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Faculté des Sciences

# MOLECULAR TOOLS APPLIED TO THE STUDY OF MICROCYSTIN-PRODUCING CYANOBACTERIA IN BELGIAN WATERBODIES

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## Abbreviations and acronyms

16S rRNA : 16S ribosomal ribonucleic acid
454 : 454 pyrosequencing
AN : accession number
B-BLOOMS : Cyanobacterial blooms : toxicity, diversity, modelling and management
BelSPo : Belgian Science Policy
bp : base pairs
C : carbon
Chl a : chlorophyll a
CV : coefficient of variation
DAN : 1,5-diaminonaphtalene
DGGE : denaturating gradient gel electrophoresis
DHB : 2,5-dihydrobenzoic acid
DNA : desoxyribonuclecleic acid
ECH : echinenone
EDTA : ethylenediaminetetraacetic acid
ELISA : enzyme-linked immunosorbent assay
GV : guideline value
HPLC : high performance liquid chromatography
ICBN : International Code of Botanical Nomenclature
ICNB : International Code of Nomenclature for Bacteria
ITS : internal transcribed spacer
KS : ketoacyl synthase
LAQUAN : Late Quaternary climate history of coastal Antarctic environments: a multi-proxy approach
m/z : mass to charge ratio
MALDI-TOF: matrix-assisted laser desorption/ionization – time of flight
MC : microcystin
MC eq : microcystin equivalent
MDA : Multiple strand displacement amplification
MIDICHIP : Microbial Diversity Chip
MLST : multilocus sequence typing
N : nitrogen
N/A : not available
nd : not determined
NRPS : non-ribosomal peptide synthase
nt : nucleotide
OD : optical density
ODEC : Organisation for Economic Co-operation and Development
OTU : operational taxonomic unit
P : phosphorus
PCA : principal component analysis
PCR : polymerase chain reaction
pH : hydrogen potential
PKS : ketoacyl synthase
qPCR : real-time quantitative polymerase chain reaction
RFLP : restriction fragment length polymorphism
sd : standard deviation
ST : sequence type

T°C : temperature in °C
TAE : tris-acetate EDTA
TE : tris-EDTA buffer
TN : total nitrogen
TP : total phospohorus
tRNA : transfer ribonucleic acid
UGent : University of Gent
ULg : Université de Liège
UNamur (FUNDP) : Université de Namur
UV : ultra-violet
VUB : Vrije Universiteit Brussel
WGA : whole genome amplification
WHO : World Health Organization
ZEA : zeaxanthin
Zeu : euphotic depth
Zm : metalimnion depth

## Abstract

'Blooms', an increasing worldwide phenomenon, are adversely affecting surface water resource, including reservoirs and lakes, used for drinking water supplies, recreation, crop, irrigation and fisheries. These amenities are affected by recurrent mass proliferations of cyanobacteria. The latter are responsible for the production of a wide range of bioactive compounds, including potent toxins (cyanotoxins). These comprise neurotoxins, cytotoxins, inflammatory agents, and hepatotoxins.

Microcystins (MCs), hepatotoxins and tumour promoters are the most documented of the cyanotoxins. The microcystin synthetase gene cluster (mcy) involved in MC biosynthesis consists of a succession of non-ribosomal peptide synthase (NRPS) and polyketide synthetase (PKS) genes. The main producers of MCs are *Anabaena, Microcystis,* and *Planktothrix*. However, it is not possible to distinguish a toxic from a non-toxic strain on the basis of their morphology.

In the present study, molecular tools were used, optimized and developed to (i) characterize the 16S rRNA gene diversity of planktonic cyanobacteria, (ii) to detect the cyanobacteria responsible for the production of MCs, (iii) to identify the MCs producing taxa, and (iv) to determine the environmental factors that influence the dynamic of toxic and non-toxic genotypes in Belgian freshwaters.

Eighty-nine strains were isolated and their 16S rRNA genes sequenced. The 16S rRNA gene diversity was studied in 32 samples by denaturing gradient gel electrophoresis (DGGE). In order to evaluate the contribution of this work to the study of the molecular diversity of cyanobacteria in Belgian waterbodies, 114 (strains and DGGE) sequences obtained during this PhD thesis were compared to Belgian sequences obtained by others. As a result, 14 previously undiscribed operational taxonomic units (OTUs) were found in the present study.

For polymerase chain reaction (PCR) detection of the *mcyA/B/E* genes, the DNA from a total of 162 environmental samples was extracted. The three genes were found together in 64.2% of the samples, whereas the *mcyB* alone was detected in 95.1% of the samples.

In order to identify the *mcyE*-carriers present in the freshwaters, a restriction fragment length polymorphism (RFLP) was performed on the *mcyE* gene. The presence of potentially toxic *Microcystis* was observed in most of the cases.

To bypass the constraint of bacterial cultivation, a combination of whole genome amplification (WGA) and enzyme-linked immunoassay (ELISA) was tested on individual colonies of members of two cyanobacteria, *Microcystis* and *Woronichinia*, directly from the natural environment. Sequences of 3 different housekeeping genes (*ftsZ*, *gltX*, and *recA*), of 3 mcy genes, and the Internal Transcribed Spacer (ITS) were analyzed for 11 colonies of *Microcystis*. MCs were detected and quantified by ELISA in 7 of the 11 *Microcystis* colonies tested, in agreement with the detection of *mcy* genes. Sequence types (ST) based on the concatenated sequences of housekeeping genes from cyanobacterial colonies from Belgian water bodies appeared to be endemic when compared to those of strains described in the literature. One colony belonged to a yet undiscovered lineage. A similar protocol was used for 6 colonies of the genus *Woronichinia*, a taxon that is very difficult to cultivate in the laboratory. The 16S rRNA analysis confirmed the colony identification based on morphology. In addition, we obtained for the first time new genetic data for this genus, such as the *rpoC1* gene sequences and the sequences and secondary structures of the ITS. The first discovery of NRPS and PKS DNA sequences in *Woronichinia* colonies highlights the need for further study of this widely occurring genus, to better assess its ability to produce MCs and/or related metabolites.

For the first time, in this study, we were able to simultaneously monitor one toxic and one non-toxic genotype of *M. aeruginosa* using real time qPCR technology during a monitoring of 2 years. Both toxic and non-toxic genotypes dynamics appeared influenced by the photoperiod. In addition, the dynamic of the toxic genotype was influenced of light intensity.

The results obtained during this PhD research show the need to characterize toxic cyanobacteria in freshwaters, as well as the conditions that influence MCs concentration dynamics. We showed that factors controlling the dynamics of toxic and non-toxic genotypes are complex. Nevertheless, detection tools can be developed to better understand these widely occurring phenomena. Therefore, efforts should go on in this field with collaborations between the scientists and the authorities.

**Résumé**

Les développements massifs de cyanobactéries, ou 'blooms', sont devenus un phénomène récurrent et global, de plus en plus important dans les eaux douces durant la dernière décennie. Ces efflorescences présentent des risques potentiels majeurs pour la santé humaine et animale et interfèrent négativement avec l'utilisation des eaux de surface, par exemple, pour le captage d'eau potable, les loisirs nautiques, l'irrigation, ou les exploitations piscicoles. En effet, les cyanobactéries sont capables de produire certaines toxines, les cyanotoxines.

La gamme de cyanotoxines comprend des hépatotoxines ou des agents inflammatoires, neurotoxines et dermatotoxines. Ces cyanotoxines sont principalement relarguées dans l'eau lors de la lyse des cellules du bloom. L'ingestion ou le contact avec de l'eau contenant des cellules de cyanobactéries ou des toxines peut poser des problèmes de santé.

Les microcystines (MCs), hépatotoxines et agents tumoraux sont les cyanotoxines les plus documentées. Leur synthèse s'effectue par l'expression des gènes qui codent pour la microcytine synthetase (*mcy*), un complexe enzymatique composé d'une succession de peptides synthétases non ribosomale (NRPS) et de polykétides synthétase (PKS). Les producteurs majeurs de MCs sont les genres *Anabaena, Microcystis*, et *Planktothrix*. Cependant, il est impossible de distinguer une souche toxique d'une souche non toxique à l'aide des critères morphologiques uniquement.

Lors de cette étude, des outils moléculaires ont été utilisés, optimisés, ou développés pour (i) caractériser la diversité moléculaire de la sous unité de l'ARN ribosomique 16S (ARNr 16S) des cyanobactéries planctoniques, pour (ii) pouvoir détecter les producteurs de MCs présents dans le milieux, pour (iii) identifier les taxons producteurs de MCs, et enfin (iv) de déterminer quels sont les facteurs environnementaux qui influencent la dynamique de génotypes toxiques et non toxiques dans les eaux de surface belges.

Quatre-vingt neuf souches de cyanobactéries ont été isolées et leur ARNr 16S ont été caractérisé. La diversité moléculaire du gène ARNr 16S a été étudiée dans 32 échantillons environnementaux via un gel en gradient dénaturant (DGGE). Pour évaluer la portée de ce travail, 114 séquences obtenues durant cette thèse (souches et DGGE) ont été comparées aux séquences Belges obtenues lors d'autres

recherches. Un total de 14 nouvelles unités opérationnelles taxonomiques (OTUs) a été obtenu au cours de ce doctorat.

Dans le cadre de la détection par réactions de polymérisation en chaîne (PCR) des gènes *mcyA/B/E*, l'ADN génomique de 162 échantillons environnementaux a été extrait. Les 3 gènes ont été retrouvé ensemble dans 64.2% des échantillons, tandis que le gène mcyB seul a été retrouvé dans 95.1% des cas.

Dans le cadre de l'identification des taxons porteurs du gène *mcyE*, une analyse de polymorphisme de longueur des fragments de restriction a été réalisé sur le gène *mcyE*. Ceci a permis de détecter la présence des génotypes potentiellement toxiques du genre *Microcystis* spp. dans la majorité des échantillons.

De manière à éviter les contraintes des méthodes d'analyse de la diversité dépendantes de la mise en culture, une approche combinant l'amplification des génomes et la méthode immuno-enzymatique ELISA a été testée sur des colonies environnementales appartenant aux genres *Microcystis* spp. et *Woronichinia* spp.. Les séquences de 3 gènes 'housekeeping' (*ftsZ, gltX, recA*), anisi que l'ITS (« internal transcribed spacer ») ont été analysé pour 11 colonies de *Microcystis*. Des MCs ont été détectées et quantifiées par ELISA dans 7 cas sur 11 en accord avec les détections des gènes mcy par PCR. Les séquences types (ST), obtenues par la concaténation des gènes 'housekeeping', se sont révélées endémiques. Une de ces colonies semble appartenir à une lignée non décrite jusqu'à présent. Un protocole similaire a été appliqué pour l'étude de 6 colonies du genre difficilement cultivable, *Woronichinia* spp.. L'analyse de la séquence du gène ARNr 16S a permis de confirmer l'identification morphologique des colonies. Cette technique a permis d'obtenir les premières séquences ITS et *rpoC1* du genre *Woronichinia* spp.. Enfin, elle a permis de découvrir la présence de séquences codant pour des NRPS et PKS dans les génomes des colonies. Ceci souligne le besoin d'apporter de nouvelles informations concernant ce genre quasi ubiquiste.

Lors de cette étude, il a été possible de suivre simultanément un génotype toxique et un génotype non toxique pendant une campagne d'échantillonnage de deux ans par PCR en temps réel. Les dynamiques de chacun de ces génotypes ont été influencées par la photopériode. De plus, la dynamique du génotype toxique était aussi influencée par l'intensité lumineuse.

Les résultats obtenus au cours de ce doctorat montrent le besoin de caractériser les efflorescences de cyanobactéries toxiques dans les eaux douces,

ainsi que d'identifier les conditions qui influencent les dynamiques des concentrations de MC. Nous avons montré que les facteurs controlant les génotypes toxiques et non-toxiques sont complexes. Néanmoins, des outils de détection peuvent être mis au point afin de comprendre ces phénomènes à incidence globale. Pour celà, les efforts devraient poursuivre en collaboration entre les scientifiques et les autorités concernées.

# Chapter 1 – Introduction

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## 1.1. Cyanobacteria

### 1.1.1. Origin of cyanobacteria

Cyanobacteria are among the oldest living organisms on Earth. They are phototrophic oxygenic bacteria that are considered to be responsible for the increase of oxygen in the atmosphere, and the change from a reductive to oxidative environment. Molecular traces (2α-methyl hopane polyols), that are hypothesized to be of cyanobacterial origin, were reported in 2.7 billion year old Australian shales and imply the existence of oxygenic photosynthesis before the oxidization of the atmosphere (Brock *et al.* 1999).

Cyanobacteria are a key microbial functional group that inhabit the modern stromatolites (Fig. 1) (Foster *et al.*, 2009), and therefore were possibly present in ancient stromatolites (Fig. 1), which were dominating the ancient Earth over 3 billion years ago (Grotzinger and Knoll, 1999).

During the last decade, microfossils attributed to cyanobacteria of different ages have been reported in literature (Schopf, 2000). However, these findings can be controversial as they are based on common morphological features found in the bacterial domain.

**Fig. 1,** left: Modern stromatolites in Shark Bay, Australia. From http://peelsoftly.blogspot.com/2009/04/setting-something-straight.html; right: Cross section of 1.8 billion year old fossil stromatolites at Great Slave Lake Canada. From: http://evolution.berkeley.edu/evosite/evo101/IIE2aOriginoflife.shtml.

### 1.1.2. Evolution of the cyanobacterial phylum

Cyanobacteria constitute one of the most morphologically diverse phylum of the bacterial domain. Cyanobacterial species occupy a wide range of habitats. There are unicellular or multicellular, coccoid or filamentous lineages. Certain species also have the capacity to differentiate cells for providing anaerobic conditions for nitrogen fixation (heterocysts), or for survival structures (akinetes). Recently, Schirrmeister and colleagues (2011) suggested that cyanobacteria descended from a multicellular ancestor. Besides, the comparison with fossil records suggested that this ancestor might occur during the great oxygenation event (2.45-2.22 billion year ago). This hypothesis was supported by a time calibrated phylogenetic study of 16S rRNA cyanobacterial sequences (Schirrmeister *et al.,* 2013; Fig. 2). Besides, it was shown that the morphological diversification process increased after the great oxygenation event.

Following the endosymbiosis theory (Gray and Doolittle, 1982), the chloroplast developed from an ancestral cyanobacterium. This is supported by molecular data, including the 16S rRNA gene phylogeny (Giovannoni, *et al*., 1988).

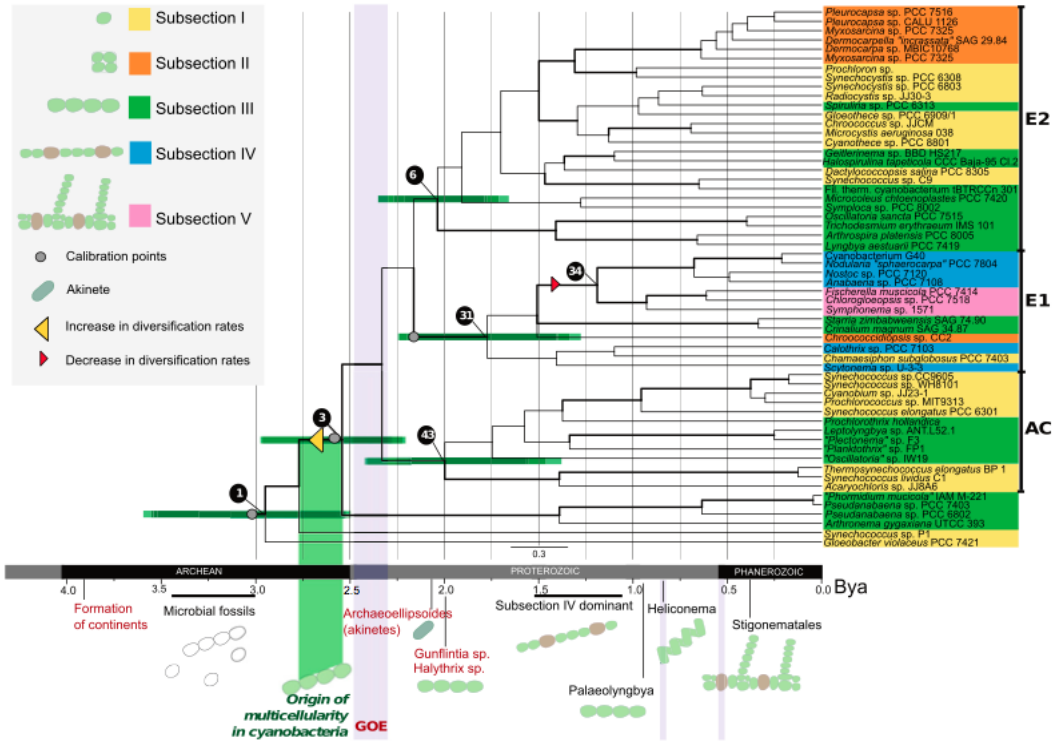


**Fig. 2,** Time calibrated phylogeny of the 16S rRNA gene from cyanobacterial sequence displaying divergence time estimates (Schirrmeister *et al.*, 2013).

### 1.1.3. Taxonomy of cyanobacteria

Cyanobacteria were first described as algae, and consequently are still referred to as 'Cyanophyta', or 'blue-green algae'. The study of cyanobacterial taxonomy started in the 18 century with the work of Linné (1753) who described the unicellular cyanobacteria. Until the end of 20 century, the nomenclature system of cyanobacteria followed the International Code of Botanical Nomenclature (ICBN). In 1978, Stanier *et al.* proposed to follow the International Code of Nomenclature for Bacteria (ICNB) because of their bacterial nature.

According to the Bergey's Manual of Systematic Bacteriology, and following the Stanier approach, cyanobacteria are divided into two groups: unicellular and filamentous cyanobacteria; and five subgroups corresponding to five cyanobacterial orders: Chroococcales, Pleurocapsales, Oscillatoriales, Nostocales, and Stigonematales (Waterbury, 2006).

To better identify a new species, the current cyanobacterial systematics is based on morphological, physiological, chemotaxonomic, and genotypic features (Oren, 2011). Studies In molecular systematics include DNA-DNA hybridization, 16S rRNA gene-based phylogeny, and the use of other markers, such as *cpcBA*-IGS, ITS, *kaiC*, *nifD*, *rbcLX*, *rpoC1* (*e.g.* Neilan *et al.,* 1995; Baurain *et al.,* 2002; Roeselers *et al.,* 2007; Lodders *et al.,* 2005; Rantala *et al.,* 2004), and now by genome sequencing (Shih *et al*., 2012). So far, the 16S rRNA gene sequences are the most represented cyanobacterial sequences in the public databases. In a recent review, Komárek (2010) proposed to apply the generic concept as described by Stackebrand and Goebel (1994) who suggested that the 16S rRNA sequence similarity threshold between bacterial genera would be of 95%. In the cyanobacterial phylum, most of the traditional genera corresponded to both genetic and phenotypic description. However, phylogenetic studies carried out during the two last decades showed that members of the Chroococcales, Pleurocapsales, and Oscillatoriales orders were not forming coherent phylogenetic lineages (Willmotte and Herdman, 2001). This observation was recently confirmed by the study of 126 genomes from the five subgroups as defined by Waterbury (2006). These incoherencies may partly be explained by the hypothesis of Shirrmeister *et al.* (2011) who suggested the occurrence of a multicellular common ancestor for all cyanobacteria, and multiple reversal events to unicellularity during the geological time scale (Fig. 2).

