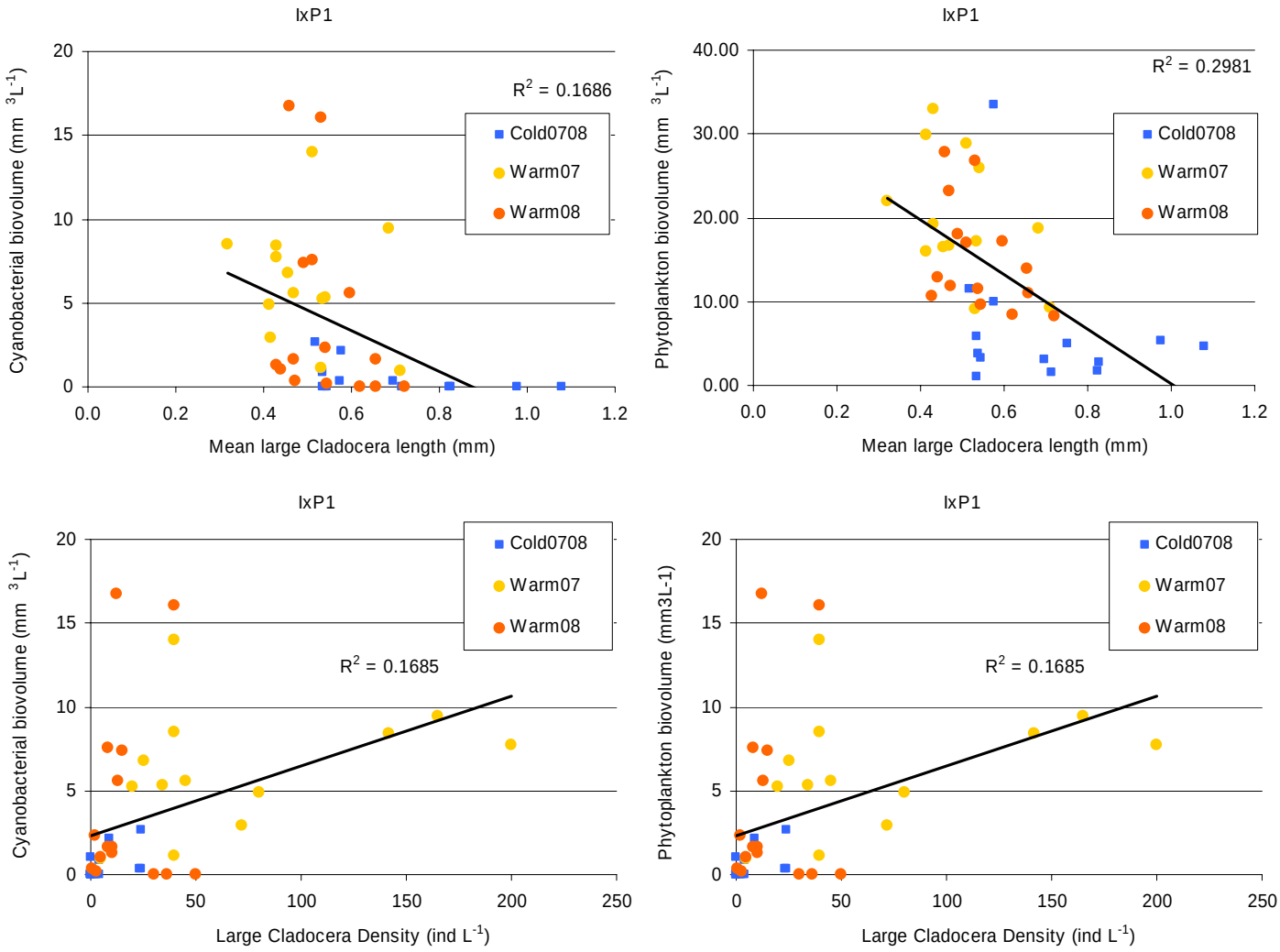



**Figure 8**: Relationships between total phytoplankton and cyanobacterial biovolume and large Cladocerans density and size. The trend lines and $R^2$ are based on all the data used in each graph

These results confirm that cyanobacteria are favoured by elevated pH. In both reference ponds pH was mostly above 8 (mean pH: IxP1-8,3, IxP2-8,4), rising up to 8,6 in IxP1 and 9 in IxP2 during cyanobacterial blooms. This could be explained by better adaptations of cyanobacteria to low $CO_2$ availability characteristic of high pH conditions.

Although very much alike in terms of environmental conditions, the two reference ponds have been very different in terms of nitrogen availability. In IxP1 DIN was generally well above the level at which phytoplankton growth could be slowed down due to nitrogen deficiency (mean DIN = 0,27 mg $L^{-1}$; May - September). Conversely, in IxP2 nitrogen concentrations were below such a level (mean DIN = 0,07 mg $L^{-1}$; May - September). This could probably explain, at least in part, the differences in cyanobacterial composition and biomass between the two reference ponds. Lower depth of IxP2 (1,3 m maximum depth as opposed to 1,8 m in IxP1) could also favour cyanobacteria that are poorly adapted to prolonged shading due to a water mixing more typical of deeper ponds (Huisman et al., 1999). The two reference ponds seem also to differ in hydrology, IxP2 being more affected by the overflows of runoff-water during heavy rains. The slow growing cyanobacteria are more sensitive to flushing than other phytoplankters. This could be the reason why IxP2 showed markedly lower biomass of cyanobacteria in more rainy 2008 than dryer 2007 (Figure 7).

It is considered that winter temperatures play an important role in determining the beginning and duration of cyanobacterial blooms in phosphorus rich ponds (Gulati and van Donk, 2002; Hudnell, 2008). Our results support this idea. After a mild winter 2007, a cyanobacterial bloom dominated by *Planktothrix* spp. was observed already in March in IxP2 and lasted till mid June continuously rising in magnitude (Figure 7). The bloom disappeared abruptly in the second half of June, probably flushed out by an exceptionally heavy rain that occurred between the two samplings. In 2008, after a cold winter, cyanobacteria have built up elevated biomass only in mid summer (Figure 6). Our March 2009 sampling of IxP2 revealed, however, an important *Aphanizomenon* spp. bloom (Secchi depth 0,45 m), following a rather cold winter. This indicates that some cyanobacteria are well adapted to low temperatures. Additional data and more in-depth analysis are necessary in order to elucidate the underlying conditions controlling cyanobacterial bloom occurence in the ponds studied. Continuing monitoring of the reference ponds as well as other ponds prone to cyanobacterial bloom development should allow identification of different cyanobacterial ecotypes and factors controlling their mass development in the ponds studied.

### 1.3. Wallonia

In Lake Falemprise, no large cyanobacteria bloom occurred in 2007. This appears to be related to the poor weather conditions of the summer 2007, which presented a maximum mean weekly air temperature of 18,5 °C, and a maximum water temperature of 21,9 °C on June 16. This contrasts with earlier reports on the same lake (Verniers & Sarmento 2004), which recorded a maximum of 27,2 °C.

The onset of stratification was observed in the beginning of June. The most obvious effect was the depletion of oxygen at 3 m depth on some occasions. Nutrient concentrations were in the same range as observed in earlier studies.

Phytoplankton consisted of cyanobacteria, chlorophytes, diatoms, chrysophytes and euglenophytes. The maximum recorded chlorophyll a concentration (average on the 0-4 m water column) in 2007 was 114.3 $\mu$g $L^{-1}$, on July 31.

Identification and counts of cyanobacteria allowed to estimate relative biovolume of the different taxa, and to combine these estimates with cyanobacteria biomass calculated from the pigment analysis (Figure 9). In 2007 *Aphanizomeno- flos aquae* developed in May - June, and from July to September, *Planktothrix agardhii* became the dominant species. Other taxa were present in the samples: *Microcystis aeruginosa*, *Anabaena* sp. and *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 on, only blooms of *Aphanizomemon flos-aquae* and *Anabaena* sp. were observed, and a few *Microcystis* colonies were recorded.

A multivariate analysis of the Falemprise data, using ACP and CCA, has been carried out. These analyses have shown a strong seasonality in the environmental variables, but some variability in the phytoplankton structure, which may be due the unstable physical structure of the lake, which undergoes summer stratification, which is however easily disrupted by weather changes. However, the CCA clearly identified zooplankton as one of the factors determining composition and biomass of phytoplankton: in particular, the development of the filamentous cyanobacteria dominant in this lake is related to the development of large cladocerans and of calanoid copepods.

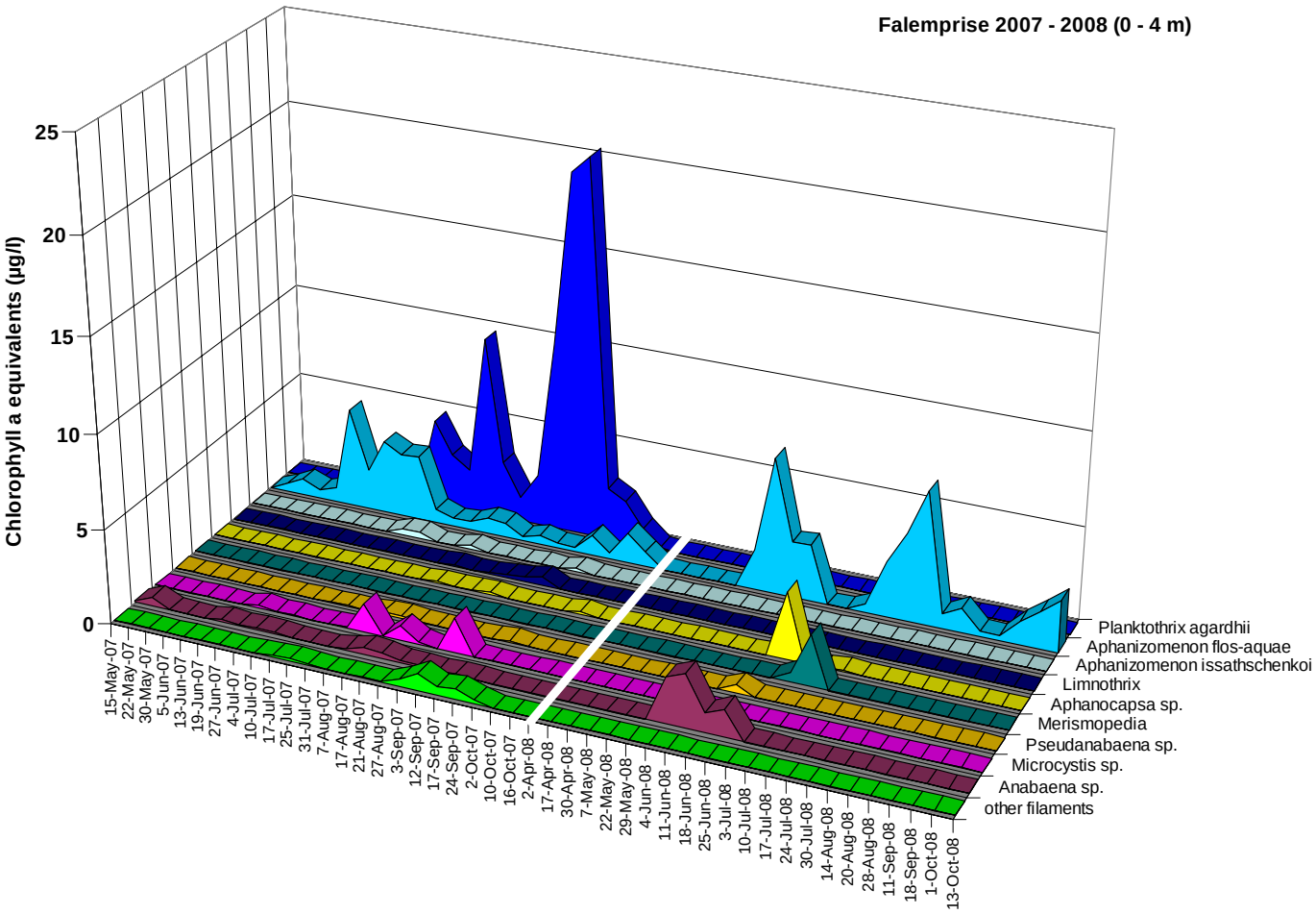



**Figure 9**: Biomass of the different cyanobacteria taxa biomass in Falemprise (2007 and 2008), averaged for the 0 – 4 m samples, expressed as chlorophyll a equivalents.

## 2. Isolation of strains

In Westveld, a total of 10 ITS genotypes were detected during the whole sampling period, although only three genotypes were dominant. In Donkmeer, isolation of strains revealed 7 different ITS genotypes in total, however, several strains possessed two or three different ITS sequences.

## 3. Phytoplankton assessment by HPLC analysis

The results of HPLC analysis of marker pigments are integrated in the results of section 1, for Westveld, Donkmeer and Falemprise (2007). For 2008, all samples from reference lakes and BLOOMNET samples have been analysed with this technique and the results were provided to the partners of Brussels and Flanders, in order to be used in the descriptions of phytoplankton composition.

## 4. Molecular ecology of cyanobacteria

### 4.1. 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 are clustering with

*Aphanizomenon*/*Anabaena* (36.4%) and *Anabaena*/*Aphanizomenon issatchenkoi* (26.1%). During the B-BLOOMS project (2003-2005), clone libraries of bloom samples showed the presence of only one to three genera. This is explained by the biased dominance of one genus during a bloom. Consequently, this result should not be taken as quantitative but as a qualitative result.

About 20 database's sequences belonged to the genus *Microcystis*. These sequences were found in 9 different Belgian lakes, which suggests a large 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 differents lakes and were present in two reference lakes (lake Falemprise and Ixelles pond II). Nevertheless, blooms of *Woronichinia* are observed in Belgium and can be associated with concentrations of microcystins 4 fold higher than in some *Microcystis* blooms (Willame et al. 2005). Picocyanobacteria, *Cyanobium*/*Synechococcus*-like sequences, were recovered from the quarry of Ecaussines, lake Falemprise, lake Ri Jaune, and lake Blaarmeersen. Picocyanobacteria play an important role in the phytoplankton and microbial loop (Becker et al., 2004). In future research, we shall apply quantitative approach to evaluate their contributions in fluxes to integrate these parameters in existing models. Other sequences belonging to clusters of *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 (Jungblut et al., 2006; Cadel-Six et al., 2007).

All databases sequences should be compared with sequences recovered from strains in order to infer their role in toxin production and concentration in Belgian freshwater bodies.

The ARB database is very useful to quickly affiliate sequences of different lengths.

**The 16S rRNA DGGE analysis**

The DGGE analysis carried out on lake Falemprise samples from 2007 showed the presence of *Microcystis*, *Planktothrix*, *Cyanobium*, *Synechococcus*, and *Snowella*. 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 quarry Ecaussinnes. All these sequences were uploaded in the ARB database. Results are summarized in Table 3.

| 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 |

**Table 3.** Affiliation of cyanobacteria based on DGGE band sequences.

### 4.2. Genotypic analyses of toxic and non-toxic cyanobacteria

#### The mcy genes detection strategy and RFLP of mcyE analysis

A total of 102 samples were extracted and tested for presence of mcy genes in the Brussels and Walloon regions between 2007 and 2008.

In 2007, the results showed that the *mcyB* detection by nested PCR was the most efficient to detect potential microcystin-producing *Microcystis* genotypes. All samples from Brussels-Wallonia, but two, were positive (Falemprise 24/09/07 and Ixelles Pond II 01/08/07). A re-extraction of the back-up sample from Ixelles Pond II was also unsuccessful and this could be due to PCR inhibition. *mcyB* was not detected as expected in the Ecaussines quarry (20/07/07) when *Microcystis* was absent, whereas it was detected in Chérapont (29/08/07) during a *Microcystis* bloom. The *mcyE* detection results showed the presence of *mcyE* in 9 Falemprise samples from 27th June to 27th August 2007, and then *mcyE* remained 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. Additional non-specific PCR products were present in several Falemprise samples. Therefore, samples and strains mixture will be tested with different PCR conditions.