Finally, the taxonomic system proposed in the Bergey's Manual of Systematic Bacteriology is based on a limited number of genera for which isolates had been cultivated in laboratory (except *Trichodesmium*). This is problematic because it does not take the entire diversity of cyanobacteria into account. Moreover, morphological features such as colony morphotypes or gas vacuoles occurrence can be lost in long-term cultures (Visser *et al*., 2005; Rippka *et al.,* 1979). Over the last few decades, several authors have combined both 'bacteriological' and 'botanical' approaches for taxonomic identifications and revisions, including ecological, morphological, molecular and physiological data (*e.g.,* Wilmotte *et al.,* 1992; Suda *et al.,* 2002; Taton *et al.*, 2006, Rajaniemi *et al.,* 2006; etc.). This 'polyphasic' approach clarified the differences between several strains that were grouped based on their similar morphology, but which were not phylogenetically related on the basis of their 16S rRNA gene sequences. For example, the unicellular genus *Synechococcus* was divided in several generic units on the basis of 16S rRNA gene sequence similarities, ecology and morphology (*i.e.* thylakoid arrangement) (Komárek, 2010). More recently, Komárek *et al.* (2011) suggested that unicellular cyanobacteria of the species *Anathece* sp. and *Aphanothece* sp. might be placed into two distinct orders (Chroococcales and Synechococcales). Thus, it is possible that the number of cyanobacterial orders is still underestimated.

While the 16S rRNA based phylogeny is more or less corresponding to generic delineations (Komarek, 2010), it is more difficult to differentiate species. In 2006, Stackebrand and Ebers proposed to separate the bacterial species by using a 16S rRNA gene sequence similarity threshold between 98.7 and 99%. This proposal was based on the comparison of 16S rRNA sequence similarities and DNA-DNA reassociation values. In addition, Komárek (2010) suggested to use slight phenotypic and ecological differences as criteria to define cyanobacterial species.

### 1.1.4. Planktonic cyanobacteria in freshwater shallow reservoirs in Belgium

Cyanobacteria are often present and dominant in freshwater bodies. The increase of cyanobacterial mass proliferations, or 'blooms', in freshwater reservoirs is a direct consequence of eutrophication by anthropogenic activities (Reynolds *et al.,* 1981).

In the Benelux, the common bloom-forming cyanobacteria are the gas-vacuolated genera *Aphanizomenon spp.* Bornet et Flahaut*, Anabaena spp.* Bornet et Flahaut*, Microcystis spp.* Lemmerman, *Planktothrix* spp. Anagnostidis et Komárek*,* and *Woronichinia spp.* Elenkin (Ibelings, 2005; Willame *et al.,* 2005). Non bloom-forming taxa such as *Aphanocapsa spp.* Nägeli, *Aphanothece spp.* Nägeli, *Cyanobium spp.* Rippka et Cohen-Bazire, *Geilterinema spp.* Anagnostidis et Komárek, *Leptolyngbya spp.* Anagnostidis et Komárek, and *Pseudanabaena spp.* Lauterborn are also occurring in the water column (Willame *et al.,* 2006).

Gas vesicles are small cylinders filled with air that act as cell ballasts. Located in the gas vacuoles (Fig. 3), they allow the regulation ofthe cell's buoyancy in the water column and are considered as an advantage for the success of bloom-forming taxa. Buoyancy varies during the daylight cycle and may be regulated in three different ways (Oliver and Ganf, 2000). One involves changes in the cell density via the accumulation of carbohydrate reserves. During the light exposure, carbohydrates formed by photosynthesis counterbalances the floating of cells ensured by the gas vesicles. The second regulation process is mainly controlled by the variation of turgor pressure in the cells. As a result of the photosynthesis, turgor pressure can provoke the collapse of the gas vesicles. Finally, the buoyancy is controlled *via* the regulation of the expression of the *gvp* genes that encode for the synthesis of the gas vesicles. Buoyancy regulations allow cyanobacteria to move vertically in the water column. This vertical migration gives cyanobacteria a better access to light or nutrients below the thermocline or near the sediment when the nutrients are limiting (Visser *et al.*, 2005). In general, colony-forming cyanobacteria move faster than single filaments (Oliver and Ganf, 2000).

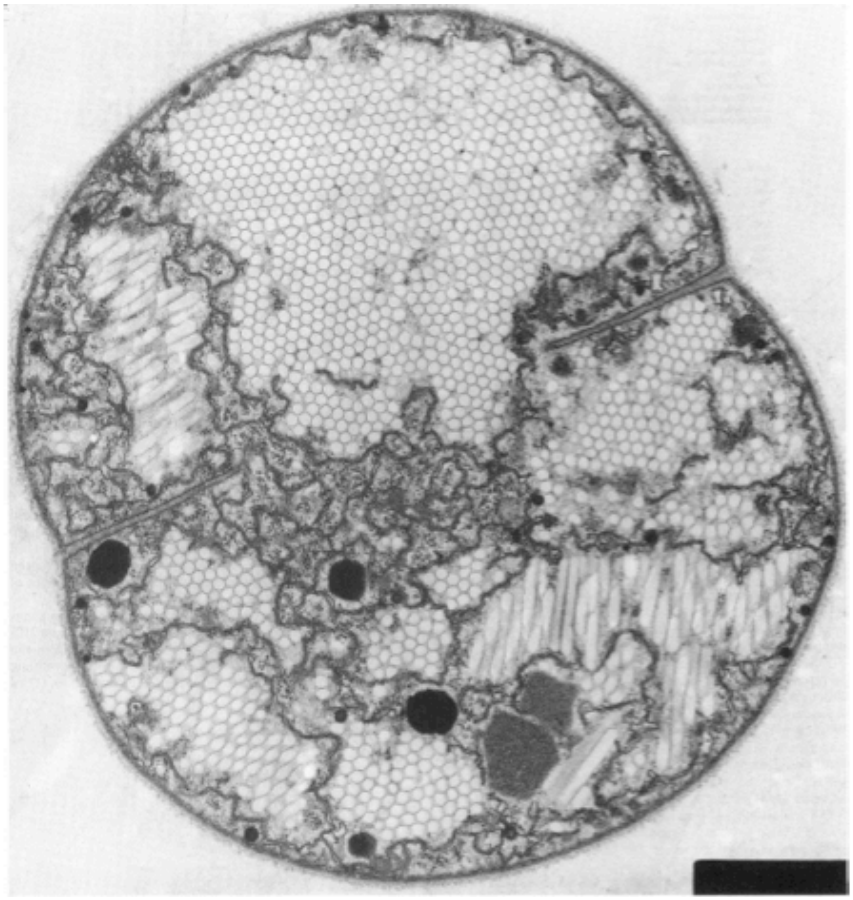

**Fig. 3,** Section of the cyanobacterium *Microcytis* sp. showing stacking of vesicles located in gas vacuoles. Magnification X 31 500 (Walsby, 1994).

**Fig. 4,** Scum formation during the collection of sample in Lake Féronval (2009).

When the water column is stable (no mixing), cyanobacteria can float up and accumulate at the surface, causing the formation of scums (Huisman *et al.*, 2004) (Fig. 4).

In shallow productive waters, the mixing regime may influence the growth of colony-forming or non-aggregated filamentous cyanobacterial taxa (Oliver and Ganf, 2000). Diurnal stratification will favour colony-forming species such as *Aphanizomenon flos-aquae* and *Microcystis aeruginosa,* whereas well mixing conditions will favour the dominance of single filamentous species such as *Planktothrix agardhii.*

Availability of nitrogen (N) and phosphorus (P) is crucial for the presence and growth of cyanobacteria. Low concentrations allow a high biodiversity of phytoplankton, whereas high concentrations may favour the growth of cyanobacteria. Unlike N, only few forms of P (*i.e.*, orthophosphates, some phosphorylated sugars, and phosphonate) are bioavailable for phytoplankton (Palenik *et al*., 2003). Hence, P is often the limiting nutrient.

## 1.2. Microcystins

### 1.2.1. Microcystin molecules

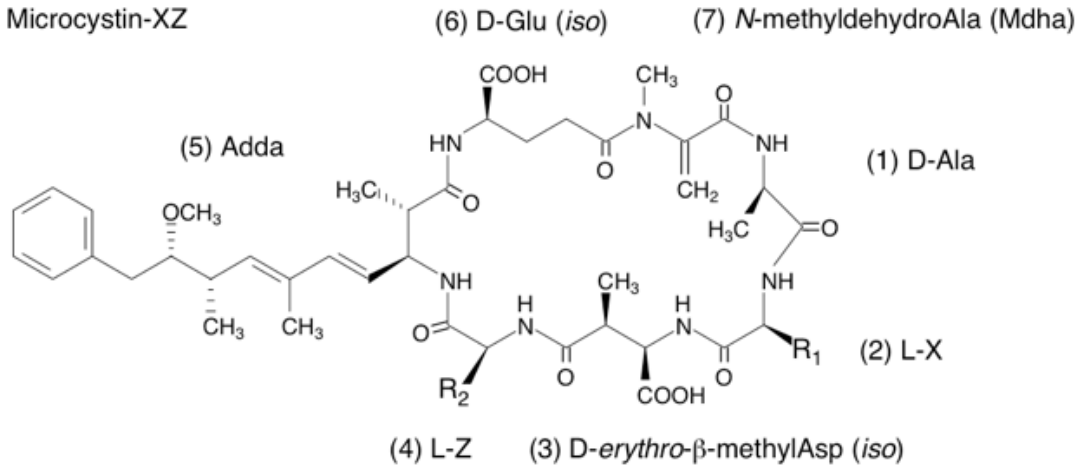


**Fig. 5,** General structure of microcystins, R1 and R2 are variable L amino acids (Nybom, 2013).

Among the known cyanotoxins, microcystins (MCs) are the most studied in the scientific literature. The molecular weight of these cyclic heptapeptides varies between 800 and 1100 Dalton. General structure of MCs is Ala(1)–X(2)–D-MeAsp(3)-Z(4)–Adda(5)–D-Glu(6)–Mdha(7) with variable L amino acid in X(2) and Z (*e.g.* for MC-LR X= Leucine (L) and Z= arginine (R)), and in which MeAsp is D-erythro-β-methylaspartic acid, MDha is N-methyldehydroalanine, Adda is 3-amino-9-methoxy-2,6,8- trimethyl-10-phenyldeca-4,6-dienoic acid (Fig. 5). So far, around 100 structural variants of MCs were reported in the literature (Nybom, 2013).

The MCs molecules are hepatotoxic and inhibit the protein phosphatases 1 and 2A (Carmichael, 1997), which have essential functions in eukaryotic cells, such as the activation of metabolic enzymes, gene expression or ion regulation.

Lipidic membranes of plants, animals, and bacteria are impermeable to MCs. Hence, toxicity occurs after ingestion and transport across the digestive system into blood and MC concentration in the liver. This may cause severe liver failure by cell structure disruption or intrahepatic bleeding, and result in the death of the organism (Kuiper-Goodman *et al.,* 1999).

Known as intracellular compound, MCs are released after the lysis of cyanobacterial cells (Young *et al.,* 2005) and may remain dissolved in the water for weeks after the collapse of blooms (Lahti *et al*., 1997).

So far, MCs were characterized in strains from different genera including *Anabaena spp., Anabaenopsis spp., Aphanocapsa spp., Fischerella spp., Gloeotrichia spp., Nostoc spp., Planktothrix spp., Radiocystis spp.,* and *Synechococcus spp.* (Sivonen, et al., 1992, Codd et al., 2005b; Domingos et al., 1999; Fiore et al., 2009; Carey et al., 2007; Genuario et al., 2010; Lombardo et al., 2006; Carmichael and Li, 2006). However, MCs are produced sporadically within strains from the same genera. Besides, it is impossible to identify a MC-producing from a non MC-producing strain simply on the basis of morphological criteria.

### 1.2.2. Genetic basis of microcystins production

Biosynthesis of microcystins is performed non-ribosomally through the thiotemplate activity of a large multifunctional modular enzymes complex composed of non-ribosomal peptide synthetases (NRPS), polyketides synthetases (PKS) and tailoring

enzymes. This enzyme complex is encoded by the *mcy* gene cluster composed of nine to ten genes depending on taxa (Rouhiainen *et al.,* 2004).

The *mcy* gene cluster sequences have been characterized in *Anabaena, Microcystis,* and *Planktothrix* (Rouhiainen *et al.,* 2004; Tillet *et al.*, 2000; Christiansen *et al.,* 2003). Soon after, it was demonstrated that these sequences were closely related to the nodularin (*nda*) synthetase genes from *Nodularia* (Moffitt and Neilan, 2004). Several gene inactivation studies showed that the presence of the *mcyA, B, D, E, F, H* genes and the gene *mcyT* (in *Plankthothrix* spp.) is necessary for MCs production (Dittmann *et al.,* 1997; Tillett *et al.,* 2000; Nishizawa *et al.*, 2000, 2001; Pearson *et al.,* 2004; Christiansen, *et al.* 2008). However, deletions in the N-methyl transferase domain of *mcyA* of *Anabaena spp.* strains resulted in the production of different MC variants (Fewer *et al*., 2008).

The *mcy* genes were probably inherited from a common ancestor, and consequently the patchy distribution of *mcy* gene in modern cyanobacteria is due to repeated loss processes (Rantala *et al*., 2004). However, an alternative explanation would be based on lateral gene transfer. Indeed, the presence of a transposase and a type IV pilus sytem (involved in DNA uptake in many bacteria) in the neighbouring regions of the *mcy* gene cluster suggested the possibility of horizontal gene transfer. A recent study of non MC-producing *Planktothrix* strains showed evidence of remnant *mcy* gene regions, which would correspond to a putative loss event (Christiansen *et al*, 2008).

An intriguing question is the role of microcystins. Indeed, if they had been selected during evolution to inhibit the eukaryotic competitors/predators, their origin would be more recent than suggested by Rantala et al. (2004). Other functions were proposed, and it was even hypothesized that their role has changed since their first origin.

To date, multiple studies were dedicated to the detection of putative MC-producing genera but only three papers were targeting different genera at the same time (Hisbergues *et al.*, 2003; Rantala *et al;* 2004, Jungblut and Neilan, 2006).

### 1.2.3. Other secondary metabolites

'Secondary metabolites' is a generic name qualifying peptides or compounds that are not essential for the life of the organism. The secondary metabolites are synthesized by the classical ribosomal pathway, by non-ribosomal peptide synthetase (NRPS), and/or polyketide synthetase (PKS) pathways. Until now, the ecological role of cyanobacterial secondary metabolites has not been solved. It may be useful for the organism against predation, for competition, fouling or resistance to extreme conditions.

Such molecules represent an increasing interest to the pharmaceutical industry (Singh et al. 2011). Indeed, they may have biological activities such as antiviral, antibacterial, antiprotozoal, antifungal, or antitumoral. During the last two decades, most of the effort was devoted to the study of toxic properties of these molecules. These include neurotoxicity (anatoxin, saxitoxin), cytotoxicity (lyngbyatoxin), inflammatory agents and hepatotoxicity (aplysiatoxin, cylindrospermospin, LPS, microcystins, nodularins) (Codd et al., 2005; Sivonen and Börner, 2008; Pearson et al., 2010).

The active quest for the discovery of cyanobacterial metabolites gave rise to a considerable amount of listed compounds, which can be organized in five major groups of molecules (Van Wagoner et. al, 2007). The first group (i) consists of polyketides, which is a large and diverse group that comprise linear or cyclic polyketides (*e.g.* oscillatoxin, etc.). Polyketides have a structurally intriguing carbon skeleton that comprises polyethers, polyenes, polyphenols, macrolides and enediynes. They are produced by the superfamily of enzyme called PKS. The second group (ii) is represented by the cyanopeptides, a large and diverse group of molecules (*e.g.* cyanopeptolins, microcystins, microginins, microviridins etc.), which can also be divided in linear and cyclic peptides weighting 300 to 2000 Da. For now, studies on the biosynthesis pathway of cyanopeptides revealed that a NRPS complex, a hybrid NRPS-PKS complex, as well as a traditional ribosomal pathway (*e.g.* cyanobactins) could code for the biosynthesis of these peptides. The third group (iii) is represented by the alkaloids (*e.g.* anatoxin, BMAA, curacin, cylindrospermopsin, saxitoxin, scytonemin, etc.) which can be divided in two subgroups: the linear-like and the ring-like alkaloids. NRPS-PKS hybrids (Nunnery *et al.*, 2010) and ribosomally expressed gene cluster (Soule *et al.*, 2009) have been

related to the biosynthesis of alkaloids. The fourth group includes the isoprenoids, which includes carotenoids and terpenoids. Finally, the fifth group gathers protein such as the potent anti-HIV molecule cyanovirin-N (Bolmstedt *et al.,* 2001) and other aromatic compounds.

The possible synergies between these secondary compounds and the cyanotoxins would deserve to be better studied.

### 1.2.4. Harmful effects of cyanobacterial populations and toxins on mammals' health

Hald first reported hazard to animals caused by cyanobacterial blooms in the eighteenth century in Denmark in 1833. Francis in Australia followed him in 1878, and Benecke in Poland in 1884 (see Codd *et al.,* 2005). Animal death, domestic (cattle, chickens, horses, sheep, pigs) and wild (birds, ducks, fish), were observed, and after examination, were associated with the drinking of poisoned water scums (Codd *et al*., 2005). Other animal death including African wild animal such as giraffe, rhinoceros, zebra or less exotic animals (frogs, squirrels, muskrat, salamanders and others) were also reported (Codd *et al*., 2005). More recent studies reported the death of dogs, which swallowed mat forming cyanobacteria (*Oscillatoria sp.* and *Phormidium sp.*) or drank the water nearby Cadel-Six, 2006).

Report of human illness or deaths are more episodic. However, the tragic outcome of Caruaru (Brazil) in 1996 where 52 over 100 patients died in a haemodialysis unit will remain in our memories (Pourria *et al*., 1998). The main outcome of this tragedy was the launching of a momentum of interdisciplinary co-operation and research, which resulted in the publication of a report on the behalf of the World Health Organization and a guideline value of 1 µg MC-LR/L for the drinking water (Chorus and Bartram, 1999).

More recently, a US coroner attributed the death of a teenager to the absorption of anatoxin-a-producing cyanobacteria (Stewart *et al*., 2006). The young boy died 48h after he swam in a golf pond in Winconsin (USA) (Fig. 6.).

4B SOUTH STAR-NEWS | TUESDAY, FEBRUARY 17, 2004

# Teen's death is a wake-up call about toxic algae

By Ramsey Campbell and Robert Sargent
*The Orlando Sentinel*

**This death happened in Wisconsin, but freshwater toxic algae abounds in the Southeast.**

ORLANDO, FLA. | Just a few hours after coming home from a pool party complaining of stomach cramps, Dane Rogers clenched his body in pain, gritted his teeth and died suddenly in his mother's arms.

What killed the active 17-year-old Wisconsin high-school soccer captain and honor student baffled medical experts for a year.

Now medical officials say Dane is the first known victim of freshwater toxic algae in the country. The death is a frightening alert for states like Florida, where warmer weather makes toxic algae more common in ponds, lakes and rivers than up North. Although Florida scientists have studied toxic algae for years, some experts think the state is too complacent about tracking illnesses and warning the public about potentially deadly threats.

"The likelihood of exposure here would seem to be greater," said John Burns, Florida's top freshwater-algae expert.

And there may be other victims whose deaths have been blamed on something different because toxic algae is rarely considered as a possibility.

"It certainly opened our eyes," said Dane County, Wis., coroner John Stanley, who had not heard of toxic algae before Dane's death in the summer of 2002.

The blue-green algae – or *cyanobacteria* – that commonly turn ponds and lakes scummy in warm weather also can trigger a variety of health problems, from abdominal distress to breathing problems to skin irritations.

Lake specialists and toxic-algae researchers are worried that medical officials haven't taken the threat seriously – especially in warm-weather states where algae levels in fresh water run wild.

Mr. Stanley, who determined that an evening dip in an algae-filled golf-course pond led to Dane's death, said there may be other victims whose demise is never explained.

Toxic algae were the last thing anyone considered when the investigation into Dane's death began. Mike Rogers said his son had come from an evening pool party with severe stomach cramps, vomiting and uncontrollable diarrhea.

Dane soon went into a series of seizures and then collapsed as his mother, Kim, tried to comfort him.

## Death a mystery

His distraught parents called paramedics, who tried to revive the teen without success. Dane died just before 3 a.m.

"There did not appear to be any logical explanation for Dane's death," Mr. Stanley said.

"It is such a bizarre thing – they couldn't pinpoint anything," said Mr. Rogers. "We thought it was something he had eaten."

Investigators ruled out drugs, alcohol or congenital heart problems. "This young person crashed and died after onset of nausea and vomiting," wrote Dr. Robert Huntington, who conducted the autopsy for the Coroner's Office.

"This is a sad, vexing case."

Mr. Stanley began retracing Dane's steps the day of his death. He had gone to soccer practice, then to a pool party that evening. In between, he ate Mexican food and pizza.

The food was analyzed, the soccer players were interviewed, and the pool water was studied. But nothing seemed to explain why Dane died. Then investigators found two other boys at the party who complained of abdominal pain, although much milder than Dane's.

Mr. Stanley discovered Dane and four friends – including the two other boys who had fallen ill – had all gone wading in a shallow golf-course pond the day before the soccer practice. It was 97 degrees that day.

"We just jumped in to cool off," said one of the boys who became only mildly ill. "We weren't in more than five, 10 minutes."

No significant pesticides or other known poisons were found in the water that could explain Dane's death.

Then a lake specialist at the University of Wisconsin sent an e-mail to Mr. Stanley with what he thought was a somewhat unlikely idea: toxic algae.

"When I read about the fatality in the newspaper, the circumstances sounded like poisoning by an algal toxin for several reasons," said Steven Carpenter, whose area of expertise is the study of freshwater lakes.

Wayne Carmichael – a professor of aquatic biology and toxicology for Wright State University in Ohio – studied blood and tissue samples from Dane and his friends. Tests showed high levels of anatoxin-a, a powerful neurotoxin produced by several species of toxic algae capable of sending the heart into arrest and causing severe abdominal distress.

Dr. Carmichael said the anatoxin-a found in Dane could only have come from blue-green algae.

The final autopsy report concluded that the likely cause of death was ingestion of toxic algae, which led to "acute diarrheal illness and subsequent death."

**Fig. 6,** Newspaper article reporting the death of a 17-year old teenager after cyanobacterial biomass exposure by Ramsey Campbell and Robert Sargent from *The Orlando Sentinel* in Star News.
http://news.google.com/newspapers?nid=1454&dat=20040217&id=530WAAAAIBAJ&sjid=3h8EAAAAIBAJ&pg=7018,185250

## 1.3. The situation in Belgium at the start of this Phd thesis

Van Hoof et al. (1994) performed the first detections of cyanotoxin in a *Microcystis aeruginosa* population in Flanders. Four years later, the first concentration of MC-LR was recorded (556 µg/g) in a *Microcystis aeruginosa* bloom near the city of Liège (Wallonia) (Wirsing *et al.,* 1998). In the framework of the European project MIDI-CHIP (EVK2-CT99-00026), Willame *et al.* measured the MC-LR concentration in samples dominated or co-dominated by *Anabaena, Aphanizomenon, Microcystis, Planktothrix,* and *Woronichinia* in 64 waterbodies across Belgium, France, and Luxembourg, and warned that 53% of the samples contained significant amounts of MC-LR. Besides, the highest concentration of MCs (2231 µg/g) recorded in a bloom dominated by *Woronichinia* was four times higher than the concentration measured by Wirsing *et al.,* 1998. In 2005, started the first national BelSPo 2-year project B-Blooms devoted to the study of MC-producing cyanobacteria in Belgian waterbodies. This project combined *mcy* genes PCR detection, genetic diversity assessment, microcystins quantifications, and a modelling approach to predict the formation of cyanobacterial proliferation. As main results, the *mcyA* and/or *mcyB* genes were detected in 79% of the samples, and 41% of the tested samples contained significant amount of MC-LR.

In 2007, started the second national BelSPo project B-Blooms2 (4 years) in which this PhD thesis was partially included. The main goals of this project were the improvement of knowledge on Belgian blooms, the reinforcement of communication and transfer of knowledge to relevant authorities, and the contribution to the establishment of guideline values and risk assessment procedures in Belgium. Among the main results of the study, it can be stressed that most of the cyanobacterial blooms consisted of potentially toxic taxa of the genera *Aphanizomenon*, *Microcystis*, *Planktothrix* and Anabaena. Improved molecular approaches demonstrated that genetic diversity within blooms can be high and that changes in strain dominance occur and can be caused by strong and specific trophic interactions. Several genes of the mcy cluster were regularly detected in bloom samples. The toxin analyses showed the presence of microcystins in all samples tested, the concentration of which exceeded World Health Organization Guideline Values for drinking- and recreational waters on several occasions. At the end of this project, the goals were partially achieved. Indeed, both the Vlaams Agentschap Zorg en Gezondheid in Flanders and the Service Public Wallonie in Wallonia engaged a communication campaign to raise public awareness. In March 2011, the result of the BBLOOMS 2 project were discussed in the Walloon parliament. In Flanders, the Vlaams Agentschap Zorg en Gezondheid is now following the 1 µg/L WHO guideline value for drinking water and 20 µg/L for recreational activities.

## 1.4. Thesis outline

The central aim of this thesis was to use, develop and optimize different molecular tools to bring insight into the genotypic diversity of MC-producing (toxic) and non MC-producing (non toxic) cyanobacteria in Belgian waterbodies, to identify the main MC-producers present in the Walloon region, and to elucidate the main factors influencing the dynamics of co-existing toxic and non toxic genotypes from the same taxa.

The MCs concentrations, and their variability during the warm season may depend on:

1. The cyanobacterial community
2. The presence of the *mcy* and identity(ies) of the *mcy* gene carriers
3. The variation of toxic and non-toxic genotypes abundances within the same population

In the chapter 2, an inventory of cyanobacterial 16S rRNA genotypes was performed to identify the putative MC-producing taxa present in the Belgian territory. Moreover, PCR and ELISA tested the potential toxigenicity of new strains.

Environmental samples were screened for the presence of three *mcy* genes in the chapter 3. Potentially toxic *Microcystis sp.* was observed in 95.1% of the samples. In order to identify the taxa of the *mcy* genes carriers, a RFLP analysis was performed. It was possible to identify *mcyE* gene carriers from the genus *Anabaena* spp., *Microcystis* spp., and *Planktothrix* spp. in Belgian waterbodies.

In chapter 4, we investigated the influence of environmental factors on two co-existing (toxic and on a non-toxic) ITS-genotypes of *Microcystis aeruginosa* in the environment. Both toxic and non-toxic genotypes appeared influenced by the photoperiod.

In the chapter 5, a cultivation independent approach combining both biochemical assay and genetic characterization was tested on colonial cyanobacteria of the genera *Microcystis.* and *Woronichinia..* This approach allowed to obtain new insights about the potential production of secondary metabolites by strains of the genus *Woronichinia.*

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# Chapter 2 – Diversity of freshwater planktonic cyanobacteria in Belgian waterbodies

Author's contribution

In this chapter, we aimed to assess the molecular diversity of planktonic cyanobacteria in Belgian waterbodies. At first, a polyphasic strategy was used to evaluate cyanobacterial diversity in the BBLOOMS-2 reference ponds by, both cultivation and cultivation independent approaches that were applied to the same samples from Lake Falemprise and Ixelles Pond I and II. However, in the wake of the relocation of the laboratory culture room and a significant decrease in the room temperature, we faced a massive loss of the strains isolated during the first two years of the PhD. Therefore, a second survey was organized by Y Lara in 2009 to collect fresh water samples from Lake Falemprise, Lake Féronval, Rénipont Pond, and Lake Ri Jaune. Thus, only the strains isolated after 2009 could be characterized.

In this section, the 2007 environmental samples, and data related to microcystins concentrations (ELISA) were acquired by the B-BLOOMS 2 partners at the University of Namur (UNamur), the University of Brussels (VUB), and the University of Dundee.

Samples from Schulensmeer Pond, Zonhoven Pond 5 were obtained by the partners of the University of Gent (UGent).

The isolation and molecular characterization work was carried out by Alexandre Lambion and Y Lara.

# Chapter 2 – Diversity of freshwater planktonic cyanobacteria in Belgian waterbodies

## 2.1. Introduction

Cyanobacteria are responsible for the production of a wide range of bioactive compounds, such as potent toxins (cyanotoxins). These include neurotoxins, cytotoxins, inflammatory agents and hepatotoxins (Codd *et al.*, 2005b; Sivonen and Börner, 2008; Pearson *et al.*, 2010).

Microcystins (MCs), hepatotoxins and tumour promoters are the most documented of the cyanotoxins. These cyclic heptapeptides were first identified from the cyanobacterium *Microcystis* (Botes *et al.*, 1984; Carmichael *et al*., 1988). Later, MCs were characterized in different planktonic and benthic natural populations and isolated strains from different genera: *Anabaena* spp.*, Aphanocapsa* spp.*, Gloeotrichia* spp.*, Fischerella* spp.*, Microcystis* spp.*, Nostoc* spp.*, Planktothrix* spp.*, Radiocystis* spp.*, Synechococcus* spp. (Sivonen, *et al*., 1992, Codd *et al*., 2005b; Domingos *et al.*, 1999; Fiore *et al.*, 2009; Carey *et al.*, 2007; Oksanen *et al.,* 2004; Genuario *et al*., 2010; Lombardo *et al*., 2006; Carmichael and Li, 2006). Over 90 structural variants have so far been characterized. MCs therefore represent good models to study cyanotoxin genes expression, and the production, function(s) and dynamics of the toxins in the environment.

In the last decade, intensive efforts have been devoted to the identification and characterization of freshwater cyanobacteria. The 16S rRNA gene sequence has been shown to be an efficient phylogenetic marker for prokaryotic classification (Rosselo-Mora and Amann, 2001). The analysis 16S rRNA gene sequence is now widely used for prokaryotic identification (Ouellette and Wilhlem, 2003) in cultivation or in the environment. Cyanobacterial 16S rRNA gene sequences can be obtained by different techniques, with or without cultivation (e.g. clone library, DGGE, 454 pyrosequencing), and they can be compared to sequences of the global database GenBank. This first comparison generally allows for a preliminary identification of the cyanobacterial genera present in the sample. On the other hand, the phylogenetic analyses give a schematic representation of the cyanobacterial evolution and diversity, and enable a more precise phylogenetic affiliation.

In Belgium, blooms are commonly dominated by the genera *Anabaena* spp.*, Aphanizomenon* spp.*, Microcystis* spp.*, Planktothrix* spp.*,* and *Woronichinia* spp. (Willame *et al,* 2005). Belgian cyanobacterial communities are also composed of 'accompanying' taxa (Willame *et al.*, 2006), which are less known and abundant such as picocyanobacteria (e.g. *Aphanocapsa* spp. and *Cyanobium* spp.), small filaments (e.g. *Leptolyngbya* spp.*, Pseudanabaena* spp.) and others (See Chapter 1 paragraph 1.1.4). In the course of the EUproject MIDICHIP and the BelSPO project BBLOOMS (Wilmotte *et al.,* 2008), a first assessment of the cyanobacterial diversity in freshwater Belgian waterbodies was performed.

In order to perform an inventory of planktonic cyanobacteria in Belgian freshwaters, we isolated strains from different waterbodies to characterize their 16S rRNA gene sequences. PCR detection of *mcy* genes and ELISA assay were carried out to assess the isolates' potential toxicity. In parallel, 16S rRNA gene sequence analysis was carried out on environmental samples using the DGGE approach. Finally, the new sequence data were compared with sequences previously obtained.

## 2.2. Material and methods

### 2.2.1. Sampling

For strain cultivation, samples were taken from the Lake Falemprise, Lake Féronval, Ixelles pond I and II, Neerpede Pond 4, Rénipont Pond, Lake Ri Jaune, Schulensmeer Pond, Zonhoven Pond 5 in 2009 (See chapter 3 for further details). From each site, 2 to 15 ml of water was collected and used for inoculation in growth media, and 2 to 15 mL of water was concentrated (three times) with a 50 µm pore sized phytoplankton net.

For environmental DNA analyses, samples were collected during the BBLOOMS-2 2007 survey, 21 from the Lake Falemprise, 9 from Ixelles Pond II, and 2 from additional lakes (quarry of Ecaussines 2007 and Lake Chérapont 2007). Water volumes were filtrated through 0.2 µm Supor filters PES Pall Life Science (NY, USA) until clogging.

### 2.2.2. Strains isolation and characterization

Fresh samples were inoculated on agar plates and in liquid medium containing 500 µg/mL$^{-1}$ of cycloheximide to avoid growth of eukaryotic algae. BG11, B11$_0$, and Z8 media (Rippka *et al.*, 1979) were used to increase the diversity of cultivated cyanobacteria and the number of strains of toxic and non-toxic genotypes. Cultures were transferred three times from liquid to solid media until a mono-cyanobacterial stage was reached. Culture were grown under constant white fluorescent illumination (40 µmol photon m$^{-2}$ s$^{-1}$) at 20°C. They were checked under epifluorescence microscopy to investigate contamination by picocyanobacteria.

The strains were observed with a Leica DM LB2 microscope equipped with a Canon Powershot S50 digital camera. Cell dimensions were measured using the AXIOVISION software from ZEISS. Taxonomic identification was based on the studies by Komarek and Anagnostidis (1998, 2005), and Perkerson *et al.* (2011).

### 2.2.3. DNA extraction

Genomic DNA of cultivated strains was extracted using the DNeasy Plant mini kit (Qiagen).

In order to extract DNA from filters (environmental samples), we used a modified hot phenol method (Giovannoni et *al.,* 1990). For optimal cell lysis, lysozyme (1.25 mg/mL) and proteinase K (0.3 mg/mL) were added to the lysis buffer. DNA extraction was performed in phenol-chloroform-isoamyl alcohol and (25:24:1, vol/vol/vol) chloroform-isoamyl alcohol (24:1, vol/vol). DNA was precipitated overnight at -20°C in sodium acetate and ethanol. Then, precipitated DNA was washed in 70% ethanol and suspended in 50 µL of TE$^{-4}$ (10 mM Tris-HCl, pH=7.4–8, 0.1 mM EDTA). Purification of extracted DNA was carried out using Promega Wizard DNA clean up system kit (Madison, WI, USA). Then, the purified genomic DNA was stored at -20°C in water. Genomic DNA was extracted from 32 filtered samples from the 2007 survey, 21 from the Falemprise Lake, 9 from Ixelles Pond II, and 2 from additional lakes (quarry of Ecaussines 2007 and Lake Chérapont 2007). These were used as templates for PCR.

2.2.4. 16S rRNA gene amplification

For strains characterization, a large segment of the 16S rRNA gene and the ITS region were amplified using primers 359F/23S30R. We used the cyanobacteria specific primers 359F as designed by Nübel *et al.* (1997) and 23S30R as described by Taton *et al*. (2003). PCR run was performed as follows: one denaturation cycle of 5 min at 94 °C; 25 cycles of 45 s at 94 °C, 1 min at 54 °C, 1 min 30 s at 68 °C. PCR products were purified with GE Healthcare GFX PCR product purification kit and sent for sequencing at GIGA facilities (ULg, Liège, Belgium).

PCR products for the DGGE experiment were obtained by performing a nested strategy. A first PCR amplified a fragment that contained a partial 16S rDNA sequence together with the ITS as described above. Then, a second run was performed with primers 359F and 781R(a) or 781r(b), as described by Boutte *et al.* (2006). The combination of 781R(a) and 781R(b) primers was used to preferentially amplify respectively filamentous and unicellular cyanobacteria. In order to ensure the melting stability of the DNA fragments, a GC clamp (5'-CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CC-3') was added at the 5' end of each reverse primer. Primers are listed in the Table 1.

For filamentous cyanobacteria, PCR programs were performed as follow: for the first run with 359F/23S30R, one denaturation cycle of 5 min at 94 °C; 25 cycles of 45 s at 94 °C, 1 min at 54 °C, 1 min 30 s at 68 °C and a final elongation step of 7 min at 68 °C; for the second run with 359F/781R(a) a first denaturation step of 5 min at 94 °C; 25 cycles of 1 min at 94 °C, 1 min at 60 °C, 1 min at 68 °C and a final elongation step of 20 min at 68 °C.

For the unicellular cyanobacteria, PCR programs were performed as follow: for 359F/23S30R reaction one denaturation cycle of 5 min at 94 °C; 10 cycles of 45 s at 94 °C, 1 min at 54 °C, 1 min 30 s at 68 °C and a final elongation step of 7 min at 68 °C; for the second run with 359F/781R(b) a first denaturation step of 5 min at 94 °C; 25 cycles of 1 min at 94 °C, 1 min at 60 °C, 1 min at 68 °C and a final elongation step of 20 min at 68 °C.

All PCR were performed with a Bio-rad Icycler thermocycler (Hercules, CA). The first reaction was done in a total PCR volume of 25 µL containing 1 µL of template DNA (20.5-477.5 µg), bovine serum albumin (1 mg/mL), 1x PCR reaction buffer (HT Biotechnologies LTD, England), deoxynucleoside triphosphate at 200 µM

each (Eurogentec, Belgium), 0.5 µM of primers 359F and 23S30R (Table 1) and 1 Unit of Super *Taq* Plus with proofreading activity (HT Biotechnologies LTD, England). The second PCR was carried out in a total volume of 50 µL containing the same reagents as the first reaction except for the reverse primers.

Equal amount of PCR products were loaded onto 6% (w/v) polyacrylamide gels in 1X TAE (20 mM Tris acetate pH= 7.4; 10 mM acetate, 0.5 mM disodium EDTA) with a denaturing gradient 45-55% (100% denaturant is 7 M urea and 40% formamide). The DGGE was run at 60V for 999 mins. The gels were post-stained with 2 µL of Gelstar (BioWhittaker Molecular Applications, Maine, USA) mixed with 15 mL of 1 pre-warmed (37°C) 1xTAE buffer. The most intense bands were associated with the major populations (Willame *et al.,* 2009).