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

In Ixelles pond II, the *mcyA* analysis showed 17/20 undoubtedly positive results while 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* was detected in 15/20 samples, but a negative detection were observed in samples from 29th April, 29th May, 25th June, 1st October and 10th October. The first step of the *mcyB* semi-nested amplification was positive for 13/20 samples. Negative results were found

for samples of 26th March, 29th April, 13th May, 29th May, 9th June, 25th June and 3rd July. 100% of the samples were positive after the second step. This suggests that potentially toxic *Microcystis* might have been less abundant in the total population at these dates. Results of the detection of the three markers in Ixelles pond II suggested that the microcystin producing cyanobacteria of Ixelles pond II are composed by *Microcystis* but also by other genera.

In Ixelles pond I, *mcyA* amplifications were observed in18/19 samples, 14/19 were positive for the first *mcyB* amplification and 19/19 after the second *mcyB* PCR. Negative reactions of the first *mcyB* detection were observed for samples 12th March, 15th April, 29th May, 9th June and 19th June. The *mcyE* amplification was observed in 17/19 samples. Negative amplification happened on the 12th March, 19th June and 7th August 2008 samples.

In general, the mcy gene analysis showed the presence of potentially toxic *Microcystis* in the majority of samples.

RFLP of *mcyE* was carried on samples from 9 filtered samples of Ixelles Pond II from June to September 2007. The analysis suggested the coexistence of two different *mcyE* genotypes of *Microcystis*. The same pattern was observed in Ixelles pond I/II 2008 and Chérapont 2007 samples. In addition, potential microcystin-producing *Planktothrix* were clearly identified in the quarry of Ecaussines (2007-2008).

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

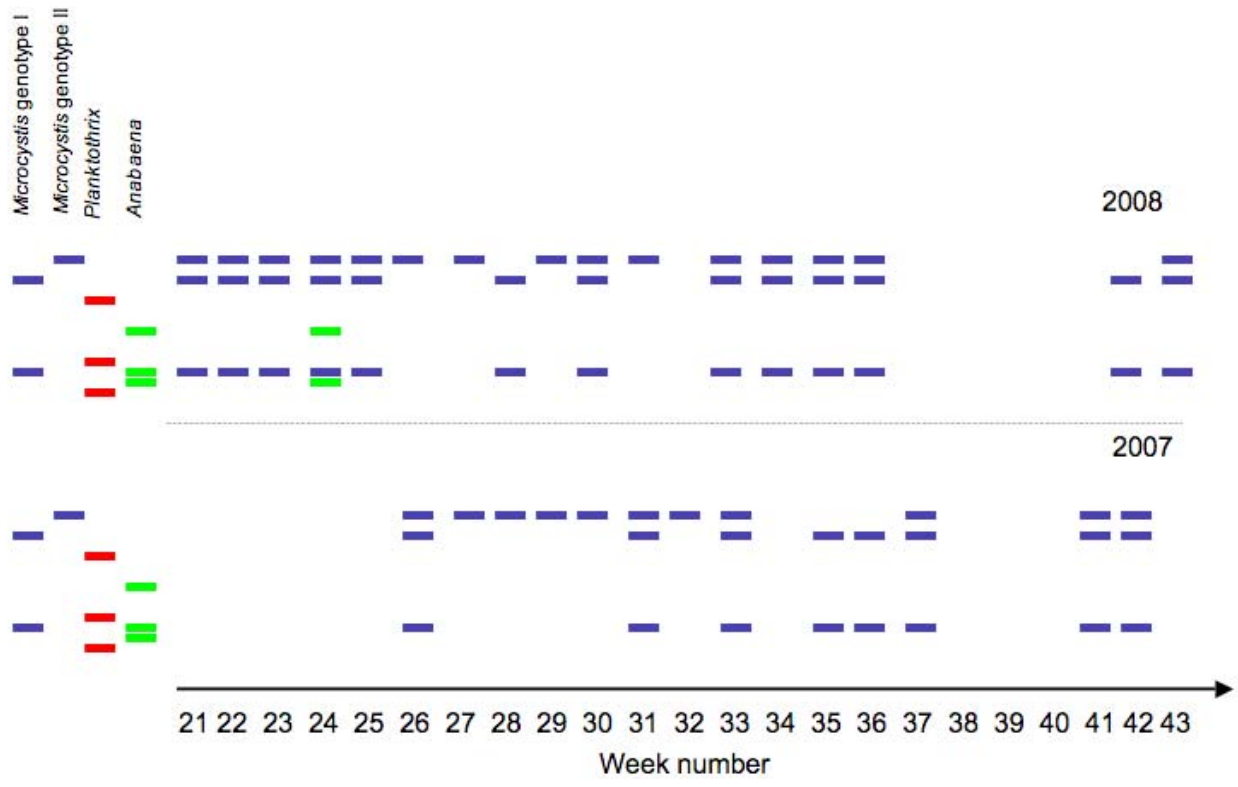



**Figure 10**: RFLP of mcyE analysis in lake Falemprise (2007-2008).

Two *mcyE* genotypes of *Microcystis* are present in the majority of our samples. To be certain that the dominance of *Microcystis* genotypes in our samples are not due to biases in the PCR reactions, we would like to verify if our PCR RFLP analysis is efficient with templates containing known mixtures of different MC producers. In the meantime, Real Time quantitative PCR will be

used to draw the dynamics of toxic genotypes during the bloom periods. Cyanobacterial strains' characterization will also give more information about the toxicity of Belgian cyanobacteria.

**The ITS genotypes analysis**

A preliminary alignment of ITS sequences of the clones agreed well with the DGGE data, as it showed a very high within-lake diversity.

In contrast to the high diversity in ITS within and among lakes for the *Microcystis* strains, all the *Planktothrix rubescens* strains isolated from Driekoningenvijver were identical. 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 Driekoningen, the single *Planktothrix* ITS genotype present was also positive. The three main ITS types present in Westveld as revealed by the ITS rDNA DGGE were all identified as potential microcystin-producers. For the strains isolated from Donkmeer in 2007, 10 of the 29 isolated strains contained *mcyA* genes.

The ITS diversity of 62 BLOOMNET samples from the Flanders region (*Microcystis* blooms: 32, *Planktothrix* blooms: 24, mixed blooms: 6) obtained from B-BLOOMS (2003-2005) was studied by 16S-23S ITS DGGE. The DGGE profiles revealed a high ITS diversity of *Microcystis* (most bloom samples contained several ITS genotypes). For *Planktothrix*, a very low ITS diversity was found (most bloom samples contained only one or two ITS genotypes).

In Westveld, a total of 10 ITS genotypes were detected during the whole sampling period, although only three genotypes 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 is clarified with the isolates of 2008 for which the original colony morphology was noted. Further analysis will be given with phase 2.

In Donkmeer, there were few changes through time. Three ITS genotypes were dominant, but in accordance to the isolated strains they probably belong to only one or two strains. This has to be clarified further.

**The Single-colony approach**

For the first time, we were able to amplify and sequence more than one locus of the genome of eleven single colonies of *Microcystis* from four different ponds. Up to now, the WGA approach was efficient for 70% of the tested colonies. 12/21 PCR reactions were carried out with success on a colony. Positive amplifications were obtained for rRNA-ITS, 16S rRNA, *rpoC1*, *recA*, *gyrB*, *rbcLX*, and *gvpA*. Detection of the microcystin synthetase operon was possible with *mcyA*, *mcyB*, and *mcyE*. *Microcystis* colony X8 corresponded to a *Microcystis ichthyoblabe* morphotype and belongs to the genus *Microcystis* based on the 16S rRNA sequence. 16S DGGE and rRNA-ITS DGGE gave respectively one band for 16S and two bands for ITS, suggesting that there are different sequences of ITS in one colony, as previously observed in Janse et al., 2004.

In addition, we have obtained the first sequences of *rpoC1, rbcLX* and rRNA-ITS from a single colony of the genus *Woronichinia* (identified by microscopy). The information is particularly interesting as Woronichinia is very difficult to isolate and keep in culture. Therefore, little data is available in Genbank.

Preliminary analysis of 16S rRNA and ITS sequences of 5 single-colonies showed the occurrence of three distincts genotypes of *Woronichinia* in Tervuren pond during the same bloom.

In conclusion, this approach allows working with a small amount of DNA, and represents a concrete answer to the lack of data on non or hardly cultivable cyanobacteria. In the future, we plan to couple our approach with ELISA analysis and chemotyping by MS (MALDI TOF). In future research, we would like to characterize *Microcystis* populations with this approach.

## 5. Determination of toxin contents

178 phytoplankton samples for microcystin analysis were received by UniDun in 2007.

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

- Wallonia: Lake Falemprise (samples from 2007 and 2008): 0.120 – 6.110 $\mu$g $L^{-1}$; Lake Chérapont: 0.524 $\mu$g $L^{-1}$; Ecaussines quarry: 7.892 $\mu$g $L^{-1}$
- Brussels: Ixelles Ponds I and II (samples from from 2007 and 2008): 0.210 - 1106 $\mu$g $L^{-1}$
- Flanders: Westveld (samples from 2007 and 2008): 0.77 - 250.7 $\mu$g $L^{-1}$; Donkmeer (samples from 2007 and 2008): 0.260 – 5.740 $\mu$g $L^{-1}$; Parkvijver Kraaimem: 0.478 $\mu$g $L^{-1}$; Spaarbekkem AWW: 49.06 $\mu$g $L^{-1}$; Schulensmeer: 7.458 $\mu$g $L^{-1}$; Kessemich: 75.22 $\mu$g $L^{-1}$.

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 is 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* have been isolated from Donkmeer and Falemprise. One series of *Anabaena* coiled filaments was isolated from Donkmeer.

120 individual colonies and filaments have been analysed: some analysis is still in progress. In some cases where samples were not received within a few days of sample collection at the lakes, microscopic evidence of colony and filament lysis was evident. It was not possible to isolate single colonies and filaments from these samples.

Microcystin quotas have so far been determined for 51 of the single colony/filament samples. *Microcystis* colony areas have also been determined to permit determination of microcystin quota per cell to be determined. *Planktothri*x filament length and width measurements will also permit 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 plastic ware but attempts will continue.

The quotas so far measured range from 45.4 to 1620 picograms per colony or filament. This range indicates diversity in microcystin quota, probably extending to per unit biovolume and is consistent with the notion of a single cell quota in the femtogram range (Orr and Jones, 1998; Akcaalan et al. 2006).

Wide variations versus sample date are being found in the compartmentation of microcystin between soluble and particulate phases. For example, at Donkmeer this ranged from 26% to 100% soluble microcystin (Figure 11). 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 11). This was the period when the Donkmeer cyanobacterial bloom was largely dominated by *Anabaena sp.* (Figure 1). From early July until late October, when the Donkmeer bloom was dominated by *Planktothrix* sp. (Figure 1), 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 8). 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 carried out.

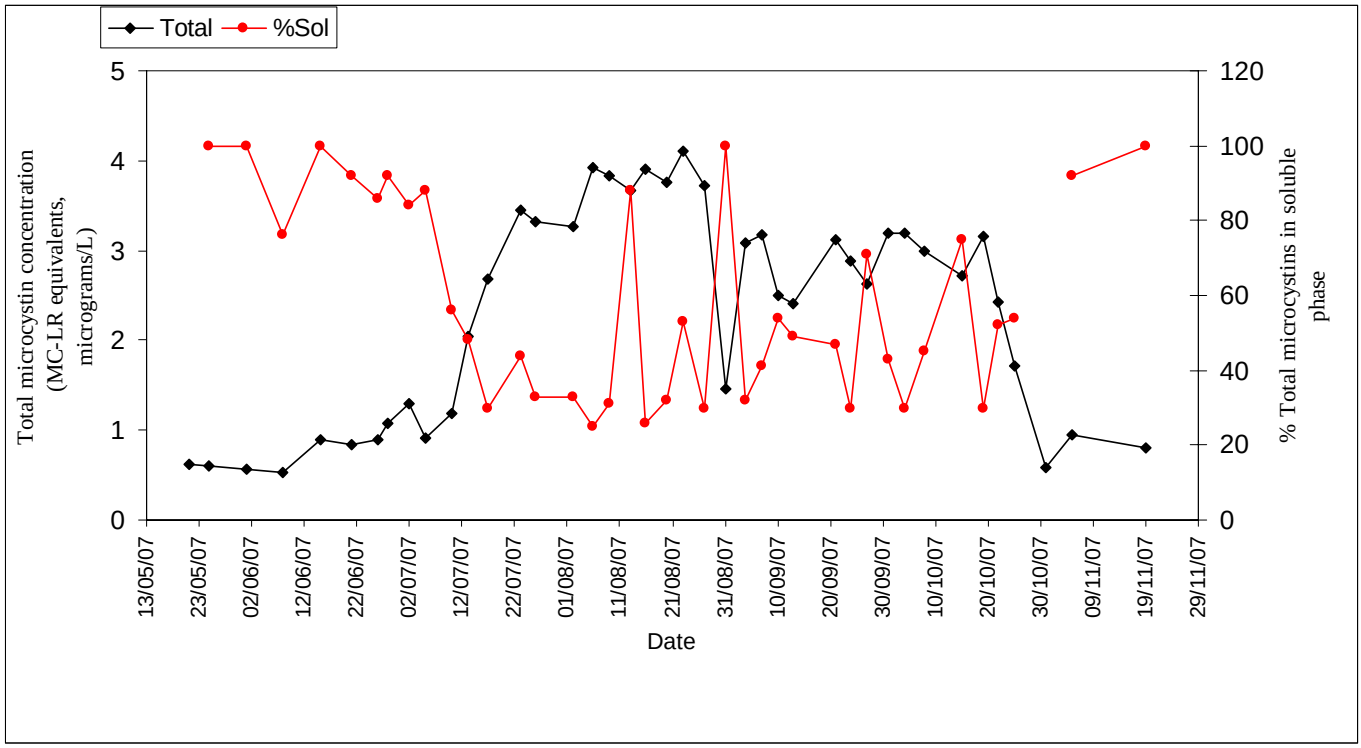



**Figure 11**: Total microcystin concentrations (determined by ELISA) and % total microcystin pool in soluble (dissolved) phase, for Donkmeer, 2007. Each point is the mean of triplicate determinations.

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 only a summary focusing of total microcystin concentration in water of the B-BLOOMS reference lakes where samples were analysed throughout 2007 an 2008 ( Figure 12), i.e. Falemprise in Wallonia and Donkmeer and Westveld in Flanders. Microcystin was detected in all samples, and many samples were well above the WHO guideline value of 1 $\mu$g $L^{-1}$. This occurred for a few summer samples in Falemprise, and for more samples in Donkmeer. These two lakes presented the same range of microcystin concentration, whereas Westveld had much higher microcystin concentration. In this lake, all measurements were above the WHO guideline. The highest concentration was measured in the Ixelles pond I during summer 2007 when the concentration in the Ixelles pond II was high as well. However, in 2008, Brussels reference ponds concentrations never reached the WHO guideline value.

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. UnivDun also produced and quantified purified microcystin-LR and anatoxin-a standards and provided analytical protocols for University of Namur for analytical methods development. HPLC analysis of anatoxin-a was developed at University of Namur. Several methods and HPLC column were tested and applied to several bloom samples but anatoxins were not detected so far.