The obtained bands were excised with a sterile scalpel and incubated in $TE^{-4}$ buffer overnight at 4 °C. The eluted DNA from each band was amplified using the primers 359F and 784R. The PCR products were sequenced as described above.

**Table 1.** Primers used in this study. F (forward) and R (reverse) refer to the primer position in relation to the rRNA.

| Primer | Sequence (3' → 5') | Position in 16S or 23S *rrn* gene | Reference |
|---|---|---|---|
| 359F | GGG GAA TTT TCC GCA ATG GG | 359 | Nübel *et al*., 1997 |
| 781R(a) | GAC TAC TGG GGT ATC TAA TCC CAT T | 781 | Modified from Nübel *et al*., 1997 |
| 781R(b) | GAC TAC AGG GGT ATC TAA TCC CTT T | 781 | Modified from Nübel *et al*., 1997 |
| 784R | GACTACTGGGGTATC TAATCCC | 784 | Modified from Nübel *et al*., 1997 |
| 23S30R | CTT CGC CTC TGT GTG CCT AGG T | 30 | Taton *et al*. 2003 |

2.2.5. Sequence analyses

For phylogenetic analyses, the closest relatives of the sequences obtained during this study were obtained by BLAST searches and downloaded from the GenBank database. All the sequences were then aligned using the MUSCLE algorithm (Edgar, 2004), and the alignments were manually corrected for each locus. Distances and phylogenetic trees were computed using MEGA 5 software (Tamura *et al.,* 2011). In

order to create a 16S rRNA sequences database of cyanobacteria from Belgian waterbodies, we collected in GenBank and in our laboratory, 142 sequences of cultivated cyanobacterial strains and clone libraries isolated from Belgian freshwater. For the 16S rRNA sequences trees, distances were computed using the Jukes and Cantor correction. Trees were built with the Neighbor-joining algorithm. Neighbor-joining bootstrap replicates (1000 replicates) were calculated, and values over 50% are indicated at the concerned nodes. The *E. coli* K12 (V00348) 16S rRNA sequence was used as outgroup. All positions containing gaps and missing data were eliminated. The bootstrap consensus tree inferred from 1000 replicates was taken to represent the evolutionary history of the taxa analyzed.

Mothur v.1.28.0 (Schloss *et al*., 2009) was used for clustering our sequences in Operational Taxonomic Units (OTU). Pairwise distances between the aligned DNA sequences were first calculated using the 'dist.seq' command. The obtained distance matrix was used afterwards by the "cluster" command to group the sequences into OTUs using the average neighbor method. OTU threshold was chosen according to Stackerbrandt and Ebers (2006) (see the General Introduction of the present manuscript for more information).

### 2.2.6. Potential toxicity of the isolated strains

The *mcyA* (mcyA-CdF/ mcyA-CdR) and *mcyE* (mcyEF2/ mcyER4) primer pairs were designed by other authors (Hisbergues *et al.,* 2003; Rantala *et al.,* 2004), on the basis of the *Anabaen*a sp., *Microcystis* sp., *Planktothrix* sp. and *Nostoc* sp. microcystin synthetase sequences. As a result, these primer sets were able to amplify the *mcyA* and *mcyE* partial sequences in these 4 genera. Thus, we used these primers to detect the presence of potential toxigenic genotypes from several microcystin-producing genera in a single reaction. (See chapter 3 paragraph 3.2.7. for further details)

The ELISA anti MC-LR assay were performed at the University of Dundee as described in chapter 3 paragraph 3.2.4..

## 2.3. Results and discussion

### 2.3.1. Isolation and characterization strains of cyanobacteria

During this thesis, we were able to cultivate the 'common' bloom-forming cyanobacteria *Microcystis,* together with non-bloom forming genera, which are less abundant in the water column, such as *Cyanobium, Nodosilinea* (formerly *Leptolyngbya*)*, Pseudanabaena* and *Snowella.* However, we were unable to isolate the dominant cyanobacteria except for *Snowella* in Lake Falemprise. This may be explained by the incubation at low temperature in our culture room (20°C). A total of 140 algal cultures were isolated (Table 2). We obtained 91 monoalgal cultures, which were mostly *Cyanobium/Synechococcus* like (40 strains) followed by small filaments (37 strains)., and *Microcystis* (10 strains) and *Snowella* (4 strains). The16S rRNA gene was successfully amplified and 89 partial sequences were obtained and analyzed. Phylogenetic analyses (Fig. 1) showed three major clades supported by 100, 99, and 65 bootstraps, respectively. For the 91 strain sequences, 21 OTUs were defined using a threshold of 98.7% similarity and partial 16S rRNA gene (*E. coli* positions 546–1212). Among these three clades, the Belgian strains were distributed into 16 OTUs (Fig. 1).

The first clade was strictly composed of picocyanobacteria. Thirty-nine strains were divided into three OTUs (OTUs 1, 2, 3) corresponding to the description of *Cyanobium* spp. genus. The OTU 1 corresponded to the *Cyanobium gracile* cluster, represented by the sequences of PCC 6307, Sai002, Sai005, Suigetsu-CG3, and 1BB04S06. The OTU 2 contained strains belonging to the formerly named 'subalpine cluster II' represented by *Cyanobium* sp. JJ21RS8 (see Callieri *et al*., 2012). One strain belonged to the OTU 3, which was also composed of cosmopolitan strains (the Arctic, Portugal, Czech Republic, Germany) that belong to the group A2 (see Callieri *et al*., 2012).

The second clade was composed of eight strains divided into five OTUs (OTU 4-8). The OTU 4 (Fig. 1) was composed of six Belgian strains corresponding to the cosmopolitan nitrogen-fixing filamentous species *Nodosilinea nodulosa* (Perkerson *et al.,* 2011). These strains were forming nodules under low light exposure. The two remaining strains were clustering together in the OTU 7 without any of the Genbank sequences.

The third clade was composed of unicellular and filamentous cyanobacteria. Two OTUs (OTU 19 and 20) corresponded to Chroococcales species. The OTU 19 was composed of strains clustering with *Snowella* sp. (Fig. 2, p, q) (Rajaniemi-Wacklin *et al.*, 2005). The OTU 20 was composed of *Microcystis* strains with two identified morphotypes (*i.e. aeruginosa, ichthyoblabe*) and strains with no distinguishable morphotype (Fig. 2, s, t, u). Eight OTUs (OTU 11, 12, 13, 14, 15, 17, 18 and 19) were composed of filamentous cyanobacteria (Fig. 1). Strains belonging to four of these OTUs (OTU 11, 12, 13 and 14) had the characteristics of the Pseudanabaenaceae family, and of *Wilmottia murrayi* (Strunecky *et al.*, 2011) and *Pseudanabaena catenata* Lauterborn 1915 (Fig. 2, i, j, k, l). Finally, the remaining strains belonged to four OTUs (OTU 15, 17,18 and 19). Strains from the OTU 15, 17 and 18 where characteristic of cyanobacteria belonging to the LPP-B group (*Lyngbya*, *Plectonema*, *Phormidium*) as described by Rippka et al. (1979), as they were thin sheath-forming filaments. Strains from OTU 15 were clustering with an uncultured clone recovered from a sample dominated by the epiphytic sulfur oxidizing *Thiothrix* spp. on marcophytes from hydrothermal vents (Konkol *et al.*, 2010). Strain FW056 from OTU 17 remained unidentified. Finally, the OTU 19 gathered Belgian strains with *Geitlerinema carotinosum* P013, and the Pseudanabaenaceae cyanobacterium HA4216-MV1.

**Table 2**. Number of strains obtained for each sampling lakes, n.d.: not determined

| Lake/pond | Dominant cyanobacteria | Number of strains | Identification |
|---|---|---|---|
| Falemprise | *Snowella* spp. (no bloom) | 28 | *Cyanobium, Snowella* |
| Féronval | *Woronichinia naegeliana* (bloom) | 42 | *Cyanobium, Microcystis, Snowella,* LPP-B*, Nodosilinea,* Pseudanabaenaceae |
| Ixelles pond I | *Planktothrix agardhii* (bloom) | 9 | *Cyanobium,* LPP-B |
| Ixelles pond II | *Woronichinia naegeliana* (bloom) | 3 | *Wilmottia murrayi* |
| Neerpede Pond IV | *Microcystis* spp. (bloom) | 1 | *Cyanobium* |
| Rénipont | *Limnothrix* spp. (bloom) | 27 | *Cyanobium, Microcystis, Nodosilinea* |
| Ri Jaune | *Microcystis* spp. (bloom) | 11 | *Cyanobium,* Pseudanabaenaceae |
| Schulensmeer | *Woronichinia naegeliana* (bloom) | 8 | *Cyanobium, Microcystis* |
| Zonhoven Pond V | n.d. | 11 | *Cyanobium,* Pseudanabaenaceae, LPP-B |

2.3.2. Characterization of potentially toxic strains

Strains were tested for the presence of *mcy* genes by PCR and/or sent to Dundee for ELISA reactions. Twenty-one cultures were identified as potential microcystin producers (Table 3). The *mcyA* and *mcyE* genes were only simultaneously detected

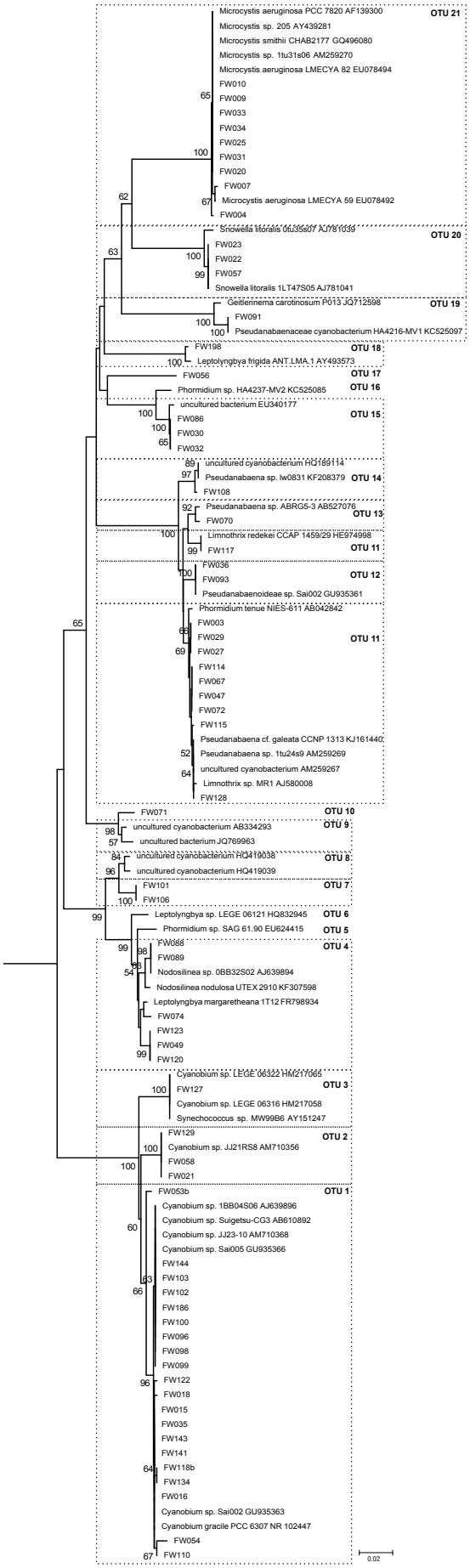
in one strain of *Microcystis* (OTU 21) and one strain of *Cyanobium gracile* (OTU 1). To date, this is the first report of a *Cyanobium gracile* with *mcy* genes. The *mcyE* gene was also found in two other *Cyanobium gracile* strains that were isolated from the same lake (Rénipont).

A significant concentration (48.7 µg/L) of MC-LR equivalent was found in another *Cyanobium* strain from lake Féronval. This is not the first time that MC-producing freshwater picocyanobacteria are chatacterized. MCs were already detected in *Aphanocapsa* cumulus by ELISA and HPLC in Caruaru, Brazil (Domingos *et al*., 1999).

Significant amounts of MC-LR eq were also detected in *Microcystis* sp. strain (OTU 1), Oscillatoriales that were clustering with *Nodosilinea* sp. (OTU 4), LPP-B like (OTU 10), *Pseudanabaena* sp. (OTU 11), *Snowella* sp. (OTU 20) and an unknown Oscillatoriaceae strain (OTU 7) (Fig. 1).

Strikingly, *Microcystis* sp. strains positive for ELISA detection were negative for PCR detection. This led to the hypothesis that either the ELISA was not specific for microcystins and reacted with some unspecific product or some mutation had occurred in the annealing region of *mcy* PCR primers sets. Recently, deletions and insertions were found to occur in the *mcy* genes cluster. Indeed, a deletion of the *mcyT* region was found in the *Planktothrix* genome, and resulted in a non-microcystin producing strain (Christiansen *et al*., 2008). In such cases, positive PCR detection is not concomitant with microcystin-based toxicity. On the other hand, deletion of other regions might have no consequence for microcystin production but could hinder the detection of *mcy* genes. Indeed, 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 was responsible for the production of a different variant of microcystin. Nevertheless, it was previously shown that the presence of the *mcyA, B, D, E, F* and *mcyH* genes is necessary for microcystin production (Dittmann *et al*., 1997; Tillet *et al*., 2000; Nishizawa *et al*., 2001; Pearson *et al*., 2004).

NRPS-like sequences were detected in a strain of *Snowella* (OTU 20). This result shows that this genus already known to carry cyanobactin genes (Leikoski *et al.,* 2009) could produce other secondary metabolites (Table 3).


Microcystis aeruginosa PCC 7820 AF139300
OTU 21
Microcystis sp. 205 AY439281
Microcystis smithii CHAB2177 GQ496080
Microcystis sp. 1tu31s06 AM259270
Microcystis aeruginosa LMECYA 82 EU078494
FW010
FW009
FW033
FW034
FW025
FW031
FW020
FW007
Microcystis aeruginosa LMECYA 59 EU078492
FW004
Snowella litoralis 0635s07 AJ781039
OTU 20
FW023
FW022
FW057
Snowella litoralis 1LT47S05 AJ781041
Geitlerinema carotinosum P013 JQ712598
OTU 19
FW091
Pseudanabaenaceae cyanobacterium HA4216-MV1 KC525097
FW198
OTU 18
Leptolyngbya frigida ANT.LMA.1 AY493573
OTU 17
FW056
Phormidium sp. HA4237-MV2 KC525085
OTU 16
uncultured bacterium EU340177
OTU 15
FW086
FW030
FW032
uncultured cyanobacterium HQ189114
Pseudanabaena sp. lw0831 KF208379
OTU 14
FW108
Pseudanabaena sp. ABRG5-3 AB527076
OTU 13
FW070
Limnothrix redekei CCAP 1459/29 HE974998
FW117
OTU 11
FW036
OTU 12
FW093
Pseudanabaenoideae sp. Sai002 GU935361
Phormidium tenue NIES-611 AB042842
FW003
FW029
OTU 11
FW027
FW114
FW067
FW047
FW072
FW115
Pseudanabaena cf. galeata CCNP 1313 KJ161440
Pseudanabaena sp. 1tu24s9 AM259269
uncultured cyanobacterium AM259267
Limnothrix sp. MR1 AJ580008
FW128
FW071
OTU 10
uncultured cyanobacterium AB334293
OTU 9
uncultured bacterium JQ769963
uncultured cyanobacterium HQ419038
OTU 8
uncultured cyanobacterium HQ419039
FW101
OTU 7
FW106
Leptolyngbya sp. LEGE 06121 HQ832945
OTU 6
Phormidium sp. SAG 61.90 EU624415
OTU 5
FW088
OTU 4
FW089
Nodosilinea sp. 0BB32S02 AJ639894
Nodosilinea nodulosa UTEX 2910 KF307598
Leptolyngbya margaretheana 1T12 FR798934
FW074
FW123
FW049
FW120
Cyanobium sp. LEGE 06122 HM217055
OTU 3
FW127
Cyanobium sp. LEGE 06316 HM217058
Synechococcus sp. MW99B6 AY151247
FW129
OTU 2
Cyanobium sp. JJ21RS8 AM710356
FW058
FW021
FW053b
OTU 1
Cyanobium sp. 1BB04S06 AJ639896
Cyanobium sp. Suigetsu-CG3 AB610892
Cyanobium sp. JJ23-10 AM710368
Cyanobium sp. Sai005 GU935366
FW144
FW103
FW102
FW186
FW100
FW096
FW098
FW099
FW122
FW018
FW015
FW035
FW143
FW141
FW118b
FW134
FW016
Cyanobium sp. Sai002 GU935363
Cyanobium gracile PCC 6307 NR 102447
FW054
FW110
0.02

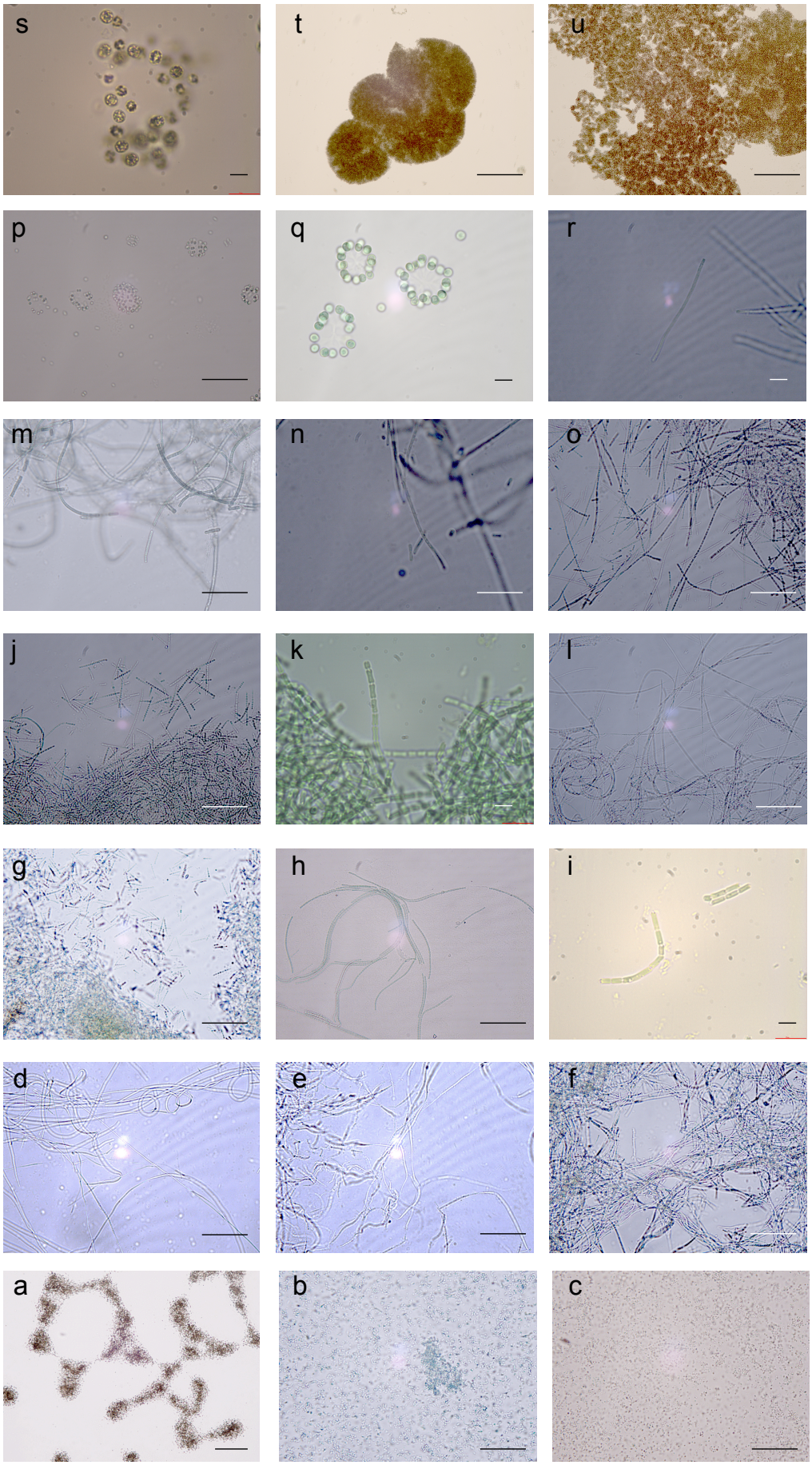

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**Fig. 1,** The 16S rRNA distance tree of Belgian cyanobacterial strains obtained during this study. Total alignment length was 666 nt. Distances were computed using the Jukes and Cantor correction and a Neighbor-joining tree was built with the software MEGA5 (Tamura *et al.,* 2011). Bootstrap replicates values are indicated at the nodes. Cutoff for OTU delineation was set at 98.7%.

**Fig. 2,** Photomicrographs of the morphospecies belonging to the Belgian's OTU obtained during this thesis. **a**; *Cyanobium* sp. FW021 (OTU 2). The scale indicates 150 µm. **b;** *Cyanobium* sp. FW058 (OTU 2). The scale indicates 50 µm. **c;** *Cyanobium gracile* FW015 (OTU 1) The scale indicates 50 µm. **d;** strain FW071 (OTU 10) The scale indicates 50 µm. **e;** strain FW106 (OTU 7). The scale indicates 50 µm. **f;** *Nodosilinea* sp. FW074 (OTU 4). The scale indicates 50 µm. **g;** *Pseudanabaena* sp. FW093 (OTU 12). The scale indicates 50 µm. **h;** *Pseudanabaena catenata* FW029 (OTU 11). The scale indicates 50 µm. **i;** *Pseudanabaena catenata* FW028 (OTU 11). The scale indicates 5 µm. **j;** *Pseudanabaena* sp. FW070 (OTU 13). The scale indicates 50 µm. **k;** *Pseudanabaena* sp. FW077 (OTU 13). The scale indicates 5 µm. **l;** *Wilmottia murrayi* FW117 (OTU 14). The scale indicates 50 µm. **m;** *Phormidium* 'like' strain FW056 (OTU 17). The scale indicates 50 µm. **n;** strain FW086 (OTU 15). The scale indicates 5 µm. **o;** strain FW108 (OTU 14). The scale indicates 50 µm. **p;** *Snowella* sp. FW022 (OTU 20) scales indicates 50 µm. **q;** *Snowella* sp. FW024 (OTU 20) scales indicates 5 µm. **r;** strain FW091 (OTU 19) scales indicates 5 µm. **s;** *Microcystis* sp. FW001 (OTU 21). The scale indicates 5 µm. **t;** *Microcystis ichthyoblabe* FW004 (OTU 21) The scale indicates 50 µm. **u;** *Microcystis aeruginosa* FW009 (OTU 21). The scale indicates 50 µm.

**Table 3**. Summary of strains that potentially produce secondary metabolites, according to PCR detection and ELISA for MC. - :absence, + : presence ; nd: not determined.

| Strain | Origin | Order | Most similar 16S rRNA sequence (analysis by BLAST) | PCR detection | | | ELISA (MC µg/L) |
|---|---|---|---|---|---|---|---|
| | | | | *mcyA* | *mcyE* | NRPS | |
| FW001 | Féronval | Chroococcales | 100% *Microcystis* PCC7820 (AF139300) | - | - | + | 2.106 |
| FW003 | Féronval | Oscillatoriales | 99.5% *Pseudanabaena* sp 1tu24s9 (AM259267) | - | - | + | nd |
| FW009 | Féronval | Chroococcales | 100% *Microcystis* LMECYA 82 (EU078494) | - | - | nd | 3.42 |
| FW024 | Falemprise | Chroococcales | 99.5% *Snowella litoralis* 1LT47S05 (AJ781041) | - | - | - | 1.081 |
| FW027 | Féronval | Oscillatoriales | 99.7% *Pseudanabaena* sp 1tu24s9 (AM259267) | - | - | + | nd |
| FW028 | Féronval | Oscillatoriales | 99.3% *Phormidium tenue* NIES-611 (AB042842) | - | - | + | 9.64 |
| FW032 | Féronval | Oscillatoriales | 99.8% Uncultured bacterium clone 2\SC\37(EU340177) | - | - | + | |
| FW038 | Féronval | Oscillatoriales | 99.5% Uncultured bacterium clone 2\SC\37(EU340177) | - | - | - | 6.83 |
| FW057 | Féronval | Chroococcales | 99.9% *Snowella litoralis* 1LT47S05 (AJ781041) | - | - | + | nd |
| FW069 | Féronval | Synechococcales | 99.8% *Cyanobium* PCC7009 (AF216945) | - | - | nd | 48.7 |
| FW101 | Féronval | Oscillatoriales | 96.5% *Leptolyngbya* sp. LGE 06121 (HQ832945) | - | - | + | nd |
| FW015 | Rénipont | Synechococcales | 100% *Cyanobium* sp. Sai002 (GU935363) | + | + | nd | nd |
| FW017 | Rénipont | Synechococcales | 100% *Cyanobium* sp. Sai002 (GU935363) | - | + | nd | nd |
| FW018 | Rénipont | Synechococcales | 100% *Cyanobium* sp. Sai002 (GU935363) | - | + | nd | nd |
| FW082 | Rénipont | Oscillatoriales | 99.0% *Nodosilinea* sp. 0BB19S12 (AJ639895) | - | - | nd | 2.217 |
| FW088 | Rénipont | Oscillatoriales | 99.8% *Pseudanabaena* sp. 1tu24s9 (AM259269) | - | - | nd | 9.61 |
| FW039 | Ri Jaune | Oscillatoriales | 98.6% Uncultured bacterium (GU935361) | - | - | nd | 3.803 |
| FW071 | Ri Jaune | Oscillatoriales | 98.5% Uncultured bacterium (JQ769963) | - | + | nd | nd |
| FW077 | Ri Jaune | Oscillatoriales | 99.9% *Pseudanabaena* sp. ABRG5-3 (AB527076) | - | - | nd | 8.598 |
| FW084 | Ri Jaune | Oscillatoriales | 99.9% *Pseudanabaena* sp. ABRG5-3 (AB527076) | - | - | nd | 7.037 |
| FW007 | Schulensmeer | Chroococcales | 99.9% *Microcystis aeruginosa* LMECYA 59 (EU078492) | + | + | nd | nd |

### 2.3.3. DGGE analyses

During this study, 32 samples were analyzed by DGGE. A total of 125 bands were excised and amplified. Forty-five sequences of the bigger size and best qualities were chosen as representative and used for the phylogenetic analysis. Recovered sequences were mainly corresponding to the 'common' bloom forming genera *Dolichospermum* spp. (formerly *Aphanizomenon* spp.) (Zapomelova *et al.,* 2012), *Microcystis* spp., and *Planktothrix* spp..

Sequence analysis of samples from Lake Falemprise (2007) showed the presence of *Microcystis* sp., *Planktothrix* sp., *Cyanobium* sp., and *Snowella* sp.. In Ixelles Pond II, sequences were affiliated to *Cupsidothrix issatschenkoi (*formerly *Aphanizomenon issatschenkoi*) (Komárek and Komárková, 2006)*, Microcystis* sp.*, Planktothrix* sp.*,* and *Woronichinia* sp. strains. In Lake Chérapont, sequences were affiliated to *Cupsidothrix issatschenkoi,* and *Microcystis* strains. Finally, in Ecaussines, sequences were assigned to *Planktothrix* sp., and *Synechococcus/Cyanobium* sp. strains (Table 4). The sequences clustered into four clades supported by 80, 100, 92, and 98 % bootstrap values. For the 45 DGGE band sequences and their GenBank relatives, 25 OTUs were defined using a threshold of 98.7% similarity on partial 16S rRNA gene (*E. coli* positions 396–740). Thirteen OTUs belonged to the Nostocales, 1 to the Oscillatoriales, 3 to the Chroococcales, and 2 to the Synechococcales (Fig. 3).

Our sequences were distributed in three distinct OTUs inside the Chroococcales clade: DG 1 composed of toxic and non toxic *Microcystis* sp. sequences, DG 2 composed of *Woronichinia* sp. sequences, and DG 4 composed of *Snowella* sp.. This is the first time that sequences from the genus *Woronichinia* spp. were recovered from Belgian waterbodies. The occurrence of this genus was reported multiple times during the course of the BelSPO project BBLOOMS-2 (Descy *et al.,* 2011), however, it was not possible to isolate any strains.

The second clade was composed of Synechococcales strains. The third was composed of sequences related to toxic and non-toxic *Planktothrix* sp. strains (DG 14). The fourth clade contained two OTUs with Belgian

sequences; DG 16 was composed of *Cupsidothrix issatschenkoi* strains, and DG 21 composed of *Dolichospermum* sp..

**Table 4.** Affiliation of cyanobacteria based on DGGE band sequences. the '*' indicates that a related band (band at the same position in the DGGE gel) was present but not successfully amplified

| Waterbody | Week sample number | Similarity | Most related strain | 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 |
| Chérapont | | | | |
| | 35 | 100% | *Aphanizomenon issatschenkoi* 473 | EU157982 |
| | 35 | 100% | *Microcystis aeruginosa* PCC 7806 | AM778951 |
| Ecaussinnes | | | | |
| | 25 | 100% | *Planktothrix agardhii* NIVA-CYA 29 | AB045931 |
| | 25 | 100% | *Synechococcus* sp. MW6C6 | AY151243 |

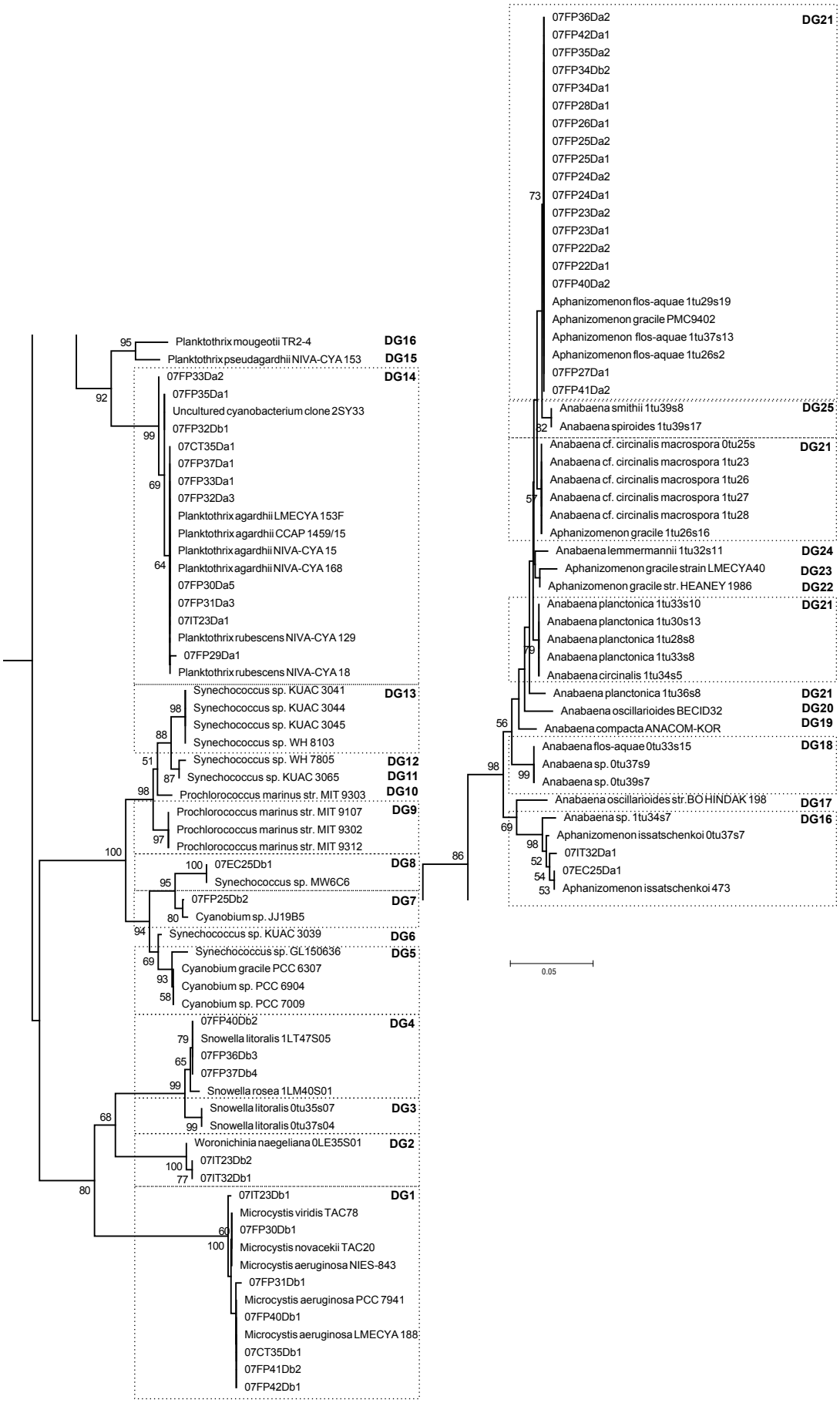
07FP36Da2
07FP42Da1
07FP35Da2
07FP34Db2
07FP34Da1
07FP28Da1
07FP26Da1
07FP25Da2
07FP25Da1
07FP24Da2
07FP24Da1
07FP23Da2
07FP23Da1
07FP22Da2
07FP22Da1
07FP40Da2
Aphanizomenon flos-aquae 1tu29s19
Aphanizomenon gracile PMC9402
Aphanizomenon flos-aquae 1tu37s13
Aphanizomenon flos-aquae 1tu26s2
07FP27Da1
07FP41Da2
DG21
Anabaena smithii 1tu39s8
Anabaena spiroides 1tu39s17
DG25
Anabaena cf. circinalis macrospora 0tu25s
Anabaena cf. circinalis macrospora 1tu23
Anabaena cf. circinalis macrospora 1tu26
Anabaena cf. circinalis macrospora 1tu27
Anabaena cf. circinalis macrospora 1tu28
Aphanizomenon gracile 1tu26s16
DG21
Anabaena lemmermannii 1tu32s11
DG24
Aphanizomenon gracile strain LMECYA40
DG23
Aphanizomenon gracile str. HEANEY 1986
DG22
Anabaena planctonica 1tu33s10
Anabaena planctonica 1tu30s13
Anabaena planctonica 1tu28s8
Anabaena planctonica 1tu33s8
Anabaena circinalis 1tu34s5
DG21
Anabaena planctonica 1tu36s8
DG21
Anabaena oscillarioides BECID32
DG20
Anabaena compacta ANACOM-KOR
DG19
Anabaena flos-aquae 0tu33s15
Anabaena sp. 0tu37s9
Anabaena sp. 0tu39s7
DG18
Anabaena oscillarioides str.BO HINDAK 198
DG17
Anabaena sp. 1tu34s7
DG16
Aphanizomenon issatschenkoi 0tu37s7
07IT32Da1
07EC25Da1
Aphanizomenon issatschenkoi 473
Planktothrix mougeotii TR2-4
DG16
Planktothrix pseudagardhii NIVA-CYA 153
DG15
07FP33Da2
07FP35Da1
Uncultured cyanobacterium clone 2SY33
07FP32Db1
07CT35Da1
07FP37Da1
07FP33Da1
07FP32Da3
Planktothrix agardhii LMECYA 153F
Planktothrix agardhii CCAP 1459/15
Planktothrix agardhii NIVA-CYA 15
Planktothrix agardhii NIVA-CYA 168
07FP30Da5
07FP31Da3
07IT23Da1
Planktothrix rubescens NIVA-CYA 129
07FP29Da1
Planktothrix rubescens NIVA-CYA 18
DG14
Synechococcus sp. KUAC 3041
Synechococcus sp. KUAC 3044
Synechococcus sp. KUAC 3045
Synechococcus sp. WH 8103
DG13
Synechococcus sp. WH 7805
DG12
Synechococcus sp. KUAC 3065
DG11
Prochlorococcus marinus str. MIT 9303
DG10
Prochlorococcus marinus str. MIT 9107
Prochlorococcus marinus str. MIT 9302
Prochlorococcus marinus str. MIT 9312
DG9
07EC25Db1
Synechococcus sp. MW6C6
DG8
07FP25Db2
Cyanobium sp. JJ19B5
DG7
Synechococcus sp. KUAC 3039
DG6
Synechococcus sp. GL150636
Cyanobium gracile PCC 6307
Cyanobium sp. PCC 6904
Cyanobium sp. PCC 7009
DG5
07FP40Db2
Snowella litoralis 1LT47S05
07FP36Db3
07FP37Db4
Snowella rosea 1LM40S01
DG4
Snowella litoralis 0tu35s07
Snowella litoralis 0tu37s04
DG3
Woronichinia naegeliana 0LE35S01
07IT23Db2
07IT32Db1
DG2
07IT23Db1
Microcystis viridis TAC78
07FP30Db1
Microcystis novacekii TAC20
Microcystis aeruginosa NIES-843
07FP31Db1
Microcystis aeruginosa PCC 7941
07FP40Db1
Microcystis aeruginosa LMECYA 188
07CT35Db1
07FP41Db2
07FP42Db1
DG1
0.05

**Fig. 3,** The 16S rRNA distance tree of DGGE band sequences obtained during this study. Total alignment length was 344 nt. Distances were computed using the Jukes and Cantor correction and a Neighbor-joining tree was built with the software MEGA5 (Tamura *et al.,* 2011). Bootstrap replicate values are indicated at the nodes. Cutoff for OTU delineation was set at 98.7%.