In addition to the program, zooplankton samples from Donkmeer and Westveld, containing small cladocerans and rotifers (*Bosmina*, *Brachionus*, unidentified ciliates, *Keratella*, *Polyarthra*) were analysed for microcystins: although a minor proportion of the samples had microcystin content below detection limits, analysis yielded a range of microcystin-LR equivalents from 23.4 to 118.4 picograms per animal. These are the first findings of microcystins (and/or microcystin detoxication products) in these freshwater animals. Similar analyses have to be repeated in order to assess the risk of transfer of microscystins in the lakes' foodweb.

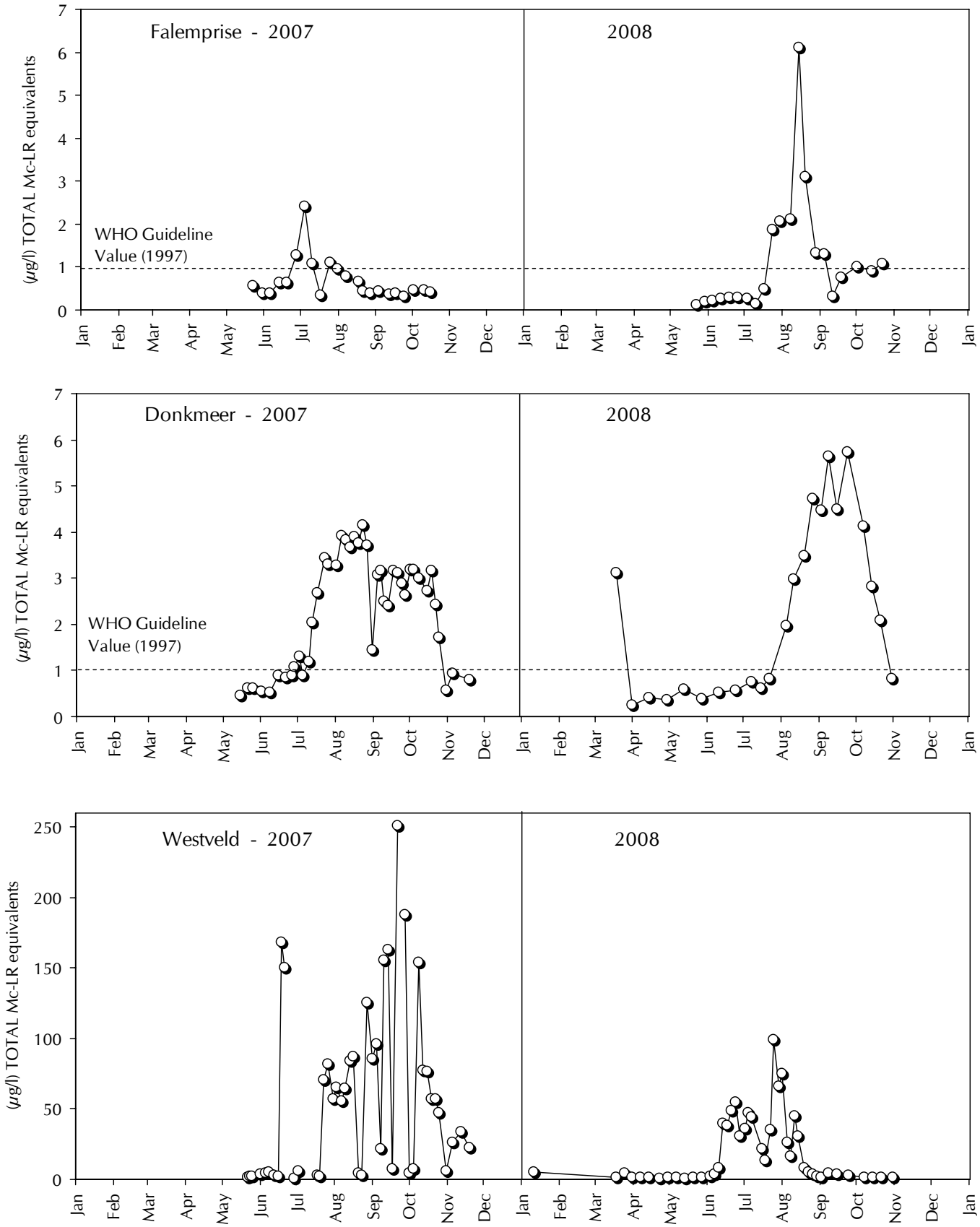



**Figure 12:** Total microcystin concentrations (determined by ELISA) for Falemprise, Donkmeer and Westveld in 2007 and 2008.

# 6. Deterministic watershed simulation modelling of Eau d'Heure Lakes

## 6.1. Deterministic and "physically based" simulations of phytoplankton in rivers and lakes

The CEME (Centre d'Etude et de Modélisation de l'Environnement) of the University of Liège (ULg) has been involved in modelling of phytoplankton in rivers since more than 20 years. These activities are now done by the Aquapole of the ULg.

The main work done during these 20 years is the following :

- first simulations of (global) phytoplankton in 1980's on the Meuse river ;
- 1990-1998 : development of a multi-species phytoplankton model : the POTAMON model (for "large rivers" : Meuse, Moselle, ...) ;
- 1999-2001 : inclusion of the POTAMON model in the integrated river/basin PEGASE model ;
- 2001-2003 : test of using the PEGASE model to simulate phytoplankton concentration in the Lakes of Eau d'Heure
- 2001-2003 : test of simulation of cyanobacteria in the Lakes of Eau d'Heure (with an "extension" of the POTAMON/PEGASE models)
- 2007-2008 : use of the existing model (with new calibration) on the Falemprise lake to simulate cyanobacteria (B-BLOOMS2)

## 6.2. The deterministic cyanobacteria model

The cyanobacteria used in this study is based on the models developed by the Aquapôle in the 20 last years (see the study : " Schéma directeur intégré pour la préservation de la qualité de l'eau et la valorisation écologique des lacs de l'Eau d'Heure dans le cadre du développement touristique et économique du site » done for INTERSUD).

### The PEGASE model

The PEGASE (Planification Et Gestion de l'ASsainissement des Eaux - Planning and management of water purification) model is devoted to the characterization of the environmental state of surface water, at the scale of a whole watershed, basin and district. The model has been used operationally in several countries like France and Benelux, but several calculations have also been performed at international level for the international commissions in charge of the Scheldt and the Meuse.

PEGASE model requires as input, at least data like digital terrain models, water flow, water level and other hydrodynamic measurements in some geo-localized points, diffuse loads properties, ecological model data (to characterize bacteria, phytoplankton, zooplankton, macrophytes, shells, etc...), chemical discharges (from industry and cities) and treatment plant effect. The output of the software consists in tables, graphs and maps, showing the state of various calculated parameters of quality (DO, BOD, COD, N, P, Chla,...) either at a specified time, along the river, or the temporal evolution at a given point. It provides printed maps or animations of the evolution of the results on the basin.

The software is able to simulate non stationary scenarios from a local scale, up to a global scale i.e. a few km² to hundreds of thousands km² and handles the entire river tree (hundreds to thousands of rivers). The model includes a hydrological and hydrodynamic sub-model, a thermal sub-model, a sub-model dedicated to releases, and a sub-model dedicated to calculate the water quality and to explicitly describe the aquatic ecosystem mechanisms.

The PEGASE model includes since year 2002 the POTAMON model (Everbecq et al., 2001), which simulates dynamics of different phytoplankton categories.

**The "Eau d'heure" model**

The existing model of the Eau d'Heure basin has been developed in the framework of a partnership between the public Water operator INTERSUD / IGRETEC and two universities (FUNDP and ULg), in connection with local and regional authorities.

The model 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 on soil and in water. The coupled model then represents the main quantities and fluxes in the global system, as well as quality variables. Further developments of the model have dealt with the representation of the phytoplankton to include specifically the cyanobacteria. The model allows running simulations of various management scenarios.

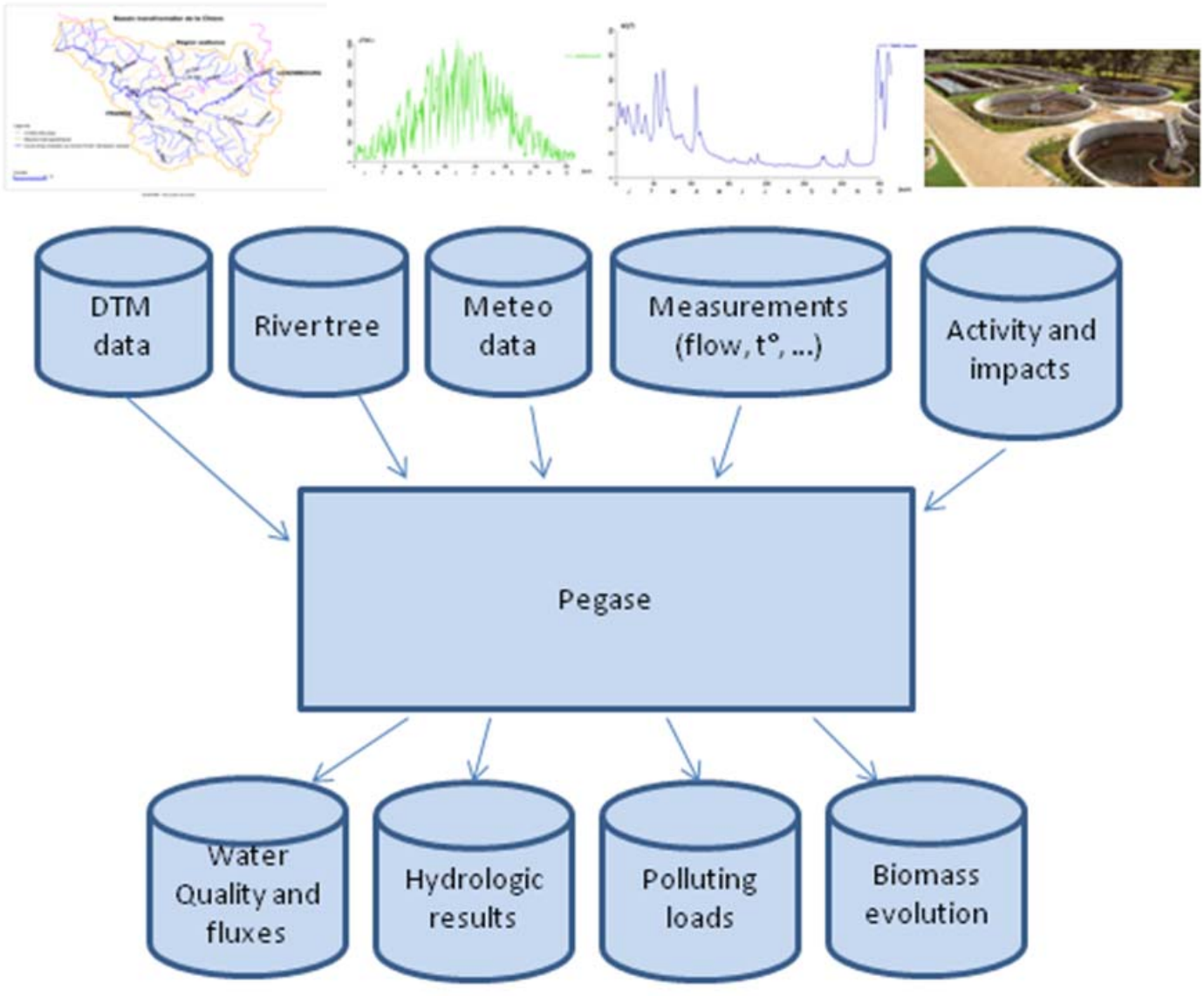



**Figure 13**: PEGASE data flow.

**The "B-BLOOMS 2" model**

In the framework of the B-BLOOMS2 project, the model has been improved thanks to the data on lake monitoring (including those from B-BLOOMS 1). The model used in this study to

simulate cyanobacteria in lake Falemprise is thus based on the POTAMON and PEGASE models, with enhancements to take into account specificities of lakes and cyanobacteria.

### 6.3. Simulations

Simulations of phytoplankton (and more specifically cyanobacteria) were made on the "Eau d'Heure lakes" Féronval and Falemprise (Figure 14) between 2001 and 2003. The model was able to represent cyanobacteria for this type of lake (Figure 15 and 16).

In this B-BLOOMS2 project, it was planned to use the model applied to Eau d'Heure lakes (which includes Falemprise, the lake monitored in Wallonia) to :

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

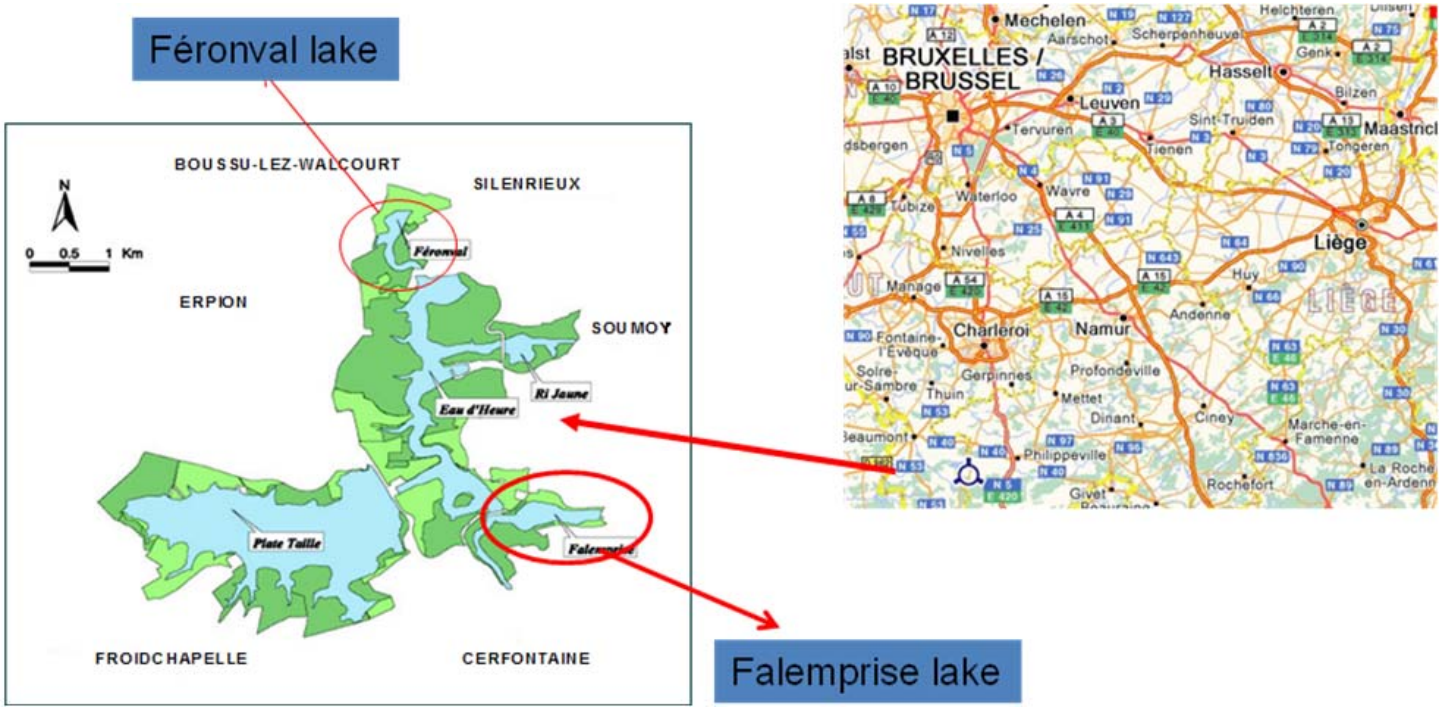
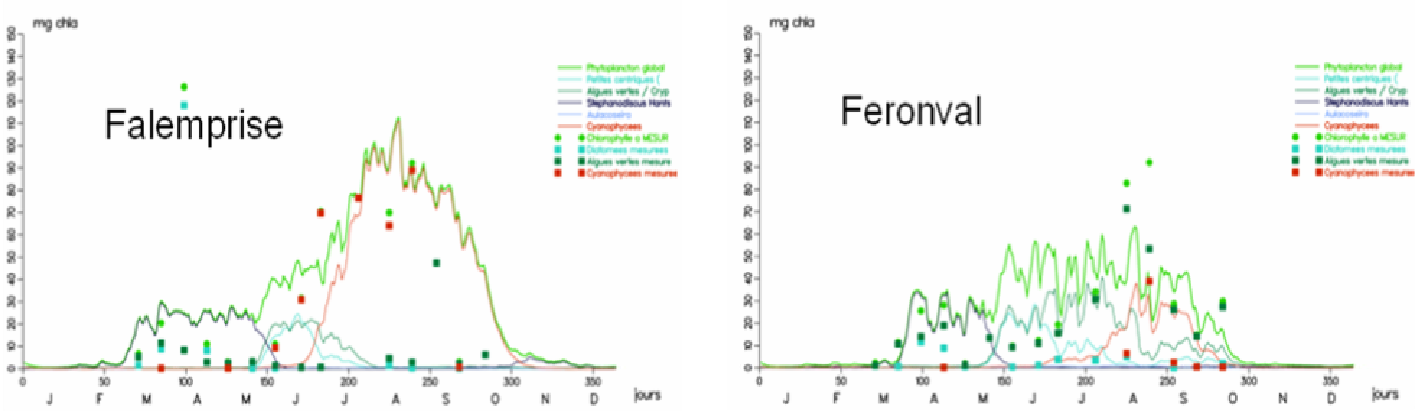