2.3.4. Comparison with Belgian sequences previously obtained

In order to evaluate the contribution of this work to the study of the molecular diversity of cyanobacteria in Belgian waterbodies, 114 (strains and DGGE) sequences obtained during this PhD thesis were compared with 142 sequences obtained by other authors (Willame *et al*., 2006; Wilmotte *et al.,* 2008; van Gremberghe *et al.,* 2008). Overlapping of sequences recovered from DGGE, clone libraries and cultivation of strains was of 183 nt. Despite the short size of this alignment, the 236 Belgian sequences were distributed into 29 OTUs using a threshold of 98.7% similarity on partial 16S rRNA gene (*E. coli* positions 546–729). Among these 29 OTUs, 14 were found during the present study. New Belgian OTUs were distributed in the Chroococcales, Oscillatoriales, Nostococcales, and Synechococcales orders, and the majority of these new OTUs were recovered by strain isolation approach. Four OTUs belonged to the Synechococcales; two of these were recovered by DGGE analysis (BE 11 and BE 12), whereas the two others (BE 9 and BE 10) were composed of strain sequences. Eight OTUs belonged to the Oscillatoriales; three OTUs (BE 18, BE 19, and BE 24) were composed of sequences of sheath producing filamentous strains members of the LPP-B group. Three OTUs (BE 29, BE 33, BE 35) were composed of sheath producing filamentous strains related to an unknown cyanobacterium and *Nodosilinea* spp.. Two OTUs were composed of filamentous strains corresponding to the description of the Pseudanabaenaceae family. Finally, two OTUs belonged to the Gomphosphaerioideae subfamily and corresponded to, *Snowella* spp. *(*BE25) and *Woronichinia* spp. (BE 27)*.*

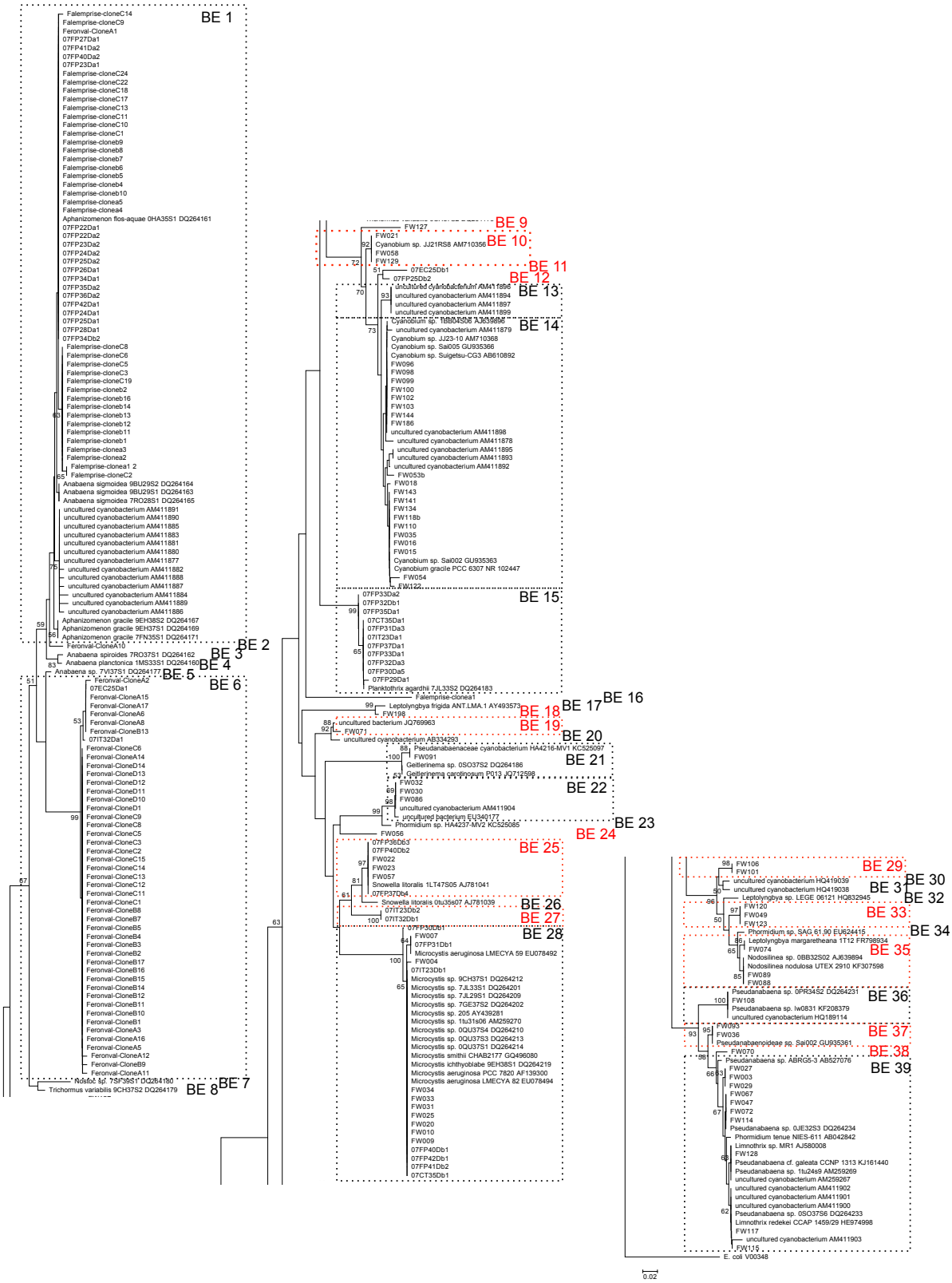


**Fig. 4,** The 16S rRNA distance tree of all Belgian sequences obtained during this study. Total alignment length was 183 nt. Distances were computed using the Jukes and Cantor correction and a Neighbor-joining tree was built with the software MEGA5 (Tamura *et al.,* 2011). Bootstrap replicates values are

indicated at the nodes. Cutoff for OTU delineation was set at 98.7%. OTUs in red font were found during this study.

## 2.4. Conclusion

In this chapter, we have studied the diversity of cyanobacteria observed in Belgian lakes and ponds by culture-dependent and –independent approaches, though on different samples. We observed an important richness of cyanobacterial taxa in the samples from 11 Belgian surface waters. Even though the two approaches could not be applied to the same samples, they appeared to be complementary. Indeed, strain isolation resulted in the acquisition of a majority of 'accompanying' taxa, which were tested for their potential to produce MCs. These taxa might be enriched by the culture conditions. For the first time, a number of these taxa (*i.e., Cyanobium* spp*., Nodosilinea* spp*.,* and *Snowella* spp.) were shown to be potentially toxic.

On the other hand, the DGGE analysis allowed to recover sequences from dominating taxa which are classically known to be bloom-forming (*i. e., Aphanizomenon* spp*., Microcystis* spp*., Planktothrix* spp., and *Woronichinia* spp.)*,* and some unicellular 'accompanying' taxa (*i.e., Cyanobium* spp*., Snowella* spp.).

Finally, the comparison with sequences from previous studies showed that we obtained 14 new OTUs distributed into four orders (Nostocales, Oscillatoriales, Chroococcales, Synechococcales).

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# Chapter 3- Microcystins concentrations, *mcy* genes detection, identification of *mcyE* gene carriers, in samples of shallow waters (Wallonia and Brussels)

*Author's contribution*

In this section, the environmental data, countings of cyanobacteria, and data related to microcystins concentrations (ELISA) were acquired by B-BLOOMS 2 partners at the University of Namur (UNamur), the University of Brussels (VUB), and the University of Dundee. However, there are some missing values in the dataset for environmental parameters and microcystins concentrations, and this could have a negative impact on the analyses presented in this chapter.

Yannick Lara has participated to the sampling in Lake Falemprise, Ixelles Pond I and II, he acquired the molecular data including PCR gene detection, RFLP analysis, DGGE, and quantitative PCR. MALDI-TOF-TOF analyses were performed in collaboration with Christelle Deleuze (PhD candidate under the supervision of Professor Edwin De Pauw at "Laboratoire de spectrométrie de masse" at the University of Liège). During this collaboration, Y. Lara has organized and participated to the collection of samples (2009), and realized the extraction of microcystins and the screening of mass spectra. Christelle Deleuze performed the post source-decay fragment mass spectra analyses, which were only possible for Lake Ri Jaune samples due to the breakdown of a device (LIFT cell).

For microcystins concentrations (ELISA), it was not possible to obtain values for each replicate but an average of OD was directly converted into a concentration. Therefore, no standard deviation will be presented here.. Y. Lara has performed all the data analyses.

# Chapter 3- Microcystins concentrations, *mcy* genes detection, identification of *mcyE* gene carriers, in samples of shallow waters (Wallonia and Brussels)

## 3.1. Introduction

Harmful cyanobacterial blooms are known to occur in freshwater bodies for more than a century. Recently, the scientific literature has suggested an increase of the frequency of this phenomenon in temperate waters (Chorus and Bratam, 1999; Paerl and Huisman, 2008). Eutrophication of waters by anthropological activities is supposed to be the trigger of the cyanobacterial proliferation increase (Paerl and Huisman, 2009). Indeed, the cyanobacterial bloom formation in freshwater environment is favored by high concentrations of P, and a low N:P supply ratio. A previous study carried out on 17 temperate lakes led to the conclusion that a total N:P (TN:TP) ratio value below 29:1 was leading to a dominance of cyanobacteria (Reynolds, 2006). In fact, other explanations for the cyanobacterial dominance in temperate lakes can be put forward (see Kardinaal and Visser, 2005 for review). Indeed, factors such as elevated water temperatures, low photon irradiance, water column stability, grazing of bigger phytoplankton by *Daphnia spp.*, or pH and carbon dioxide concentrations, have already been suggested to play a role.

MCs are known to be produced by most of the common bloom-forming cyanobacteria found in the temperate zone: *Anabaena spp., Microcystis spp.,* and *Plankthotrix spp.*. Application of different methodological approaches to quantify MCs in samples revealed wide ranges in MCs concentrations over the world (Kardinaal and Visser, 2005; Chorus, 1999). During a study of 55 German water bodies, highest concentrations of microcystins per dry weight were observed in *P. rubescens* and *P. agardhii* (Fastner *et al.,* 1999). Later, in a study of 64 bloom samples taken across Belgium and Luxembourg, the highest concentration of microcystins was found in a *Woronichinia naegeliana* dominated bloom (Willame et al., 2005).

The factors influencing the MCs concentration dynamics are still quite elusive. However, the succession of cyanobacterial genotypes in the environment may explain differences in microcystins production (Kardinaal

and Visser, 2005). Moreover, significant negative relationships between the microcystin concentrations and cyanobacterial biomass in German and Dutch lakes suggested that the toxic cells were more abundant at the onset of *Microcystis spp.* blooms (Kardinaal and Visser, 2005; Welker *et al*., 2003). Recently, total phosphorus and ammonium concentrations appeared strongly related to MC-LR concentrations in Lake of the Woods, Ontario, Canada (Chen *et al.*, 2009).

The knock out of one peptide synthase gene was the first evidence of the presence of the operon involved in the MCs biosynthesis in *Microcystis sp.* PCC7806 (Dittmann *et al.,* 1997). This was followed by the characterization of MCs biosynthesis gene clusters, the *mcy* genes, in *Anabaena spp.*, *Microcystis spp.,* and *Planktothrix spp.* (Rouhiainen *et al.,* 2000; Tillet *et al.,* 2000; Christiansen *et al.*, 2003). The *mcy* genes are coding for a mixed non-ribosomal peptide synthase (NRPS)/ketoacyl synthase (PKS) enzymatic complex. Characterization of this complex led to the development of strategies to detect the *mcy* gene in strains and the environment. Initially, most of the PCR primers were designed to target *Microcystis spp*. *mcy* genes (Fewer *et al.*, 2008; Kurmayer *et al.,* 2002; Tillett *et al.,* 2001; Nonneman and Zimba, 2002; Pan *et al.,* 2002; Yoshida *et al.,* 2005). It allowed to distinguished potentially toxic from non-toxic *Microcystis spp.* strains grown in the laboratory, or genotypes co-occurring in environmental samples. Similar results were obtained with PCR strategies designed for MC-producing *Anabaena spp.* and *Planktothrix spp.* (Kurmayer *et al.*, 2004; Mbedi *et al.,* 2005; Rantala *et al.*, 2006). Later, PCR primers were designed to target different genera at the same time (Hisbergues *et al.*, 2003; Rantala *et al.*, 2004, Jungblut and Neilan, 2006). These more 'universal' primer strategies gave the possibility to identify the *mcyA* carriers by PCR-RFLP (Hisbergues *et al.*, 2003), or the *mcyE* carriers by PCR-DGGE (Fewer *et al.*, 2009). During the BelSPo project B-BLOOMS (2003-2005) (Wilmotte et al., 2008), *mcyB* and *mcyE* were detected together in 79% of the BLOOMNET samples from 63 different waterbodies located in the Flemish part. In Wallonia and Brussels, both genes were detected into 10/11 samples from seven different waterbodies. In a previous study carried out in Finland using the same primers, the *mcyE* gene was detected in 84% of samples from 70 different

lakes (Rantala et al., 2006). In the same study, the authors designed primers specific to *Anabaena spp., Microcystis spp.,* and *Planktothrix spp..* They showed that potential *Microcystis spp.* MCs-producing strains were present in 70% of the samples; potential *Planktothrix spp.* MCs-producing strains were present in 63% of the samples, and *Anabaena spp.* MCs-producing strains were present in 37% of the lakes.

In the present chapter, the trophic status, the cyanobacterial biomass, and MCs concentrations were determined. The influence of environmental factors on cyanobacterial biomass and MCs concentrations was investigated. We also have characterized the presence of potentially MC-producing cyanobacteria in Walloon and Brussels shallow waters by PCR. Then, in order to characterize the potential MC-producing genera present in water samples, we tested a PCR-RFLP strategy on *mcyE* genes carriers.

## 3.2. Materials and methods

### 3.2.1. Sampling

Sampling was carried out in 19 waterbodies located in Wallonia or in the surroundings of Brussels, in Belgium. All the waterbodies were sampled following a common protocol developed by the coordinator of the BelSPO project B-BLOOMS2. For the measure of abiotic variables, the water temperature, oxygen concentrations, pH and conductivity were measured using the probe available to the sampling partners (VUB and FUNDP).

For the reference waterbodies, at Lake Falemprise, a sampling was carried out on the 3rd May 2007, on a weekly basis from June to October 2007, on the 17 April 2008, on a weekly basis from 22nd May 2008 to 22nd October 2008. Finally, two extra samples were taken on the 16 September 2009 and on the 23rd September 2009.

In Brussels, Ixelles Pond I and Ixelles Pond II, both located next to Flagey Square in the Ixelles area, were only sampled five times in 2007 between 26 June to 11 September, and nine times between 7 June to 11 September, respectively. Moreover, both ponds were sampled the 19 December 2008, and irregularly from 30 January 2008 to 4 November 2008.

Additional samplings were performed in 2007 at Ecaussines Quarry the 20 June and in Lake Gouvy the 29 August, in 2008 at Ecaussines Quarry the 1st February and in Lake Bambois the 30 July 2008 and 9 September 2009, the 25 June 2009 at Lake Gouvy, and the 20 August 2009 at Lake Rénipont. Eight ponds were sampled in the surroundings of Brussels: Cambre Wood Pond, Clementine Square Pond, Karreveld Castle Pond, Leybeek Pond, Mellaerts Klein Pond, Neerpede Pond 2 and 4, Tercoigne Pond, and King Baudoin Park Pond I.

In 2009, additional monitoring was organized at Lake Féronval, and Lake Ri Jaune from 28 July to 21st of October, and Virelles Ponds from 7 July to 16 October 2009.

Water samples were processed in the laboratory. For chemical characterization, 0.2 mL $H_2SO_4$ at 5N were added to 50 ml of sampled water in order to measure total phosphorous (TP). Water was filtrated through a previously incinerated Whatman (Kent, USA) GF/C filter until the filter gets clogged in order to measure particulate C and N.

For phytoplankton pigments characterization, 15 to 200 ml, were filtered through Macherey-Nägel (Düren, Germany) GF/3 filters, until filters clogged, then filters were conserved at -80°C until processed.

In order to measure the total microcystins and soluble microcystins concentrations present in the sample by ELISA, 2 x 1 ml of raw sample were collected in eppendorf tube and stored at -20°C. Then, samples were sent to the University of Dundee for ELISA analysis.

Finally, for genetic analyses, water volumes were filtrated through 0.2 µm Supor filter PES Pall Life Science (NY, USA). Filters were placed in 2 mL lysis buffer composed with 40 mM EDTA, 400 mM NaCl, 0.75 M sucrose, and 0.50 mM Tris (HCl; pH 8.3). The filters were kept at -20°C until DNA extraction.

### 3.2.2. Pigments analysis

The BBLOOMS2 partner in Namur processed the filters for pigment analysis as described by Descy and colleagues (2000). Extraction was performed in 8

mL of 90% HPLC grade acetone. Two times 15 minutes incubation in an ultra sonication water bath were followed by a 4°C overnight incubation in the dark. The analysis was performed following the gradient elution method (Wright et al., 1991) using a Waters 600E (Milford, USA) comprising a Waters 996 PDA and a Waters 470 fluorescence detectors. Commercial external standards (DHI, Denmark) were used for calibration. Carotenoids were approximated using relative response of fucoxanthin by the ratio of the specific absorbance coefficients at 440 nm (Jeffrey et al., 1997). Characterization of pigments was carried out using a library of pigments spectra, which were obtained by acetone extraction from pure cultures of eukaryotic algae and cyanobacteria. Algal classes abundances were calculated using CHEMTAX software, which estimates contributions of phytoplankton pigments classes to the total Chl a concentration (Mackey et *al.*, 1996). A unique initial ratio was used for all lakes. For the purpose of this thesis only the results for total phytoplankton and total cyanobacteria are presented.

### 3.2.3. Cyanobacterial composition in reference ponds

For microscopy countings, 250 mL of samples were immediately fixed with Lugol and concentrated by settling for 24h. Then, the concentrates were preserved by addition of neutral formaldehyde (2-4% final concentration) for long-term storage in the dark. The FUNDP and the VUB groups on the basis of specialized taxonomic literature performed identification and counts.

In Ulg, 50 mL samples concentrated by a 50 µm plankton net were observed one day after sampling. 1 mL samples were fixed with 0.3% glutaraldehyde and stored in the dark at 4°C for long-term conservation. Identification of cyanobacteria was performed using the identification key of unicellular cyanobacteria (Komarek and Anagnostidis, 1999).

### 3.2.4. Microcystins concentrations by ELISA

The B-BLOOMS 2 partner at the University of Dundee carried out the microcystins analysis by ELISA. Polyclonal antibodies were conceived to

recognize microcystin-LR (Metcalf et *al.*, 2000). Their cross-reactivity were confirmed for seven microcystins variant (-LR, -LA, -LY, -LW, -LF, -RR, YR, -D-Asp3-RR, and -Asp3(Z)-Dhb7-HtyR) and nodularin-R. In addition, specificity to microcystins and nodularin was confirmed by testing other cyanobacterial metabolites such as anabaenopeptine–A, anabaenopeptin-B, cyanostatin-A, cyanostatin-B, microviridin J, and nostocylin, and other protein phosphatase inhibitors such as calyculin A, okadaic acid, and tautomycin (Metcalf et *al*., 2001). In order to quantify the total amount of microcystins, 1 mL of water sample was boiled for 1 minute in a water bath (Metcalf et *al*., 2001). The amount of soluble microcystins was estimated after 1 mL of raw sample was centrifuged at 10 000 rpm for 10 minutes by taking the supernatant for analysis. Toxin concentrations were expressed in µg toxin per L water.

### 3.2.5. Microcystins analyses by MALDI-ToF-ToF

Characterization of microcystins by MALDI-ToF-ToF analyses were performed after extraction of the GF/C filters according to a modified version of the standard operational procedure as described in Meriluoto and Codd (2005). Briefly, filters were lyophilized and stored at -20°C after sample filtration. For extraction, filters were transferred into 15 ml Falcon tubes with 3 mL of 75% methanol solution. Tubes were shacked using a tube vortexer for 5 minutes. The mixed solution was incubated in a Branson 2210 ultrasonicator Branson Ultrasonics (Danbury, USA), bath for 15 minutes. A supplementary ultrasonication step of 30 sec to 1 minute was performed on ice to each of the samples using a MSE ultrasonicator probe. Tubes were centrifuged at 9000 rpm for 10 min. Volumes of 400 µL of the supernatant were taken transferred to Epprendorf tubes. Finally, samples were dried using a speedVac and suspended in 75% methanol right before analysis.

In order to perform a fast screening of MCs, samples were separately mixed with two different matrices and spotted. The first matrix was made of a fresh solution of 20 mg $mL^{-1}$ of 2,5-dihydrobenzoic acid (2,5-DHB)..The second matrix was made of a saturated 1,5-diaminonaphtalene (1,5-DAN) in 0.1% 50:50 ACN:FA. To record the mass spectrum with the standard

procedure (Fastner et al., 2001), 1 µL microliter 2,5-DHB was added to 1 µL extracted sample. To detect the presence of MCs variants with Dha or Mdha at the site of the 7 amino acid, we added 1 µL 1,5-DAN as described by Deleuze et al., (2011). Indeed, as reduction property, 1,5 DAN is able to reduce the carbon-carbon double bond selectively. In the case of MCs, only one carbon-carbon double bond is observed in the 7 amino acid position when there is Dha or MDha. The reduction can be observed on the spectrum as a modification of the isotopic pattern. An increase in the intensity of the [M + H + 2] peak is observed when the carbon-carbon double bond is present.

Analyses were performed using a Bruker Ultraflex II MALDI-ToF-ToF mass spectrometer, which was equipped with a Smartbeam laser. MS/MS experiments were performed using a LIFT cell (Suckau et al., 2003). It allowed to post-accelerate the metastable fragments formed during the extraction of the ions.

### 3.2.6. DNA extraction and Purification

In order to extract DNA from filters, we used a modified hot phenol method (Giovannoni et *al.,* 1990). For the lysis of cells, lysozyme (1.25 mg/mL) and proteinase K (0.3 mg/mL) were added to the lysis buffer. DNA extraction was performed in phenol-chloroform-isoamyl alcohol and (25:24:1, vol/vol/vol) chloroform-isoamyl alcohol (24:1, vol/vol). DNA was precipitated overnight at -20°C in sodium acetate and ethanol. Then, precipitated DNA was washed in 70% ethanol and suspended in Tris-EDTA (10:1, vol/vol). Purification of extracted DNA was carried out using Promega Wizard DNA clean up system kit (Madison, WI, USA). Then, the purified genomic DNA were stored at -20°C.in water.

### 3.2.7. PCR for detection

In order to verify the presence of cyanobacterial DNA in the extracted samples, we performed a PCR using the cyanobacterial specific primers

359F/781R that are targeting 16S rRNA (Nübel *et al.*, 1997). Then, three different PCR protocols were used to detect the *mcy* genes operon. Those protocols are targeting three different loci: *mcyA, mcyB,* and *mcyE* genes.

The mcyA-CdF/mcyA-CdR primer pair is targeting the coding part of the condensation domain of the *mcyA* NRPS module (Hisbergues *et al.*, 2003). The size of the amplified fragment is about 291 to 297 bp length. The mcyE-F2/mcyE-R4 primer set is amplifying a fragment of *mcyE* (809 to 812 bp) that contains a partial region of the adenylation domain and a phosphopantetehine-binding site, which is responsible for the activation of the glutamic acid (Rantala et al., 2004). Both primer sets were designed in rather conserved regions using sequences from different orders. This implies that each primer set might anneal with *mcyA* or *mcyE* sequences from diverse cyanobacterial taxa.

In order to detect potential MC-producing *Microcystis spp.* genome in our samples, we used the semi-nested approach as developed by Nonneman and Zimba (2001), which targets the *mcyB* specific to *Microcystis*. The first primer set, mcyF1/mcyR1, was amplifying a fragment of 319 bp starting at the position 1740 and ending at the position 2059 of the NRPS gene *mcyB* (Z28338). The second primer set, mcyF1/mcyR2, was amplifying a fragment of 287 bp. The primer mcyR2 is located at position 2027.

Polymerase Super *Taq* (HT Biotechnology, Cambridge) (0.8 U/reactions) was used to perform PCR gene detection of *mcyA, mcyB* and 16S rRNA*,* whereas the proofreading polymerase Super *Taq* Plus (HT Biotechnology, Cambridge) (0.8 U/reactions) was preferred for the detection of *mcyE* (see next paragraph). PCR for *mcyA/B* and 16S were performed in a 25 µL (total volume) reaction mixture containing 1 µl of DNA template, 1 X Super *Taq* PCR buffer, with each deoxynucleoside triphosphate at a concentration of 0.2 µM, 0.5 µM of each forward and reverse primers. For the detection of the *mcyE*, PCR were performed in 50 µL (total volume) reaction mixture containing 1 µl of DNA template, 1 X Super *Taq* PCR buffer, with each deoxynucleoside triphosphate at a concentration of 0.2 µM, 0.5 µM of each forward and reverse primers.

All PCR were performed in a Bio-rad MJmini or a Bio-rad Icycler (Hercules, CA), and the products were screened using 1.5% (w/v) agarose gel electrophoresis.

The PCR cycles were performed as follow:

- For *mcyA,* 94°C for 5 min; 35X (94°C for 1min, 54°C for 1min, 72°C for 1 min); 72°C for 7 min.
- For *mcyB* first step*,* 94°C for 3 min; 28X (94°C for 30 sec, 57°C for 45 sec, 72°C for 1 min); 72°C for 7 min.
- For *mcyB* second step, 94°C for 3 min; 35X (94°C for 30 sec, 57°C for 45 sec, 72°C for 1 min); 72°C for 7 min.
- For *mcyE*, 94°C 3 for min; 35X (94°C for 30 sec, 57°C for 45 sec, 72°C 1 min); 72°C for 7 min.

### 3.2.8. Design and testing of the mcyE-RFLP

In order to identify the *mcyE* carriers in the environment, a RFLP approach was developed in the laboratory by Dr. Annick Wilmotte during the BelSPO project B-BLOOMS (Wilmotte et al, 2008). To perform the digestion of the *mcyE* fragment, the enzyme AluI was chosen *in silico* using the "restriction of DNA sequences" tool of the online software available at: http://insilico.ehu.es/restriction/. For this work, I have carried out an, *in silico* analysis with the 27 *mcyE* strains sequences from Rantala et al. (2004) (AY382531-AY382556) from genus *Anabaena spp., Microcystis spp., Nostoc spp., Nodularia spp.* and *Planktothrix spp.*. Also, DNA from different strains were tested: *Planktothrix sp.* Lux1121, *Microcystis sp.* PCC7806, *Microcystis sp.* Lux66, *Microcystis sp.* Lux151, *Microcystis sp.* Lux0114.

Finally, in order to optimize the PCR program, the annealing temperature was tested in a gradient PCR protocol, and the annealing temperature was increased to 57°C. For the purpose of PCR-RFLP, we used the Super *Taq* plus (HT Biotechnology, Cambridge) (0.8 U/reactions) polymerase, and an elongation temperature of 68°C.

Digestion was performed in a thermocycler for 3 h at 37°C, followed by 20 minutes at 65°C. Then, the samples were loaded onto a 2% Nusieve 3:1

(Lonza, Rockland, USA) agarose gel in TAE for 80 minutes at 100 V. DNA was stained with ethidium bromide and visualized under UV.

### 3.2.9. 16S rDNA DGGE analysis at lake Falemprise 2007

PCR products for the DGGE run were obtained by performing a nested strategy as described in the chapter 2 (paragraph 2.2.4.).

### 3.2.10. Real time multiplex qPCR assay

In order to quantify the ratio of *mcyB* in *Microcystis spp.* populations, we used a multiplex real time PCR strategy using TaqMan probes (Briand et, al. 2008), which was adapted to BioRad MiniOpticon real time PCR detection system (Hercules, CA USA). The targeted genes were *mcyB* and *cpcBA*. The *mcyB* gene (MCY) was chosen to quantify potential MCs-producing *Microcystis,* and the *cpc* region (PC) was chosen to follow the dynamic of entire *Microcystis spp.* population. Primers and probes were previously designed (Kurmayer and Kutzenberger, 2003). In order to discriminate the two amplifications in a multiplex assay, fluorescent reporter and quencher dyes were different for both reactions (MCY: 5'-HEX and 3'-BHQ1; PC: 5'-6'FAM and 3'-TAMRA). The genomic DNA of the strain PCC7806 was used to perform the standard curves. For each run of samples, serial dilution triplicates containing approximately $5x10^{1}$, 5, $5x10^{-1}$, $5x10^{-2}$ ng of the genomic DNA $\mu g.L^{-1}$ were prepared from DNA freshly extracted from PCC7806.

The multiplex reaction was performed in 20 µL (final volume), it contained 2X KAPA PROBE FAST BioRad iCycler qPCR mix with fluorescein as reference dye, 100 nM of PC-probe, 300 nM for each of forward and reverse primer for the PC reaction, 250 nM of MCY probe, and 900 nM for each of forward and reverse primer for the MCY reaction.

The *mcyB* ratio was determined using the *∂*Ct approach as described by Briand et al. (2009). The theoretical equation for determination of the *mcyB* ratio values was y= 3.32 *X* log(x)- 8.78 as defined by the original study.

Multiplex assays were performed on samples from Lake Falemprise in 2007/2008, Lake Féronval and Lake Ri Jaune in 2009, and samples from Lake Gouvy 2007 and 2009. All samples were analyzed in triplicate.

### 3.2.11. Data analysis

A correlation test and a principal component analysis (PCA, Legendre and Legendre, 1998) were performed on samples from Lake Falemprise, Ixelles Pond I and II. In order to approximate to normal distribution, three data matrices were treated with the square root transformation. The matrices contained 46, 37, and 39 samples for which 15 variables were available, respectively.

In order to find relationships between cyanobacterial biomass and environmental conditions, a Spearman rank correlation test was applied on the three matrices.

After exclusion of samples with missing values, the variables were standardized. The realization of a quantile-quantile plot controlled the approximation of the normality. In order to group samples from the three waterbodies on the basis of environmental variables and cyanobacterial biomass, a PCA was performed.

All analyses were performed using FactoMineR from the R package.

## 3.3. Results

### 3.3.1 Trophic status of sampling sites

In order to study the influence of the trophic status on the occurrence of the MCs genes, the samples were grouped according to their TP, *Chl a*, secchi disc depth and the oxygen saturation. The oxygen is depleted during the collapse of the phytoplankton blooms when cells are dying and decomposing. Thus, low rates of oxygen saturation mean that the bloom phenomenon is ending whereas high rates mean that photosynthetic cells are active and healthy. The trophic status was then characterized as recommended by the

Organisation for Economic Co-operation and Development (OECD) (1982), see Table 1.

**Table 1**. Determination of the trophic status

| Trophic status | TP (µg $L^{-1}$) | % O2 minima | Chl *a* maxima (µg $L^{-1}$) | Secchi disc depth (m) |
|---|---|---|---|---|
| Ultra oligotrophic | 4 | <90 | 2.5 | 6 |
| Oligotrophic | 10 | <80 | 8 | 3 |
| Mesotrophic | 10 -35 | 40-89 | 8-25 | 3-1.5 |
| Eutrophic | 35-100 | 40-0 | 25-75 | 1.5-0.7 |
| Hypereutrophic | 100 | 10-0 | <75 | >0.7 |

In 2007, the trophic status of Lake Falemprise evolved from eutrophic to hypereutrophic according to TP, oxygen saturation, and Chl *a* values (table 2). A shift of the TP concentration was observed after the 25 July 2007. A remarkable growth of phytoplankton was shown by a peak of Chl *a* between the 25 and 31$^{st}$ July 2007 when the TP concentration increased 1.9-fold (Fig. 2). In Brussels, Ixelles Pond I and II were both eutrophic during the summer period.

In 2008, the trophic status of Lake Falemprise remained eutrophic during the sampling period with very high oxygen saturation values (table 2). According to the data available for the Bambois and Virelles samples in 2008, both waterbodies were hypereutrophic. Environmental data from the Lake Féronval samples suggest that it was eutrophic. Ixelles Pond I was mesotrophic during the cold season and eutrophic from the spring to the fall 2008. Strikingly, Ixelles Pond II was hypereutropic all year long. Moreover, a bloom dominated by *Woronichinia spp.* was observed under the ice cover during the winter.

In 2009, Lake Bambois was characterized as hypereutrophic on the basis of the high values of 3 criteria (TP, total Chl *a*, and water transparency). Lake Gouvy and Lake Rénipont were also diagnosed as hypereutrophic with a high rate of oxygen saturation. This indicates that the samples were taken at an early stage of the bloom process.

Lake Féronval, Lake Ri Jaune, and Virelles Pond samples were diagnosed as eutrophic.

**Table 2**. Summary of the characteristics of waterbodies

| Waterbody | Sampling year | TP (µg $L^{-1}$) | % O2 | Chl *a* (µg $L^{-1}$) | Secchi disc depth (m) |
|---|---|---|---|---|---|
| | **2007** | | | | |
| Lake Falemprise | | 40-160 | 8-9 | 10-114 | 1-5 |
| Ixelles Pond I | | 63-323 | - | 28-134 | 1-3 |
| Ixelles Pond II | | 180-460 | - | 31-309 | 1 |
| | **2008** | | | | |
| Lake Bambois | | 1988 | - | - | 1 |
| Lake Falemprise | | 30-100 | 43-138 | 50-68 | 2-6 |
| Lake Féronval | | 63-71 | 64-69 | 30-55 | - |
| Ixelles Pond I | | 40-190 | - | 17-76 | 1-4 |
| Ixelles pond II | | 73-513 | - | 31-309 | 1 |
| Virelles Pond | | 172 | 56 | 145 | - |
| | **2009** | | | | |
| Lake Bambois | | 144-263 | 95.6-189.3 | 91.2-279.7 | 0.54-0.59 |
| Lake Gouvy | | 217 | 103.1-158.5 | 80.57 | - |
| Lake Féronval | | 65-185 | 57.8-157.8 | 43.4-176.4 | 0.04 -0.86 |
| Lake Rénipont | | 217 | 145.9 | 108.3 | - |
| Lake Ri Jaune | | 20-49 | 85.4-149.5 | 15.5-25.1 | 0.59-1.18 |
| Virelles Pond | | 73-108 | 78.7-90.7 | 3.1-22.6 | 0.4-2.5 |

3.3.2. Characterization of environmental conditions in reference waterbodies

The reference waterbodies were characterized by Principal Component Analysis (PCA). The purpose of PCA is to reduce the number of variables and to identify hidden patterns in the dataset.

Table 3. Correlations between each variable and loading score of components for the three main components of the PCA.

| | PC 1 | | PC 2 | | PC 3 | |
|---|---|---|---|---|---|---|
| Environmental variable | Corr. | p-value | Corr. | p-value | Corr. | p-value |
| Conductivity | -0.45 | $2x10^{-3}$ | | | -0.63 | $7x10^{-6}$ |
| Cyanobacterial biomass | | | -0.47 | $2x10^{-3}$ | | |
| Dissolved nitrogen | | | 0.84 | $2x10^{-12}$ | 0.33 | $3x10^{-2}$ |
| Dissolved oxygen | | | -0.32 | $4x10^{-2}$ | 0.81 | $5x10^{-11}$ |
| Epilimnion temperature | 0.87 | $3x10^{-14}$ | | | | |
| Light intensity | 0.85 | $4x10^{-13}$ | | | | |
| NH4+ | -0.37 | $1x10^{-2}$ | | | | |
| NO3- | | | 0.82 | $1x10^{-11}$ | 0.37 | $2x10^{-2}$ |
| Outside temperature | 0.84 | $3x10^{-12}$ | | | -0.32 | $4x10^{-2}$ |
| pH | 0.44 | $3x10^{-3}$ | -0.55 | $1x10^{-4}$ | 0.49 | $8x10^{-4}$ |
| Photoperiod | 0.86 | $3x10^{-14}$ | 0.41 | $6x10^{-3}$ | | |
| Secchi disk depth | | | 0.68 | $6x10^{-7}$ | | |
| Total phosphorus | | | -0.80 | $1x10^{-10}$ | | |

In Lake Falemprise, the samples were grouped on the basis of 14 physicochemical variables, and the total cyanobacterial biomass. The three main components (factors) represented 60.69% of the variance. The two first factors represented 24.80% and 23.38% of the variance, respectively. The correlations between each variable and the loading score of components are shown for the three main components in Table 3.

Briefly, the first factor showed a correlation of the seasonal conditions (light intensity, temperature, and photoperiod). The second factor correlated with nutrient concentrations and Secchi. Based on their scores for the first two

factors, the samples were distributed in three groups (Fig. 2), which were mainly characterized by nutrient limitation and temperature. The first group of samples was characterized by a higher N/P ratio (N/P ratio average of 25.74 ± 11.85), which could indicate a co-limitation of N and P, and by an epilimnion temperature average of 18.45°C ± 1.76°C. The corresponding samples were taken during spring and early summer. The second group, composed by samples from mid to late summer, was characterized by N limitation, a lower N/P ratio (N/P ratio average of 8.10 ± 3.63, and DIN average of 0.206 $mgL^{-1}$) and an epilimnion temperature average of 18.9°C ± 1.86°C. The sample of 17 September 2007 is isolated from the rest of the group. Finally, the third group was composed by samples taken during fall, which were N limited (N/P ratio average of 8.51 ± 2.41) and showed a low temperature average of 13.58°C ± 1.42°C, except for the sample of 10 October which was warmer.

It is known that weak summer stratification is occurring in Lake Falemprise (Verniers *et al*., 2005). Indeed, wide variations in the transparency dynamics occurred during the two-year monitoring. Episodically, the Zeu was observed under the Zm value, while both were respectively ranging from 0.92 to 5.41 m and 1 to 4.5 m in 2007, and 1.84 to 6.39 m and 1 to 5 m in 2008.