**Figure 14**: The Eau d'Heure Lakes.




**Figures 15 & 16**: Annual simulations of cyanobacteria in the Falemprise and Feronval lakes for the year 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 6 measurements 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/m².day) at the IRM Dourbes Station
- Calculation of semi-hourly values for each day

Determination of pollution inputs (punctual and diffuse loads): urban releases : 2 purification plants + sewers systems; diffuse loads : use of semi-statistic input functions

In the watershed of lake Falemprise, two sewage treatment plants are operating (Figure 17) :

- 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 year 2015 are planned to be 90% at Soumoy and 82% at Senzeille.

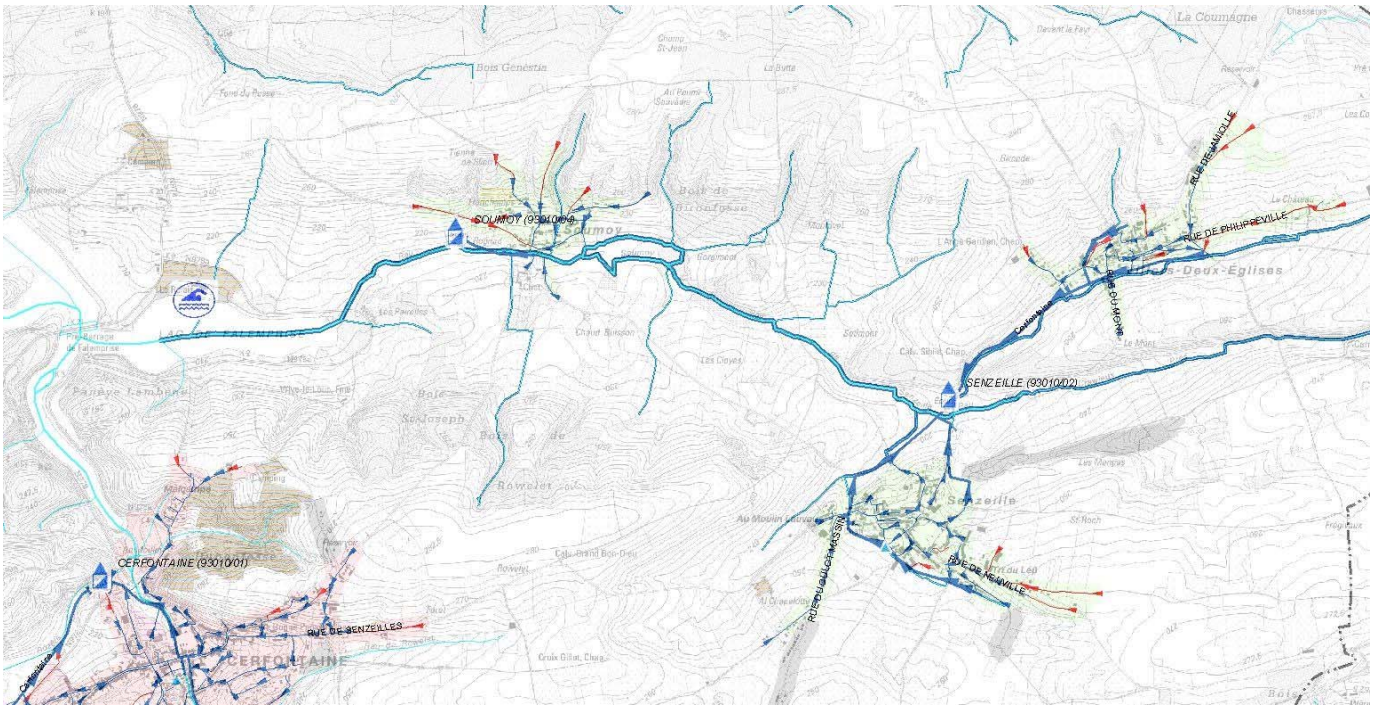



**Figure 17** : Sewage treatment plants and sewage networks in the Falemprise lake basin.

Figure 18 shows the results of a simulation made for the year 2007, with the parametrizations used for the 2002 simulations. We can see that cyanobacteria simulated in Lake Falemprise (red lines) were much greater that the measured concentration (red markers). Presumably, this resulted from a bad calibration of the phosphorus dependence of cyanobacteria, not detected in the 2002 simulations, as P inputs were greater at that time, when the Senzeille plant was not working yet. Therefore, P kinetics of cyanobacteria were modified and new simulations were run with this revised calibration (Figures 19 and 20).

The simulations show that the validation for the year 2007 is much better, while the validation for the year 2002 is still consistent. Additional simulations were run, Based on a the P inputs

expected for the year 2015, assuming the same weather conditions as in 2002 and 2007 (in particular, for irradiance and temperature).

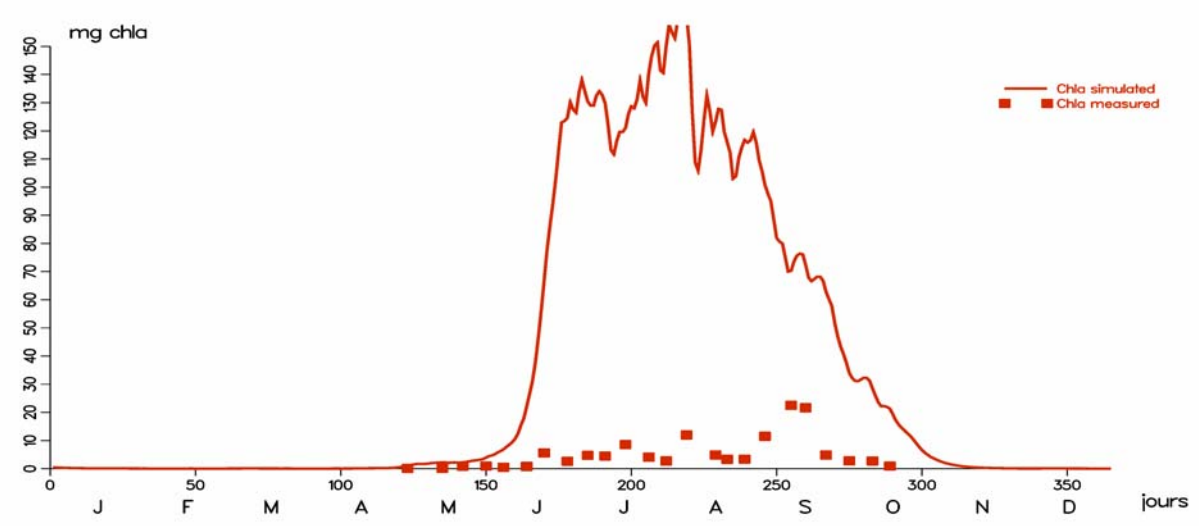
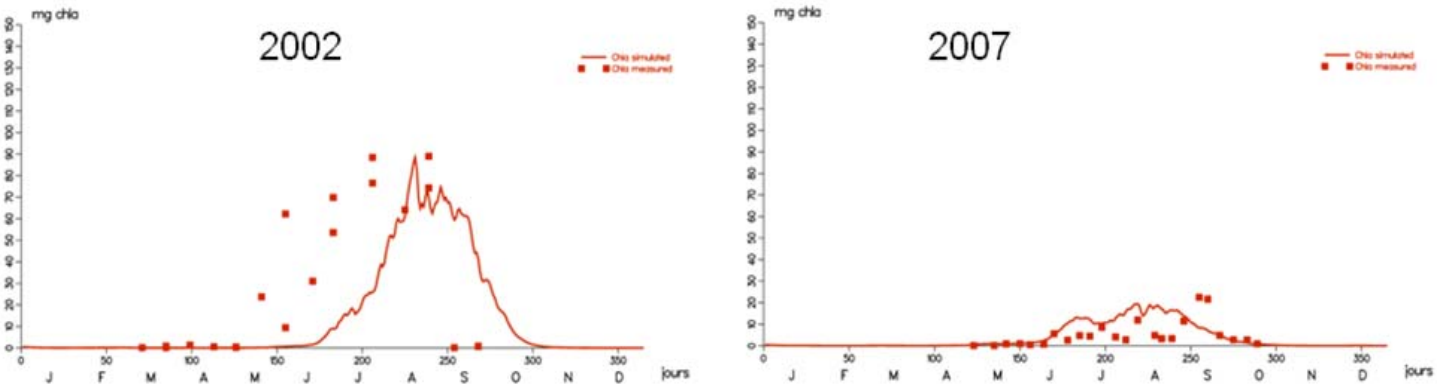
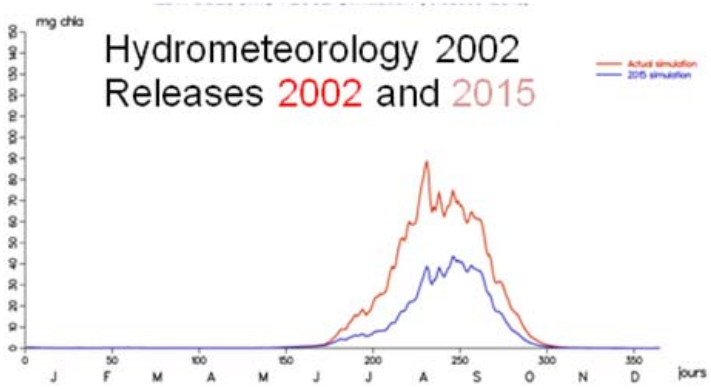


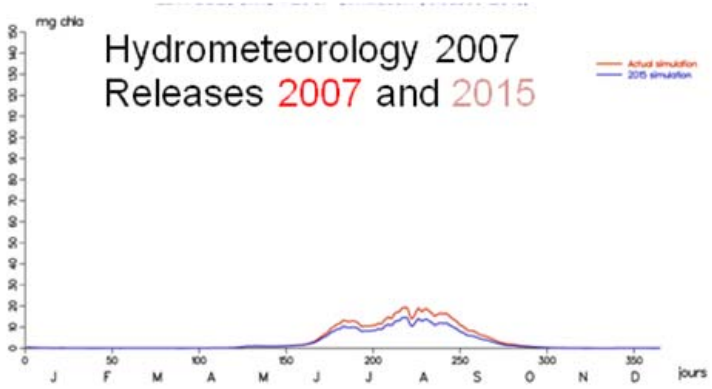

**Figure 18**: 2007 simulations of cyanobacteria in the Falemprise lake with 2002 calibration.




**Figures 19 & 20**: 2002 and 2007 simulations of cyanobacteria in the Falemprise lake with new parametrization of phosphorus kinetics.

The simulations with this scenario allowed prediction of a decrease in cyanobacteria concentrations (Figures 20 & 21), mainly for the « 2002 » situation for which the expected P reduction in 2015 is comparatively much higher than for the 2007 situation.




**Figures 20 & 21** : 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 silmulate cyanobacterial blooms in lake Falemprise, and it can be used as predictive tool for future sewage treatment scenarios. However, improvements are still needed in order to fully adapt the model to lakes deep enough to develop summer stratification. Obviously, a complete representation of the lake vertical structure should be achieved, with al least two layers; a vertical migration sub-model for cyanobacteria should be developed, and the sedimentation-resuspension processes should be represented.

## 7. Predictive models

The data from the 22 Brussels ponds sampled in 2007 and 33 ponds sampled in 2008 were combined with the data from the same or other Brussels ponds acquired between 2003 – 2006 according to the same methodology into a single data matrix comprising 48 ponds sampled on 482 occasions for the development of a statistical predictive model. First, linear relationships between different phytoplankton groups (divisions) and environmental variables were investigated by multivariate statistical method (Figure 22). RDA results showed that pH, submerged vegetation cover large Cladocerans length and density and SRP and DIN had significant relationship with phytoplankton biomass and explained the greater part of the variation in the phytoplankton data (Table 4). Significant negative relationship of dissolved nutrients (SRP, DIN) with the phytoplankton data indicates phytoplankton control of nutrients rather than the other way around. 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 $NL^{-1}$.

**Table 4**. RDA forward selection results. Marginal effects show the variance explained by each environmental variable alone (Lambda1); conditional effects show the significance of the addition of a given variable (P) and the variance explained by the selected variables at the time they were included in the model (LambdaA).

| Marginal effects | | Conditional effects | | |
|---|---|---|---|---|
| Variable | Lambda1 | Variable | LambdaA | *p* |
| pH | 0.17 | pH | 0.17 | 0.002 |
| LCL | 0.16 | SV | 0.13 | 0.002 |
| SV | 0.15 | LCL | 0.03 | 0.002 |
| T | 0.06 | SRP | 0.03 | 0.012 |
| LCD | 0.03 | MD | 0.02 | 0.016 |
| DIN | 0.03 | DIN | 0.02 | 0.022 |
| SRP | 0.02 | LCD | 0.01 | 0.034 |
| MD | 0.02 | RT | 0.01 | 0.200 |
| RT | 0.01 | T | 0.01 | 0.466 |

Cyanobacteria are separated from most of the other phytoplankton groups on the RDA triplot (Figure 22). This suggests that they have different environmental adaptations than most of the other phytoplankters. Generally, only euglenophytes and dinophytes were abundant in ponds dominated by the bloom-forming cyanobacteria. This is supported by the RDA results; Euglenophyta and Dinophyta arrows are located close to that of cyanobacteria.

Cyanobacteria showed negative relationship with large Cladocerans length ($r = -0.38$) and submerged vegetation ($r = -0.30$) and positive relationship with pH ($r = 0.78$) and temperature ($r = 0.27$) thus supporting the idea that the former inhibit and the latter favour cyanobacterial growth.

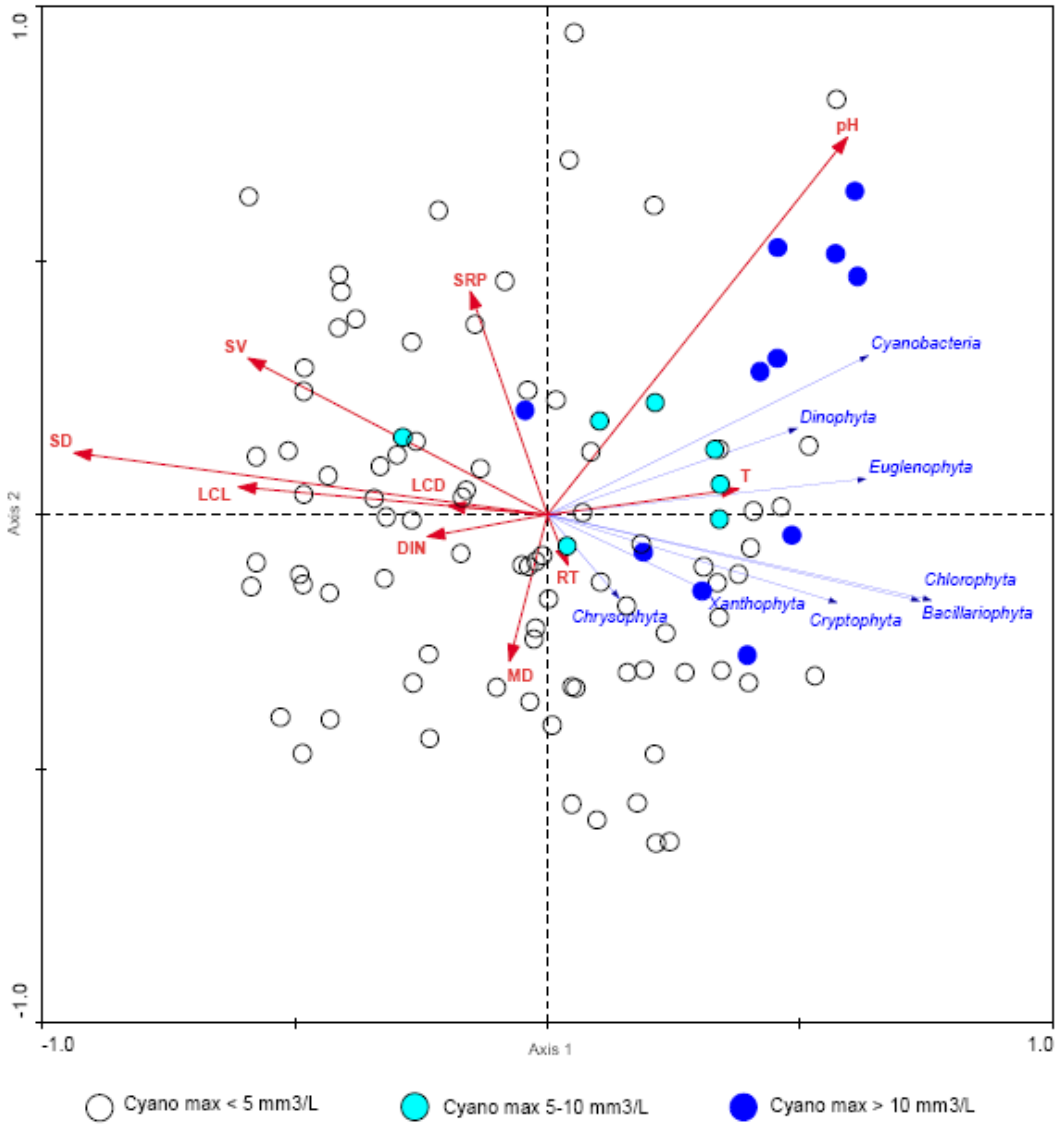



**Figure 22**: RDA triplot (phytoplankton divisions, sites and environmental variables) based on averaged per year 2005-2008 data from 48 Brussels ponds. Phytoplankton biovolumes are aggregated to division level; site symbols colored according to the maximum cyanobacterial biovolume. LCL – large Cladocerans length, SV – % submerged vegetation cover, T – temperature, SRP – soluble reactive phosphorus, LCD – large Cladocerans density, MD – maximum depth, RT – hydraulic retention time.

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 threshold rather than linear nature (Figure 23).

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

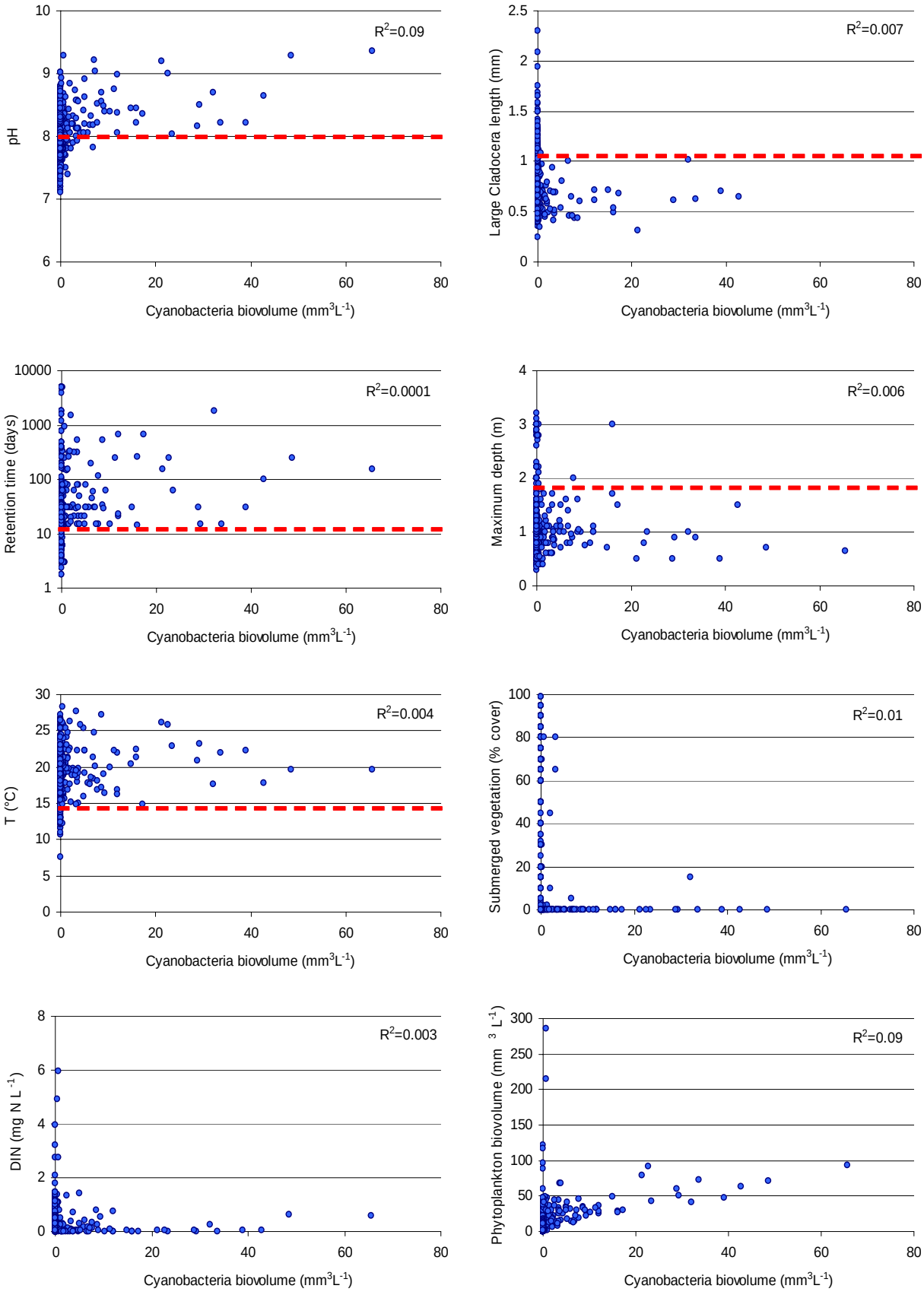



**Figure 23**: Scatter plots based on 2003-2008 data from 48 Brussels ponds showing relationships between cyanobacteria and environmental variables; dashed lines indicate thresholds in the distribution of cyanobacterial biomass along a given gradient.

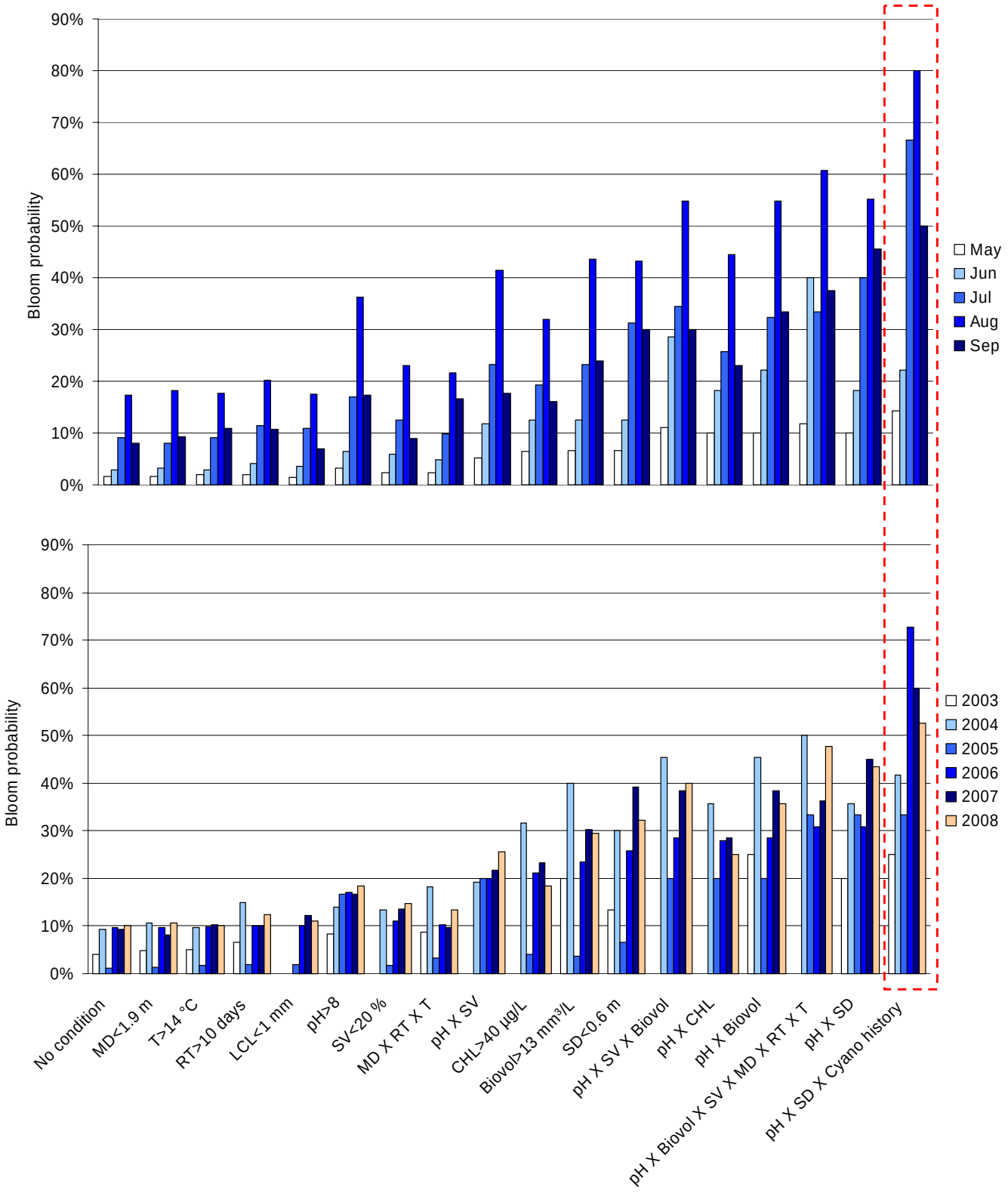



**Figure 24**: Seasonal and inter-annual variations in the unconditional and conditional probabilities of cyanobacterial bloom (5 $mm^3$ of cyanobacteria) occurrence in the ponds studied. Higher probability values indicate higher predictive capacity of a given condition. Red rectangle indicates the probability of bloom occurrence in ponds with the history of cyanobacterial blooms given the conditions that pH > 8 and SD < 0.6.

The probability value is 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 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 pH and Secchi depth used as conditions for probability calculation rendered probability values that are not significantly different from the latter (Wilcoxon matched pares test). This implies that the large number of variables difficult to measure might be substituted by a few easily measured variables for the rapid assessment of cyanobacterial bloom risk.

The probability values and thus predictive capacity of the model markedly increased when only ponds with the history of cyanobacterial blooms were taken into account (dash-line frame; Figure 8). It shows that the probability of a bloom occurrence in a Brussels pond previously affected by cyanobacteria is the highest in August (80%).

This approach permits to identify the ponds prone to cyanobacterial bloom development and thus can help managers to focus their monitoring program on the most problematic ponds. Such ponds can be more frequently monitored during the time of elevated risk of bloom occurrence 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.

# CONCLUSIONS AND RECOMMENDATIONS

Several key results were obtained during phase 1 of the B-BLOOMS2 project, and the main objectives were met. In this phase, the project is mainly based on collecting blooms samples throughout 2007 and 2008, both from reference lakes and from other water bodies through BLOOMNET network involving water managers and users in the project.

The three teams involved in regular field sampling (FUNDP, UGent and VUB) have 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 exploitation of BLOOMNET, the network of water-users and managers, further expands 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 in a standardised way and entered in 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 remains to exploit the data and correlate environmental, taxonomical, genetic and toxicity information. So far, the B-BLOOMS database contains all data collected in 2007 and 2008 in the reference lakes, and some additional data from BLOOMNET and parallel studies. The processing with the final data analysis could yield prediction models, using adequate, presumably non-linear, modelling techniques. 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 urban lakes.

In Flanders, both reference lakes suffered heavily from cyanobacterial blooms, but with contrasting bloom dynamics and species. The intensive sampling at both the cyanobacterial community and population level (both selectively neutral and toxin genes) allows 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 genotypic composition in Westveld 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 Cladocerans 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, 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 with nutrient level reduction, because when nutrient loadings are too high, biomanipulation might not bring the desired results.

In Wallonia, the survey of lake Falemprise, which was followed by the Namur team since 2002 in the framework of other programs, is of particular interest as it is an official swimming 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 to the most recent European directive on bathing waters, which includes the need for monitoring cyanobacterial proliferation and identifying health risks, and for taking adequate measures to

prevent exposure of the public. According to our survey, cyanobacteria blooms regularly occurred in lake Falemprise in the period 2002-2008. These blooms are dominated by *Aphanizomenon flos-aquae*, but other potentially toxic taxa occur, such as *Microscystis aeruginosa* and *Planktothrix agardhii*. The presence and the potential toxicity of these taxa has been confirmed by the genetic analyses, and microcystin was detected at levels above the WHO guideline value in 2007 and 2008. 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 to the EC directive.

A major result of this project, so far, 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 water bodies sampled in 2007 and 2008, 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. Understanding the processes behind toxin production and their dependence on environment and genetic diversity is also a key component of the project, which will be tackled with the data gathered during phase 1 of the B-BLOOMS2 project.

Similarly, toxin analysis of the BLOOMNET samples collected in Flanders showed that cyanobacterial toxin concentrations can be high enough to require risk management and possible intervention. Temporary closures of recreational waterbodies in 2007 occurred in 'Schulensmeer', 'de Gavers' and 'Dongelberg' where fish mass mortalities also occurred, although a direct link with cyanobacterial toxins was not established. In Wallonia, detection of cyanotoxins was also achived in a few cases thanks to BLOOMNET. An example is the development of a toxic *Planktothrix* population at depth in the Ecaussines quarry exploited by VIVAQUA provides a reminder that blooms can go undetected if they do not occur at the water surface, and yet can result in potential toxicity in the water.

The second phase of the B-BLOOMS2 project, started in 2009, focuses even more on practical and science policy objectives: developing BLOOMNET, transferring knowledge to the official laboratories in charge of water quality control, and improving information and awareness of the authorities and the general population.

# ACKNOWLEDGEMENTS

The team involved in B-BLOOMS2 wish to express their thanks to the members of the users committee and to BLOOMNET active members, such as VMM, ISSeP and IBGE.BIM, who provided information, feed-back and an adequate framework for the collection of cyanobacteria blooms.

We also thank Ralitsa Dimitrova and Maya P. Stoyneva for the microscopic phytoplankton examination of Lake Falemprise samples.

# REFERENCES

Akcaalan R., Young F. M., Metcalf J. S., Morrison L. F., Albay M. and Codd G. A. (2006). Microcystin analysis in single filaments of Planktothrix spp. In laboratory cultures and environmental blooms. *Water Research* 44(8):1583-1590.

Becker S., Kumar Singh, A., Christine Postius, C., Böger, P., and Ernst A. (2004) Genetic diversity and distribution of periphytic *Synechococcus spp.* in biofilms and picoplankton of Lake Constance FEMS *Microbiology Ecology* 49(2):181-190.

Bell, S. G. and G. A.Codd (1996). Cyanobacterial toxins and human health. *Rev. Med. Microbiol.* 5:256-264.

Benndorf J., Boing W., Koop J. and Neubauer I. (2002) Top-down control of phytoplankton: the role of time scale, lake depth and trophic state. *Freshwater Biology*. 47:2282-2295.

Boutte C., Grubisic S., Balthasart P., and Wilmotte A. (2006). Optimisation of the DGGE technique for the study of cyanobacterial molecular ecology. *Journal of Microbiological Methods* 65:542-550.

Brunberg A.K. and Blomqvist P. (2003) Recruitment of Microcystis (Cyanophyceae) from lake sediments: The importance of littoral inocula. *J. Phycol.* 39(1):58-63.

Cadel-Six S., Peyraud-Thomas C., Brient L., Tandeau de Marsac N., Rippka R., and Méjean A. (2007) Different Genotypes of Anatoxin-Producing Cyanobacteria Coexist in the Tarn River, France. *Appl. Environ. Microbiol*. 73(23):7605-7614.

Carmichael W.W., S.M.F.O. Azevedo, J.S. An, R.J. Molica, E.M. Jochimsen, S. Lau, K.L. Rinehart, G.R. Shaw and G.K. Eaglesham (2001). Human fatalities from cyanobacteria: chemical and biological evidence for cyanotoxins*, Environ. Health Perspect*. 109:663-668.

Carpenter S. R., Cole J. J., Hodgson J.R., Kitchell J. F., PaceM. L., Bade D., Cottingham K. L., Essington T. E., Houser J. N. and Schindler D. E. (2001) Trophic cascades, nutrients, and lake productivity: whole-lake experiments. *Ecological Monographs* 71:163-186.

Chorus, I. (2001). Cyanotoxins occurrence in freshwaters - a summary of survey results from different countries. In: I. Chorus (ed.) Cyanotoxins: Occurrence, Causes, Consequences. Springer-Verlag: Berlin/New York, 357 p.

Codd G. A., Lindsay J., Young F. M., Morrison L. F., and Metcalf J. S. (2005a). Harmful Cyanobacteria: from mass mortalities to management measures, p.1-24. In Huisman J, Matthijs HCP, and Visser PM (ed.), Harmful cyanobacteria. Springer Dordrecht, The Netherlands.

Codd G. A., Azevedo SMFO, Bagchi S. N., Burch M. D., Carmichael W. W., Harding W. R., Kaya K. and Utkilen H. C. (2005b). CYANONET A global network for cyanobacterial bloom and toxin risk management. IHP-VI. Technical Documents in Hydrology, n° 76.UNESCO, Paris, France.

Christiansen G., Fastner J., Erhard M., Börner T., and Dittmann E. (2003) Microcystin biosynthesis in Planktothrix: genes, evolution, manipulation. *J. Bact.* 185:564-572.

Christiansen G., Molitor C., Philmus B. and Kurmayer R. (2008) Non-toxic strains of cyanobacteria are the result of major gene deletion events induced by a transposable element. *Molecular Biology and Evolution* 25:1695-1704.

de Figueiredo D.R., U.M. Azeiteiro, S.M. Esteves, F.J.M. Gonzalves and M.J. Pereira (2004). Microcystin-producing blooms - a serious global public health issue, *Ecotoxicol. Environ. Saf.* 59:151-163.

Descy J.-P., Higgins K. W., Mackey D. J., Hurley J. P. and Frost T. M. (2000). Pigment ratios and phytoplankton assessment in North Wisconsin lakes. *Journal of Phycology*, 36:274-286.

Dittmann E., Neilan B. A., Erhard M., von Döhren H. and Börner T., (1997) Insertional mutagenesis of a peptide synthetase gene that is responsible for hepatotoxin production in the cyanobacterium Microcystis aeruginosa PCC 7806. *Molecular Microbiology* 26:779-787.

Dittmann E. and Börner T. (2005) Genetic contributions to the risk assessment of microcystin in the environment. *Toxicology and Applied Pharmacology* 203:192-200.

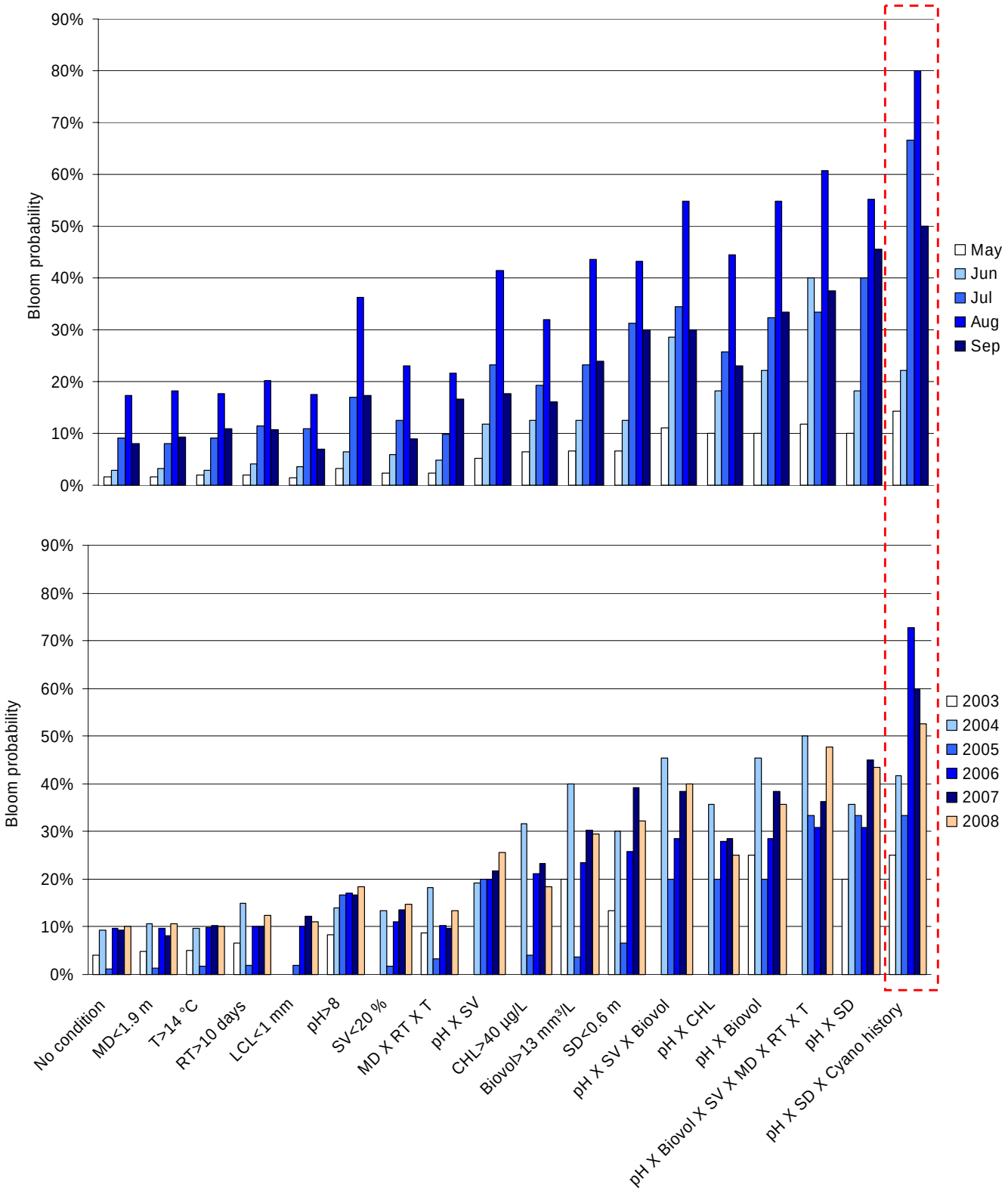



**Figure 24**: Seasonal and inter-annual variations in the unconditional and conditional probabilities of cyanobacterial bloom (5 $mm^3$ of cyanobacteria) occurrence in the ponds studied. Higher probability values indicate higher predictive capacity of a given condition. Red rectangle indicates the probability of bloom occurrence in ponds with the history of cyanobacterial blooms given the conditions that pH > 8 and SD < 0.6.

The probability value is 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 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 pH and Secchi depth used as conditions for probability calculation rendered probability values that are not significantly different from the latter (Wilcoxon matched pares test). This implies that the large number of variables difficult to measure might be substituted by a few easily measured variables for the rapid assessment of cyanobacterial bloom risk.

The probability values and thus predictive capacity of the model markedly increased when only ponds with the history of cyanobacterial blooms were taken into account (dash-line frame; Figure 8). It shows that the probability of a bloom occurrence in a Brussels pond previously affected by cyanobacteria is the highest in August (80%).

This approach permits to identify the ponds prone to cyanobacterial bloom development and thus can help managers to focus their monitoring program on the most problematic ponds. Such ponds can be more frequently monitored during the time of elevated risk of bloom occurrence 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.

# CONCLUSIONS AND RECOMMENDATIONS

Several key results were obtained during phase 1 of the B-BLOOMS2 project, and the main objectives were met. In this phase, the project is mainly based on collecting blooms samples throughout 2007 and 2008, both from reference lakes and from other water bodies through BLOOMNET network involving water managers and users in the project.

The three teams involved in regular field sampling (FUNDP, UGent and VUB) have 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 exploitation of BLOOMNET, the network of water-users and managers, further expands 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 in a standardised way and entered in 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 remains to exploit the data and correlate environmental, taxonomical, genetic and toxicity information. So far, the B-BLOOMS database contains all data collected in 2007 and 2008 in the reference lakes, and some additional data from BLOOMNET and parallel studies. The processing with the final data analysis could yield prediction models, using adequate, presumably non-linear, modelling techniques. 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 urban lakes.

In Flanders, both reference lakes suffered heavily from cyanobacterial blooms, but with contrasting bloom dynamics and species. The intensive sampling at both the cyanobacterial community and population level (both selectively neutral and toxin genes) allows 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 genotypic composition in Westveld 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 Cladocerans 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, 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 with nutrient level reduction, because when nutrient loadings are too high, biomanipulation might not bring the desired results.

In Wallonia, the survey of lake Falemprise, which was followed by the Namur team since 2002 in the framework of other programs, is of particular interest as it is an official swimming 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 to the most recent European directive on bathing waters, which includes the need for monitoring cyanobacterial proliferation and identifying health risks, and for taking adequate measures to

prevent exposure of the public. According to our survey, cyanobacteria blooms regularly occurred in lake Falemprise in the period 2002-2008. These blooms are dominated by *Aphanizomenon flos-aquae*, but other potentially toxic taxa occur, such as *Microscystis aeruginosa* and *Planktothrix agardhii*. The presence and the potential toxicity of these taxa has been confirmed by the genetic analyses, and microcystin was detected at levels above the WHO guideline value in 2007 and 2008. 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 to the EC directive.

A major result of this project, so far, 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 water bodies sampled in 2007 and 2008, 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. Understanding the processes behind toxin production and their dependence on environment and genetic diversity is also a key component of the project, which will be tackled with the data gathered during phase 1 of the B-BLOOMS2 project.

Similarly, toxin analysis of the BLOOMNET samples collected in Flanders showed that cyanobacterial toxin concentrations can be high enough to require risk management and possible intervention. Temporary closures of recreational waterbodies in 2007 occurred in 'Schulensmeer', 'de Gavers' and 'Dongelberg' where fish mass mortalities also occurred, although a direct link with cyanobacterial toxins was not established. In Wallonia, detection of cyanotoxins was also achived in a few cases thanks to BLOOMNET. An example is the development of a toxic *Planktothrix* population at depth in the Ecaussines quarry exploited by VIVAQUA provides a reminder that blooms can go undetected if they do not occur at the water surface, and yet can result in potential toxicity in the water.

The second phase of the B-BLOOMS2 project, started in 2009, focuses even more on practical and science policy objectives: developing BLOOMNET, transferring knowledge to the official laboratories in charge of water quality control, and improving information and awareness of the authorities and the general population.

# ACKNOWLEDGEMENTS

The team involved in B-BLOOMS2 wish to express their thanks to the members of the users committee and to BLOOMNET active members, such as VMM, ISSeP and IBGE.BIM, who provided information, feed-back and an adequate framework for the collection of cyanobacteria blooms.

We also thank Ralitsa Dimitrova and Maya P. Stoyneva for the microscopic phytoplankton examination of Lake Falemprise samples.

# REFERENCES

Akcaalan R., Young F. M., Metcalf J. S., Morrison L. F., Albay M. and Codd G. A. (2006). Microcystin analysis in single filaments of Planktothrix spp. In laboratory cultures and environmental blooms. *Water Research* 44(8):1583-1590.

Becker S., Kumar Singh, A., Christine Postius, C., Böger, P., and Ernst A. (2004) Genetic diversity and distribution of periphytic *Synechococcus spp.* in biofilms and picoplankton of Lake Constance FEMS *Microbiology Ecology* 49(2):181-190.

Bell, S. G. and G. A.Codd (1996). Cyanobacterial toxins and human health. *Rev. Med. Microbiol.* 5:256-264.

Benndorf J., Boing W., Koop J. and Neubauer I. (2002) Top-down control of phytoplankton: the role of time scale, lake depth and trophic state. *Freshwater Biology*. 47:2282-2295.

Boutte C., Grubisic S., Balthasart P., and Wilmotte A. (2006). Optimisation of the DGGE technique for the study of cyanobacterial molecular ecology. *Journal of Microbiological Methods* 65:542-550.

Brunberg A.K. and Blomqvist P. (2003) Recruitment of Microcystis (Cyanophyceae) from lake sediments: The importance of littoral inocula. *J. Phycol.* 39(1):58-63.

Cadel-Six S., Peyraud-Thomas C., Brient L., Tandeau de Marsac N., Rippka R., and Méjean A. (2007) Different Genotypes of Anatoxin-Producing Cyanobacteria Coexist in the Tarn River, France. *Appl. Environ. Microbiol*. 73(23):7605-7614.

Carmichael W.W., S.M.F.O. Azevedo, J.S. An, R.J. Molica, E.M. Jochimsen, S. Lau, K.L. Rinehart, G.R. Shaw and G.K. Eaglesham (2001). Human fatalities from cyanobacteria: chemical and biological evidence for cyanotoxins*, Environ. Health Perspect*. 109:663-668.

Carpenter S. R., Cole J. J., Hodgson J.R., Kitchell J. F., PaceM. L., Bade D., Cottingham K. L., Essington T. E., Houser J. N. and Schindler D. E. (2001) Trophic cascades, nutrients, and lake productivity: whole-lake experiments. *Ecological Monographs* 71:163-186.

Chorus, I. (2001). Cyanotoxins occurrence in freshwaters - a summary of survey results from different countries. In: I. Chorus (ed.) Cyanotoxins: Occurrence, Causes, Consequences. Springer-Verlag: Berlin/New York, 357 p.

Codd G. A., Lindsay J., Young F. M., Morrison L. F., and Metcalf J. S. (2005a). Harmful Cyanobacteria: from mass mortalities to management measures, p.1-24. In Huisman J, Matthijs HCP, and Visser PM (ed.), Harmful cyanobacteria. Springer Dordrecht, The Netherlands.

Codd G. A., Azevedo SMFO, Bagchi S. N., Burch M. D., Carmichael W. W., Harding W. R., Kaya K. and Utkilen H. C. (2005b). CYANONET A global network for cyanobacterial bloom and toxin risk management. IHP-VI. Technical Documents in Hydrology, n° 76.UNESCO, Paris, France.

Christiansen G., Fastner J., Erhard M., Börner T., and Dittmann E. (2003) Microcystin biosynthesis in Planktothrix: genes, evolution, manipulation. *J. Bact.* 185:564-572.

Christiansen G., Molitor C., Philmus B. and Kurmayer R. (2008) Non-toxic strains of cyanobacteria are the result of major gene deletion events induced by a transposable element. *Molecular Biology and Evolution* 25:1695-1704.

de Figueiredo D.R., U.M. Azeiteiro, S.M. Esteves, F.J.M. Gonzalves and M.J. Pereira (2004). Microcystin-producing blooms - a serious global public health issue, *Ecotoxicol. Environ. Saf.* 59:151-163.

Descy J.-P., Higgins K. W., Mackey D. J., Hurley J. P. and Frost T. M. (2000). Pigment ratios and phytoplankton assessment in North Wisconsin lakes. *Journal of Phycology*, 36:274-286.

Dittmann E., Neilan B. A., Erhard M., von Döhren H. and Börner T., (1997) Insertional mutagenesis of a peptide synthetase gene that is responsible for hepatotoxin production in the cyanobacterium Microcystis aeruginosa PCC 7806. *Molecular Microbiology* 26:779-787.

Dittmann E. and Börner T. (2005) Genetic contributions to the risk assessment of microcystin in the environment. *Toxicology and Applied Pharmacology* 203:192-200.

Dittmann E., and Wiegand. C. (2006). Cyanobacterial Toxins - Occurrence, Biosynthesis and Impact on Human Affairs. Invited Review. *Molecular Nutrition and Food Research* 50:7-17.

Everbecq E., Gosselain V., Viroux L. and Descy J.-P. (2001) POTAMON: a dynamic model for predicting phytoplankton composition and biomass in large rivers. *Wat. Res.* 35(4):901-912.

Fastner J., Codd G. A., Metcalf J. S., Woitke P., Wiedner C. and Utkilen H. (2002) An international intercomparison exercise for the determination of purified microcystin-LR and microcystins in cyanobacterial field material. *Analytical and Bioanalytical Chemistry* 374:437-444.

Fewer D., Tooming-Klunderud A., Jokela J., Wahlsten M., Rouhiainen L., Kristensen T., Rohrlack T., Jakobsen K. S., and Sivonen K. (2008) Natural occurrence of microcystin synthetase deletion mutants capable of producing microcystins in strains of the genus Anabaena (Cyanobacteria). *Microbiology* 154: 1007–1014.

Fischbach, M. A. and Walsh, C. T. (2006) Assembly-line enzymology for polyketide and nonribosomal peptide antibiotics: logic, machinery, and mechanisms. *Chemical Reviews* 106, 3468-3496

Gugger M., Lenoir S., Berger C., Ledreux A., Druart J. C., Humbert J. F., Guette C. and Bernard C. (2005) First report in river in France of the benthic cyanobacterium Phormidium favosum producing anatoxin-a associated with dog neurotoxicosis. *Toxicon* 45:919-928.

Gulati R. and van Donk E. (2002) Lakes in the Netherlands, their origin, eutrophication and restoration: state-of-the-art review. *Hydrobiologia*. 478:73-106.

Hisbergues M., Christiansen G., Rouhiainen L., Sivonen K., and Börner T. (2003) PCR-based identification of microcystin producing genotypes of different cyanobacterial genera: application to environmental samples. *Arch. Microbiol.*180:402-410.

Hudnell K. H., Ed. (2008) Cyanobacterial Harmful Algal Blooms. New York, Springer.

Huisman J., van Oostveen P. and Weissing F. J. (1999) Species dynamics in phytoplankton blooms: incomplete mixing and competition for light. *American Naturalist.* 154:46-68.

Janse I., Meima M., Kardinaal W. E. A., and Zwart G. (2003) High-resolution differentiation of cyanobacteria by using rRNA-internal transcribed spacer denaturing gradient gel electrophoresis. *Appl. Environ. Microbiol*. 69:6634-6643.

Janse I., Kardinaal W. E. A., Meima M., Fastner J., Visser P. M., and Zwart. G. (2004) Toxic and Nontoxic Microcystis Colonies in Natural Populations Can Be Differentiated on the Basis of rRNA Gene Internal Transcribed Spacer Diversity. *Appl. Environ. Microbiol.* 70: 3979-3987.

Jeffrey S. W., Mantoura R. F. C. and Wright S.W. (eds) (1997) Phytoplankton pigments In oceanography guidehnes to modern methods UNESCO Pans.

Jungblut A., Hoeger S. J. Mountfort D. O., Hitzfeld B., Dietrich D. R. and Neilan B. A. (2006) Characterization of microcystin production in an Antarctic cyanobacterial mat community. *Toxicon* 47:271–278.

Kardinaal W. E. A. and Visser P. M. (2005) Dynamics of cyanobacterial toxins, In: Harmful Cyanobacteria, Huisman, J., Matthijs, H.C.P. & Visser P.M. (Eds.), Aquatic Ecology Series, Vol.3, Springer, Dordrecht, pp. 41-64.

Kardinaal W. E. A., Janse I., Kamst-van Agterveld M., Meima M., Snoek J., Mur L. R., Huisman J., Zwart G., and Visser P. (2007) Microcystis genotype succession in relation to microcystin concentrations in freshwater lakes. *Aquat. Microb. Ecol.* 48:1-12.

Mackey M. D., Mackey D. J., Higgins H. W. and Wright S. W. (1996) CHEMTAX-a program for estimating class abundances from chemical markers: Application to HPLC measurements of phytoplankton. *Marine Ecology-Progress Series* 144:265-283.

Metcalf J. S., Bell S. G. and Codd G. A. (1990). Colorimetric Immuno-Protein Phosphatase Inhibition Assay for Specific Detection of Microcystins and Nodularins of Cyanobacteria. *Applied and Environmental Microbiology*, 67(2): 904-909.

Metcalf, J. S., Bell, S. G. and Codd, G. A. (2000). Production of novel polyclonal antibodies against the cyanobacterial toxin microcystin-LR and their application for the detection and quantification of microcystins and nodularins *Water Research* 34: 2761-2769.

Moss B., Stephen D., Alvarez C., Becares E., Van de Bund W., Collings S. E., Van Donk E., De Eyto E., Feldmann T., Fernandez-Alaez C., Fernandez-Alaez M., Franken R. J. M., Garcia-Criado F., Gross E. M., Gyllstrom M., Hansson L. A., Irvine K., Jarvalt A., Jensen J. P., Jeppesen E., Kairesalo T., Kornijow R., Krause T., Kunnap H., Laas A., Lille E., Lorens B., Luup H., Miracle M. R., Noges P., Noges T., Nykanen M., Ott I., Peczula W., Peeters E., Phillips G., Romo S., Russell V., Salujoe J., Scheffer M., Siewertsen K., Smal H., Tesch C., Timm H., Tuvikene L., Tonno I., Virro T., Vicente E. and Wilson D. (2003) The determination of ecological status in shallow lakes - a tested system (ECOFRAME) for implementation of the European Water Framework Directive. *Aquatic Conservation-Marine and Freshwater Ecosystems*. 13:507-549.

Nishizawa T., Ueda A., Asayama M., Fujii K., Harada K., Ochi K. and Shirai M. (2000) Polyketide synthase gene coupled to the peptide synthetase module involved in the biosynthesis of the cyclic heptapeptide microcystin. *Journal of Biochemistry* 127:779- 789.

Nubel, U., Garcia-Pichel, F., and Muyzer G. (1997) PCR primers to amplify 16S rRNA genes from cyanobacteria. *Appl. Environ. Microbiol.* 63:3327-3332.

Nonemann D., and Zimba P. (2001) A PCR-based test to assess the potential for microcystin occurrence in channel catfish production ponds. *J. Phycol.* 38: 230-233.

Ouellette A. J. A. and Wilhelm S. W. (2003) Toxic cyanobacteria: the evolving molecular toolbox. *Frontiers in Ecology and Environment* 1:359-366.

Orr P. T. and Jones G. J. (1998) Relationship between microcystin production and cell division rates in nitrogen-limited Microcystis aeruginosa cultures*, Limnol. Oceanogr.* 43(7):1604–1614.

Pinel-Alloul B. (1995) Impacts des prédateurs invertébrés sur les communautés aquatiques. In Limnologie Générale, Pourriot R, Meybeck M (eds). Masson: Paris; 628-686.

Pearson L. A., Hisbergues M., Borner T., Dittmann E. and Neilan B. A. (2004) Inactivation of an ABC transporter gene, mcyH, results in loss of microcystin production in the cyanobacterium Microcystis aeruginosa PCC 7806. *Appl Environ Microbiol.* 70(11):370-378.

Rantala A., Rajaniemi-Wacklin P., Lyra C., Lepisto L., Rintala J., Mankiewicz-Boczek J., and Sivonen K. (2006) Detection of Microcystin-Producing Cyanobacteria in Finnish Lakes with Genus-Specific Microcystin Synthetase Gene E (mcyE) PCR and Associations with Environmental Factors *Appl. Envir. Microbiol.* 72(9):6101 - 6110.

Reynolds CS. (1984) The ecology of freshwater phytoplankton. Cambridge University Press: Cambridge.

Rouhiainen L., Vakkilainen T., Siemer B. L., Buikema W., Haselkorn R. and Sivonen K. (2004) Genes coding for hepatotoxic heptapeptides (microcystins) in the cyanobacterium Anabaena strain 90. *Applied and Environmental Microbiology* 70:686-692.

Rossello-Mora R. and Amann R. (2001) The species concept for prokaryotes. *FEMS Microbiology Reviews* 25:39-67.

Rudi K., Skulberg O. M., and Jakobsen K. (1998) Evolution of cyanobacteria by exchange of genetic material among phyletically related strains. *Journal of Bacteriology* 180(13):3453-3461.

Sivonen, K. (1996) Cyanobacterial toxins and toxin production. *Phycologia* 35(6):12-24.

Tanabe Y., Kasai F. & Watanabe M. (2007) Multilocus sequence typing (MLST) reveals high genetic diversity and clonal population structure of the toxic cyanobacterium Microcystis aeruginosa. *Microbiology* 153 :3695-3703.

Taton A., Grubisic S., Brambilla E., De Witt E., and Wilmotte A. (2003) Cyanobacterial diversity in natural and artificial microbial mats of Lake Fryxell (McMurdo Dry Valleys, Anarctica): morphological and molecular approach. *Appl. Environ. Microbiol.* 69:5157-5169.

Teissier S. (2008) Aquatic ecology as a tool for the management of eutrophic to hypereutrophic peri-urban ponds from the Woluwe catchment: internal-externalnutrient loadings. Report, VUB, Brussels.

Tillet D., Parker D. L., and Neilan B. A. (2000) Detection of toxigenicity by a probe for the microcystin synthesis A gene (mcyA) of the cyanobacterial genus Microcystis: comparison of toxicities with 16S rRNA and phycocyanin operon (phycocyanin intergenic spacer) phylogenies. *Appl. Environ. Microbiol.* 67:2810-2818.

Verniers G. and Sarmento H. (2004) – Schéma directeur intégré pour la préservation de la qualité de l'eau et la valorisation écologique des lacs de l'Eau d'Heure – brochure de vulgarisation – Igretec / Région wallonne – 20 p.

Verspagen J.M.H., Snelder E.O.F.M., Visser P.M. (2004) Recruitment of benthic Microcystis (Cyanophyceae) to the water column. *J. Phycol.* 40(2):260–270.

Via-Ordorika L., Fastner J., Kurmayer R., Hisbergues M., Dittmann E., Komarek J., Erhard M., and Chorus I. (2004) Distribution of microcystin-producing and non-microcystin-producing Microcystis sp. in European freshwater bodies: detection of microcystins and microcystin genes in individual colonies. *Syst. Appl. Microbiol.* 27:592-602.

Willame R., Jurczak T., Iffly, J. F., Kull T., Meriluoto J., and Hoffmann L. (2005). Distribution of toxigenic cyanobacterial blooms in Belgium and Luxembourg. *Hydrobiologia* 551 : 99-117.

Willame R, Boutte C, Grubisic S, Wilmotte A, Komárek J and Hoffmann L. (2006). Morphological and molecular characterization of planktonic cyanobacteria from Belgium and Luxembourg. *Journal of Phycology* 42:1312-1332.

Wilmotte A., Descy, J.-P. & Vyverman, W. (2008) Algal blooms: emerging problem for health and sustainable use of surface waters (B-BLOOMS) SPSD 2, Research project EV/13/34B. Final report (2003-2005) Bruxelles: Science Policy Office, Belgium. 77 p.

Wright S. W., Jeffrey S. W., Mantoura R. F. C., Llewellyn C. A., Bjsrnland T., Repeta D. and Welschmeyer N. (1991) Improved HPLC method for the analysis of chlorophylls and carotenoids from marine phytoplankton. *Mar. Ecol. Prog. Ser.* 77:183-196.

Yoshida M., Yoshida T., Kashima A., Takashima Y., Hosoda N., Nagasaki K. and Hiroishi, S. (2008) Ecological dynamics of the toxic bloom-forming cyanobacterium Microcystis aeruginosa and its cyanophages in freshwater. *Applied and Environmental Microbiology* 74:3269-3273.

Young F., Morisson L. F., James J. and Codd G.A. (2008).Quantification and localization of microcystinsincolonies of a laboratory strain of Microcystis(Cyanobacteria) using immunological methods. *Eur. J. Phycol.* 43(2): 217-225.

# ANNEX I : List of parameters and Field protocol

- **Meteorological data**

  Gather these data from the nearest meteorological station (KMI) from May –October :

  - Air temperature (°C)
  - direction and velocity of wind (m/s)
  - Rainfall (mm)
  - Irradiance ($W/m^2$)

- **Physical variables** (surface in shallow lakes < 3 m; vertical profile from different depths in deeper lakes)
  - Temperature(°C)
  - pH
  - Oxygen (mg/l, % saturation)
  - Conductivity ($\mu S$/cm at 25°C)
  - Depth (at the sampling site ; mean depth only once) Do we have to make a bathymetric map?NO Just mesure depth at the sampling site, and sample the lake at different places to have an estimate of mean depth
  - Euphotic depth (Secchi depth or irradiance profile with light sensors)
  - Depth of the mixed layer (in lakes > 3 m) : determined from T / $O_2$ vertical profiles

- **Chemical variables** :

Measurements on filtered water, unfiltered water or filter of:

  - $NO_3^-$ on GF/C or GF/F filtered water (for ex: we determine N-$NO_3$ by an adapted cadmium reduction method to reach a minimum detectable concentration of about 10 $\mu$g N-$NO_3$/L)
  - $NO_2^-$ on GF/C or GF/F filtered water (for ex: we determine N-$NO_2$ by the sulfanilamide method to reach a minimum detectable concentration of about 10 $\mu$g N-$NO_2$/L
  - $NH_4^+$ on GF/C or GF/F filtered water (for ex: we determine N-NH4 by the salicylate-dichlorocyanurate method to reach a minimum detectable concentration of about 20 $\mu$g N-NH4/L)
  - $PO_4$ (SRP),on GF/C or GF/F filtered water (for ex: we determine P by the molybdate method to reach a minimum detectable concentration of about 10 $\mu$g P/L);
  - P total, see P after persulfate digestion (**unfiltered water**).
  - PON (particulate organic N) (elemental analysis, Carlo Erba NA 1500, nitrogen/carbon analyser), **filtration of a known volume on GF/F previously incinerated at 500 °C**
  - POC Particulate organic C) (elemental analysis, Carlo Erba NA 1500, nitrogen/carbon analyser); **carried out on the same filter as for PON**
  - Dissolved Si, on GF/C or GF/F filtered water

- **Plankton examination and biomass** :
  - Phytoplankton composition and biomass
    - Chla content and pigments composition (HPLC),
    - microscope examinations
  - Zooplankton composition and biomass (microscope examinations)

## In the field

- Measure physical variables from the water column :

(a depth profile in deep stratified lakes or only at the surface in shallow, well mixed lakes)

  - Temperature
  - pH
  - Oxygen (mg/l, % saturation)
  - Conductivity
  - depth

- Transparency : use a Secchi disc (do not measure in the shadow of the boat)

- Gather water samples with a Van Dorn bottle on regular depths in the stratified lakes or with a tube samples in the shallow lakes from at least three different localities in each lake. From the integrated sample (gathered in a big container of 100L) take subsamples for phytoplankton microscopy and laboratory analysis and transport these to the lab in an ice-cooled box.

- Take a zooplankton sample with a Schindler Patalas sampler at regular depths in the deep lakes at each locality; alternatively, filter water from the big container with the integrated sample over a 64 $\mu$m mesh size net in case of shallow lakes, write down the volume filtered and fix with sugared formaldehyde to an end concentration of ~ 10%. (add 10 ml formaldehyde (35 %) in 100 ml sample).

- Take extra water samples in case of a clear phytoplankton bloom :
  - Gather water from the surface in a graduated bucket of 10L
  - Filter this water over a net of 20-25 $\mu$m meshsize until the net cloggs, take care to remove organic debris with tweezers.
  - Rinse the concentrate from the net with a small amount of filtered water (in a rinsing bottle) and gather in a glass beaker
  - In case of a scum layer on the water surface, sample directly with a beaker, no concentration over a net is necessary
  - Bring the concentrate in a 100 ml PE vial with a broad opening (to fasten lyophilisation) and store in the deepfreeze (-20°C).

- In case of evident blooms, collect fresh sample of water (simply 15 ml of "green" water in a sterile Falcon tube or equivalent) and as soon as possible deliver (prior) to ULG in a brown envelope for isolation by culture methods. (not on Friday or just before a day-off)

- Fill in the sampling form

## In the laboratory (the same day) :

**For each collected sample :**

- **Whole water :**
  - Fill a **500ml bottle (we take 250 ml and fix it with 125 µl alkaline lugol, 6.25 ml borax buffered formaldehyde and 250 µl sodium thiosulphate)** with concentrated Lugol (final color « tea ») for counting / for identification of phytoplankton, settle for at least 24h and concentrate; add formaline according final volume for a 2% final concentration.

  - Fill a 50 ml PE bottle (we use Falcon-tubes) for total phosphorus , add **0,2 ml $H_2SO_4$ 5N**, to be kept at 4°C

  - Into 4 labelled 1.5ml microcentrifuge tubes, pipette 1ml of mixed environmental water sample. (see Codd laboratory detailed protocols for further operations).

    a) Store 2 tubes at -20°C. (TM by ELISA)
    b) Store 1 tubes at +4°C. (Samples for single colony and filament ELISA, we sometimes concentrate the samples first to make isolation easier)
    c) Centrifuge 1 tube and remove supernatant and place into new labelled 1.5ml microcentrifuge tube, store sample at -20°C. (SM by ELISA)

- **Filtrations :**
  - **1 filter GF/C whatman previously incinerated** (500°C during 4h) **for particulate C and N**

    Filter a sufficient amount of water (until the filter gets clogged and/or gets a clear colour), place the filter in a special filter cap, note volume filtered and dry weight and store in the deep freezer(-20°C)

  - **1 filter GF/C whatman for chlorophyll a and pigments analysis by HPLC**

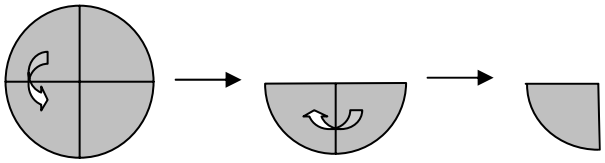
    Filter an adequate volume of a well-mixed environmental water (do not exceed 200mbar of vacuum pressure). Roll the filter on itself and place it in a glass or PE tube. Pigments have to be extracted using 8 mL of 90% aqueous acetone in an adequate vial (glass vial, as those used for scintillation); extraction is carried out with 15 minutes sonication in a sonication bath with ice (~ 0-2°C) (for ex.: bath style sonicator, Branson. The vials are then stored overnight in a refrigerator (4°C); then a second 15 min sonication is performed; ); alternatively, a one-step sonication can be performed with a point sonicator; centrifuge the extract and keep at least 2 ml in an amber vial for

HPLC analysis. If you have to store the filter before extraction, keep them in a deep freezer or liquid nitrogen but pigment extraction should de done within 2 weeks of collection. It's definitely better to carry out the extraction on the fresh filters and to store the 2 mL extract after centrifugation. The pigments analysis will be done at Namur laboratory. Store the extracts in the deep freezer at -80°C.

- **2 filters 0.2 $\mu$m for molecular analysis** (PCR for microcystin genes)

  A volume from 100 to 500 ml (or part if the filter is severely clogged, in that case record the volume filtered!) is filtered onto a 0.2 $\mu$m Supor Filter, PES, Pall Life Science, 47 mm, 0,2 $\mu$m, VWR, (Catalogue #: 60 300).

  Do this twice – so we will have one filter in reserve. Pay attention not to contaminate the filter by putting it on the floor or taking it with fingers: manipulate it carefully with a tweezers all time. After filtration, fold it into four parts, put in lysis buffer, label it properly and immediately frozen at –20°C (if possible). The samples have to be sent, still frozen, to Ulg for DNA extraction. (see ULG sampling procedure for details).



- **1 filter GF/C whatman for particulate microcystin content** (PM)

  Filter 1 liter of a mixed environmental water or an appropriate reduced volume on a pre-weighted GF/C filter (precision 0.1 mg), and record volume filtered. Retain the filtrate in a labelled 1L glass. (see below); Place the filter disc in a labelled plastic Petri dish and store at -20°C. (see Codd laboratory detailed protocols for further operations).

- **Filtrated water :**
  - 1 or more PE bottle for storage of an adequate volume of water for analysis of dissolved nutrients (volume depending of your laboratory test needs, store at -20°C if needed) We store these in a Falcon tube of 50 ml.

  - 1 L glass bottle for soluble microcystin content (SM), store the filtrate (from the filtration for particulate microcystin) at +4°C until SPE. (see Codd laboratory detailed protocols for further operations).

# ANNEX II : List of samples

| Reference Lakes in 2007 and 2008: | | | | |
|---|---|---|---|---|
| Flanders | | Brussels | | Wallonia |
| Westveld | Donkmeer | Ixelles Pound 1 | Ixelles Pound 2 | Falemprise |
| 1/05/2007 | 1/05/2007 | 27/03/2007 | 27/03/2007 | 3/05/2007 |
| 15/05/2007 | 15/05/2007 | 26/04/2007 | 26/04/2007 | 15/05/2007 |
| 21/05/2007 | 21/05/2007 | 22/05/2007 | 22/05/2007 | 22/05/2007 |
| 25/05/2007 | 25/05/2007 | 26/06/2007 | 7/06/2007 | 30/05/2007 |
| 29/05/2007 | 1/06/2007 | 3/07/2007 | 18/06/2007 | 5/06/2007 |
| 1/06/2007 | 8/06/2007 | 10/07/2007 | 26/06/2007 | 13/06/2007 |
| 5/06/2007 | 15/06/2007 | 19/07/2007 | 3/07/2007 | 19/06/2007 |
| 8/06/2007 | 21/06/2007 | 24/07/2007 | 10/07/2007 | 27/06/2007 |
| 12/06/2007 | 25/06/2007 | 3/08/2007 | 19/07/2007 | 4/07/2007 |
| 15/06/2007 | 28/06/2007 | 10/08/2007 | 24/07/2007 | 10/07/2007 |
| 18/06/2007 | 2/07/2007 | 17/08/2007 | 3/08/2007 | 17/07/2007 |
| 21/06/2007 | 5/07/2007 | 24/08/2007 | 10/08/2007 | 25/07/2007 |
| 25/06/2007 | 10/07/2007 | 29/08/2007 | 17/08/2007 | 31/07/2007 |
| 28/06/2007 | 13/07/2007 | 11/09/2007 | 24/08/2007 | 7/08/2007 |
| 2/07/2007 | 17/07/2007 | 26/09/2007 | 29/08/2007 | 17/08/2007 |
| 5/07/2007 | 23/07/2007 | 30/01/2008 | 11/09/2007 | 21/08/2007 |
| 10/07/2007 | 26/07/2007 | 26/02/2008 | 26/09/2007 | 27/08/2007 |
| 13/07/2007 | 2/08/2007 | 12/03/2008 | 30/01/2008 | 3/09/2007 |
| 17/07/2007 | 6/08/2007 | 26/03/2008 | 26/02/2008 | 12/09/2007 |
| 19/07/2007 | 9/08/2007 | 15/04/2008 | 12/03/2008 | 17/09/2007 |
| 23/07/2007 | 13/08/2007 | 29/04/2008 | 26/03/2008 | 24/09/2007 |
| 26/07/2007 | 16/08/2007 | 13/05/2008 | 15/04/2008 | 2/10/2007 |
| 30/07/2007 | 20/08/2007 | 29/05/2008 | 29/04/2008 | 10/10/2007 |
| 2/08/2007 | 23/08/2007 | 9/06/2008 | 13/05/2008 | 16/10/2007 |
| 6/08/2007 | 27/08/2007 | 19/06/2008 | 29/05/2008 | 2/04/2008 |
| 9/08/2007 | 31/08/2007 | 25/06/2008 | 9/06/2008 | 17/04/2008 |
| 13/08/2007 | 4/09/2007 | 3/07/2008 | 19/06/2008 | 30/04/2008 |
| 16/08/2007 | 7/09/2007 | 9/07/2008 | 25/06/2008 | 7/05/2008 |
| 20/08/2007 | 10/09/2007 | 16/07/2008 | 3/07/2008 | 22/05/2008 |
| 23/08/2007 | 13/09/2007 | 24/07/2008 | 9/07/2008 | 29/05/2008 |
| 27/08/2007 | 17/09/2007 | 30/07/2008 | 16/07/2008 | 4/06/2008 |
| 31/08/2007 | 21/09/2007 | 7/08/2008 | 24/07/2008 | 11/06/2008 |
| 4/09/2007 | 24/09/2007 | 13/08/2008 | 30/07/2008 | 18/06/2008 |
| 7/09/2007 | 27/09/2007 | 20/08/2008 | 7/08/2008 | 25/06/2008 |
| 10/09/2007 | 1/10/2007 | 27/08/2008 | 13/08/2008 | 3/07/2008 |
| 13/09/2007 | 4/10/2007 | 17/09/2008 | 20/08/2008 | 10/07/2008 |
| 17/09/2007 | 8/10/2007 | 1/10/2008 | 27/08/2008 | 17/07/2008 |
| 21/09/2007 | 15/10/2007 | | 17/09/2008 | 24/07/2008 |
| 24/09/2007 | 19/10/2007 | | 1/10/2008 | 30/07/2008 |
| 27/09/2007 | 22/10/2007 | | | 7/08/2008 |
| 1/10/2007 | 25/10/2007 | | | 14/08/2008 |
| 4/10/2007 | 31/10/2007 | | | 20/08/2008 |
| 8/10/2007 | 5/11/2007 | | | 28/08/2008 |
| 12/10/2007 | 19/11/2007 | | | 4/09/2008 |
| 15/10/2007 | 11/01/2008 | | | 11/09/2008 |
| 19/10/2007 | 18/03/2008 | | | 18/09/2008 |
| 22/10/2007 | 31/03/2008 | | | 1/10/2008 |
| 25/10/2007 | 14/04/2008 | | | 13/10/2008 |
| 31/10/2007 | 29/04/2008 | | | 22/10/2008 |
| 5/11/2007 | 13/05/2008 | | | |
| 12/11/2007 | 27/05/2008 | | | |
| 19/11/2007 | 10/06/2008 | | | |
| 26/11/2007 | 24/06/2008 | | | |
| 11/01/2008 | 7/07/2008 | | | |
| 18/03/2008 | 15/07/2008 | | | |
| 25/03/2008 | 22/07/2008 | | | |
| 31/03/2008 | 5/08/2008 | | | |
| 7/04/2008 | 11/08/2008 | | | |
| 14/04/2008 | 19/08/2008 | | | |
| 22/04/2008 | 26/08/2008 | | | |
| 29/04/2008 | 2/09/2008 | | | |
| 6/05/2008 | 8/09/2008 | | | |
| 13/05/2008 | 15/09/2008 | | | |
| 20/05/2008 | 24/09/2008 | | | |
| 27/05/2008 | 7/10/2008 | | | |
| 3/06/2008 | 14/10/2008 | | | |
| 6/06/2008 | 21/10/2008 | | | |
| 10/06/2008 | 30/10/2008 | | | |
| 13/06/2008 | | | | |
| 17/06/2008 | | | | |
| 20/06/2008 | | | | |
| 24/06/2008 | | | | |
| 27/06/2008 | | | | |
| 1/07/2008 | | | | |
| 4/07/2008 | | | | |
| 7/07/2008 | | | | |
| 15/07/2008 | | | | |
| 18/07/2008 | | | | |
| 22/07/2008 | | | | |
| 25/07/2008 | | | | |
| 29/07/2008 | | | | |
| 1/08/2008 | | | | |
| 5/08/2008 | | | | |
| 8/08/2008 | | | | |
| 11/08/2008 | | | | |
| 14/08/2008 | | | | |
| 19/08/2008 | | | | |
| 22/08/2008 | | | | |
| 26/08/2008 | | | | |
| 29/08/2008 | | | | |
| 2/09/2008 | | | | |
| 8/09/2008 | | | | |
| 15/09/2008 | | | | |
| 24/09/2008 | | | | |
| 7/10/2008 | | | | |
| 14/10/2008 | | | | |
| 21/10/2008 | | | | |
| 30/10/2008 | | | | |

# ANNEX III : B-BLOOMS 2 database

All data from the B-BLOOMS 2 project (BELSPO) are available on the website: **http://www.bblooms.be**

Please, contact the coordinator (jpdescy@fundp.ac.be) to create a login