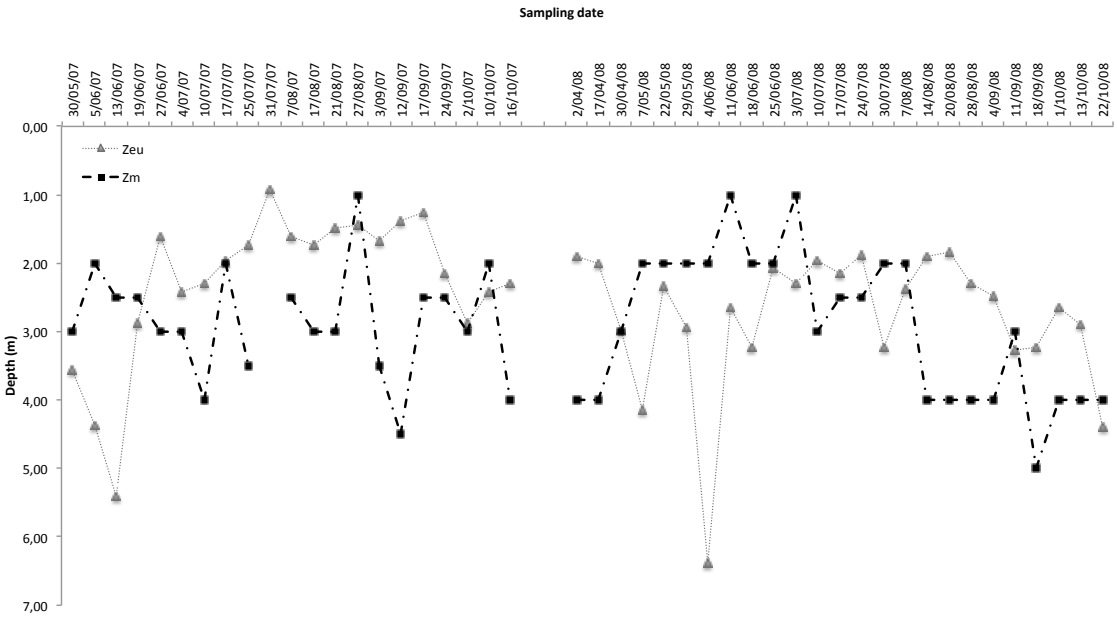



**Fig 1**. Localization of the euphotic zone versus the thermocline

In 2007, we observed six phytoplankton peaks between 19 June and 4 July, 25 July and 7 August, 7 and 21st August, 21st August and 3rd September,

3rd and 24 September, and 2nd and 16 October. Maximum Chl *a* was observed on the 31st July. Four peaks of cyanobacterial biomass were observed between the 5 and 27 June, 10 and 25 July, 31st July and 17 August, 27 August and 24 August (Fig. 2, d). All four peaks were occurring in samples belonging to the PCA group 2.

In 2008, the phytoplankton peaks were more frequent but with a rather low Chl *a* value as compared to 2007. Seven Chl *a* peaks were distinguished between the 7 and 29 May, 4 and 18 June, 18 June and 3rd July, 17 and 30 July, 30 July and 14 August, 20 August and 11 September, and 1st and 22nd October.

Peaks of cyanobacterial biomass occurred between 29 May and 11 June, and 24 July and 7 August (Fig. 2, d.).

The normal distribution of variables was not verified. Therefore, in order to find relationships between the cyanobacterial biomass and environmental variables, we performed a Spearman rank correlation test. Significant correlations were observed for temperatures, light intensity, Secchi depth, and TP (Table 4).

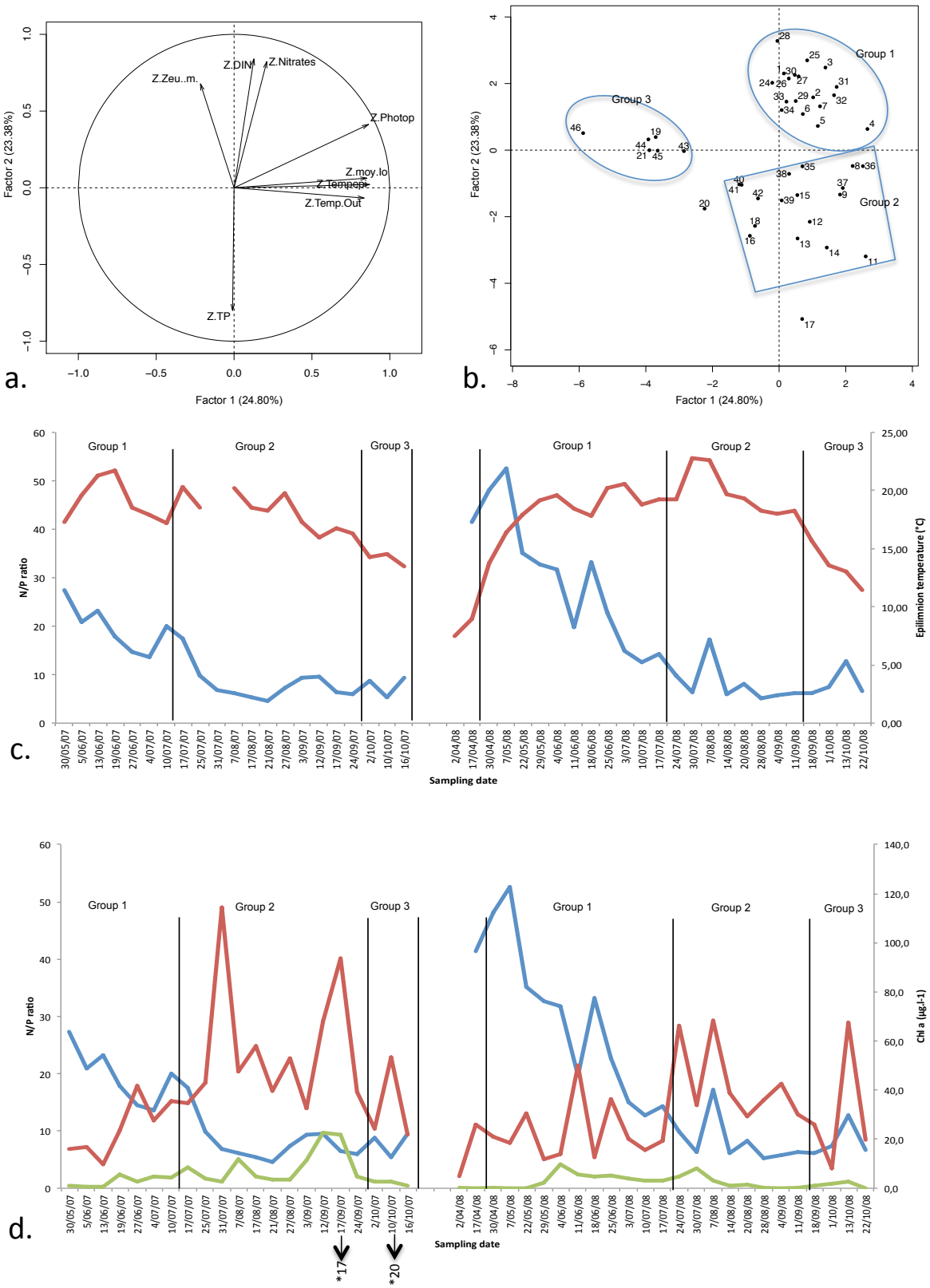



**Fig. 2a.** Contribution of variables to factor 1 and 2; **b.** Distribution of samples along factor 1 and factor 2; **c.** N/P ratio and epilimnion temperature at Lake Falemprise (blue: N/P ratio, red: epilimnion temperature in °C); **d.** Trends of phytoplankton biomass and N/P ratio at Lake Falemprise (blue: N/P ratio, red: total Chl a in µg $L^{-1}$, green: cyanobacterial biomass in µg. $L^{-1}$).

**Table 4.** Relationship between cyanobacteria total biomass and environmental variables assessed by e Spearman correlation test. (ρ : Spearman correlation coefficient)

| Environmental variable | Lake Falemprise | | Ixelles Pond I | | Ixelles Pond II | |
|---|---|---|---|---|---|---|
| | ρ | p-value | ρ | p-value | ρ | p-value |
| Ammonium | | | | | -0.54 | $6x10^{-4}$ |
| Conductivity | | | -0.74 | $5x\ 10^{-7}$ | -0.45 | 0.005 |
| Dissolved nitrogen | | | -0.50 | 0.002 | -0.51 | 0.001 |
| Epilimnion temperature | 0.38 | 0.010 | 0.39 | 0.023 | | |
| Light intensity | 0.32 | 0.033 | | | | |
| Nitrate | | | -0.50 | 0.003 | -0.48 | 0.003 |
| Outside temperature | 0.38 | 0.011 | 0.59 | $2x10^{-4}$ | | |
| pH | | | | | 0.42 | 0.010 |
| Photoperiod | 0.32 | 0.032 | | | | |
| Secchi depth | -0.38 | 0.008 | -0.82 | $3x10^{-9}$ | -0.69 | $3x10^{-6}$ |
| Total phosphorus | 0.43 | 0.003 | 0.57 | $5x\ 10^{-4}$ | | |

During the two years survey, the TP concentrations were higher in Ixelles Pond II with a range of concentrations of 0.073 to 0.513 mg $L^{-1}$, whereas they ranged from 0.04 to 0.323 mg L-1 in Ixelles Pond I (see table 2).

Due to missing data, only 21/37 samples for Ixelles Pond I and 22/39 samples for Ixelles Pond II could be analyzed by PCA. Separate PCA were performed for each sample set.

In Ixelles Pond I, the first two components of PCA covered 78.19% of the variance. The first component (Factor 1), which explained 50.34% of the variance, included both the phytoplankton growth (nitrogen concentrations, Secchi depth, TP, and cyanobacterial biomass) and seasonal conditions (temperatures and photoperiod) (Fig 3,a).

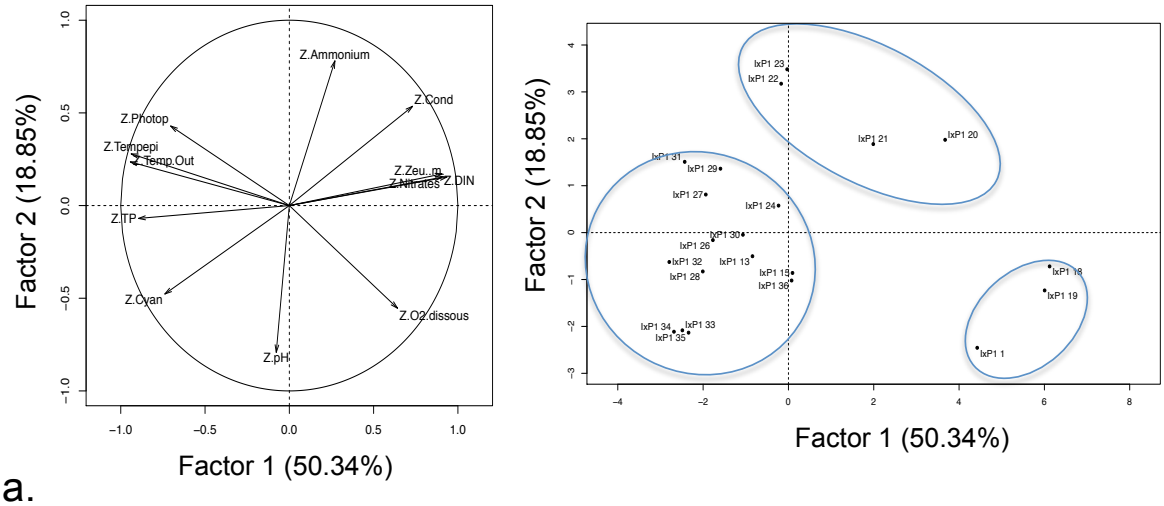
The samples were divided into three groups. The first group contained samples taken during late winter and early spring (27 March 2007, 12 and 26 March 2008). Samples from the group I were characterized by the absence or small numbers of cyanobacteria according to the pigment analysis results (0 to 6,24% of the total concentration of Chl *a*), low epilimnion temperatures with a maximum of 8.9°C, and a high transparency characterized by a mean of 3,07 m for Secchi depth. The second group was composed by samples taken during spring 2008 from the 15 March to 29 May with an epilimnion

temperature ranging from 10.5 to 21.1°C. No cyanobacteria were detected according to the pigment analysis, but an average of 2.24 m for Secchi depth was observed, which suggests an increase of the turbidity. The increase in turbidity was mainly caused by an increase of the zooplankton biomass (data not shown). Due to missing values for the measurement of the dissolved oxygen during the 2007 survey, the last group was mainly composed by samples of the 2008 survey (except two samples taken on 29 August and 26 September 2007). Warmer epilimnion temperatures (average of 18.96°C), a rather high turbidity (average of 1.32 m Secchi depth), and the presence of cyanobacteria (except for the 9 June 2008) characterized those samples.

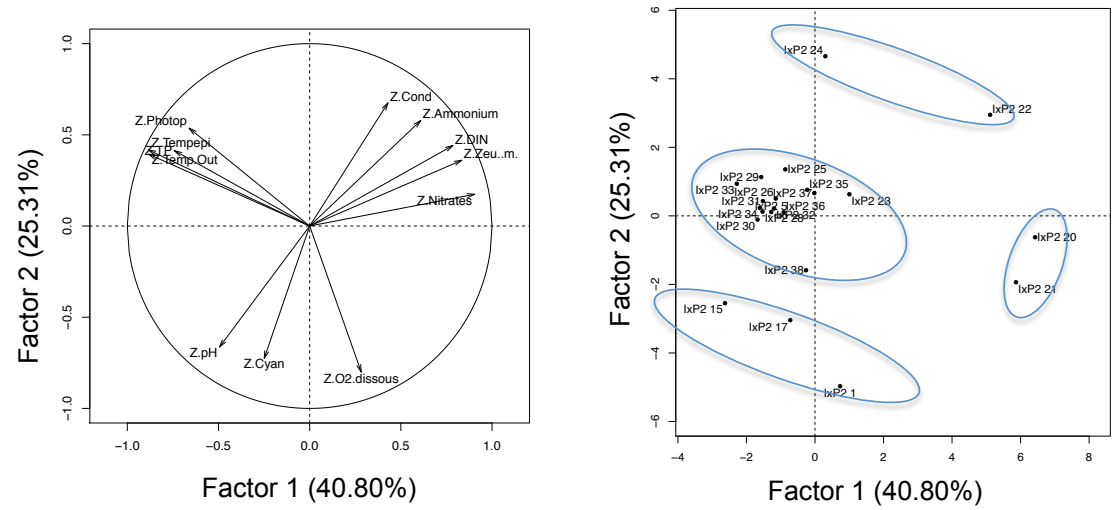
For Ixelles Pond II, PCA Factor 1 and 2 together explained 66.11% of the variance of the data set (Fig. 3, b). The first factor included dissolved nitrogen (ammonium, and nitrates), total phosphorus, temperatures, photoperiod, and turbidity. Factor 2 mainly represented dissolved oxygen concentrations and cyanobacterial biomass. Due to missing values, only four samples from 2007 and 19 samples from 2008 were present in the final data matrix used for PCA. The samples were divided into four groups. The first group was composed by three samples from 2007 taken on the 27 March, 29 August, and 26 September, which were characterized by an average Chl *a* concentration of 174.37 ± 20.6 µg $L^{-1}$ with a contribution of cyanobacterial biomass of 50.59 ±7.74 %. The second group was composed by two samples taken on the 12th and 26th March 2008. The samples were characterized by low epilimnion temperatures (5.4 and 7.6°C), dissolved oxygen concentration of 11.1 and 11.9 mg $L^{-1}$, and pH value of 8 and 8.42. The third group was composed by two samples taken on the 15 April and 13 March 2008, which were essentially characterized by the lowest values of total Chl *a* (10.6 and 14.9 µg $L^{-1}$). Finally, the last group was composed by samples taken on 18 June 2007, on 29 April 2008, and between 29 May and 17 September. A low cyanobacterial Chl *a* proportion characterized this group, at the exception of sample taken on 25 June 2008 where a cyanobacterial Chl *a* proportion of 54.81% but with a rather low concentration of total Chl *a* (54.4 µg $L^{-1}$).

The Spearman correlation tests showed significant relationships between the cyanobacterial dynamics and the conductivity, dissolved nitrogen, nitrates and turbidity for both ponds (table 4). The TP was correlated

with cyanobacterial Chl *a* only in Ixelles Pond I whereas the ammonium and pH were significantly correlated with cyanobacterial Chl *a* only in Ixelles Pond II.




a.




b.

**Fig. 3**, a. Principal component analysis of Ixelles Pond I samples; b. Principal component analysis of Ixelles Pond II. (on the left : the principal components explained by the variables or Factors ; on the right : Samples distribution according to principal components)

### 3.3.3. Relative abundances of cyanobacterial genera and cyanobacterial 16S rRNA DGGE analysis in Lake Falemprise 2007

Because of the heterogeneities in the dataset and missing data, we have chosen to present here only the data concerning Lake Falemprise in 2007.

The countings performed at the University of Namur have shown the existence of several shifts in the cyanobacterial diversity. The nitrogen-fixing colony-forming species *Aphanizomenon flos-aquae,* and the filamentous species *Planktothrix agardhii,* shared the dominance of the cyanobacterial community during the 2007 survey (Fig. 4). A shift occurred in the community composition, probably as a result of a change in nutrient limitation. *Aphanizomenon flos-aquae* represented more than 77% of the cyanobacteria in all samples of the group 1 (as defined previously by PCA), when N and P were limited. *Anabaena spp.* were present from the beginning of the survey, but in reduced concentrations. The first appearance of *Planktothrix spp.* was on the third sampling date, and afterwards, it maintained a concentration ranging from 1% to 18% of the total population. Then, on the 17 July, N became limiting and the relative abundance of *Aphanizomenon flos-aquae* dropped to 41 and *Planktothrix spp.* abundance increased over 54%.

Genus richness was rather high during the N limited period. Indeed, during this period, we could observe the co-occurrence of *Anabaena spp., Aphanizomenon spp., Aphanocapsa spp., Microcystis spp., Planktothrix spp.,* and small filamentous cyanobacteria such as *Limnothrix spp.* and *Pseudanabaena spp. A Microcystis spp.* population also appeared in this period.

At the molecular level, the DGGE patterns for filamentous cyanobacteria followed more or less the microscopical observations (Fig. 5). Briefly, a total of 125 bands were excised, successfully amplified, and sequenced. Based on our own quality criteria system (*i. e.* were kept only sequences above 350 bp with less than 3 ambiguities), 37 sequences were used for analysis. Only the intense bands gave sequences of good qualities. Less intense band gave usually mixtures of sequences with a higher proportion of sequence identical to the ones of the intense bands. Affiliations of DGGE bands by BLAST analysis are summarized in table 5. *Aphanizomenon spp.* related sequences were detectable at the beginning of the survey, whereas *Planktothrix spp.* sequences were detected after 17 July. At this date, the decrease of the intensity of the *Aphanizomenon spp.* band corresponded to a decrease the *Aphanizomenon spp.* in population. Many variations occurred in band intensities along the survey. Unicellular

cyanobacteria (corresponding to the reverse primer 381Rb) were only detectable on the DGGE gel from 19 June. Bands related to *Microcystis* were detectable in all samples except on 27 August and 3rd September. Bands related to *Cyanobium spp.* were detected from 27 June to 10 July. *Snowella spp.* was detected from 3rd September to 2nd October.

The Neighbor Joining analysis of 103 sequences (Fig. 6), which was composed of the 37 DGGE bands, 66 strains sequences obtained from GenBank database, plus *E. coli* as an outgroup yielded a tree that clearly separated filamentous cyanobacteria from the unicellular ones. Bands sequences were placed into five different clusters supported by high bootstrap values. A total of 19 sequences belong to a clade that contained *Aphanizomenon flos-aquae* strains (1TU26S2, 1TU29S19, and 1TU37S13) and *Aphanizomenon gracile* PMC 9407, which was already shown to belong to the *A. flos-aquae* typical cluster by Rajaniemi et al. (2005). Nine sequences were part of a clade that contained *Planktothrix spp.* strains. Both filamentous clades were related and clustering together. The remaining three clades included unicellular cyanobacteria. One sequence belonged to the clade of Synechococcales and clustered with the strain *Cyanobium* JJ19B. Five sequences were clustering with strains from the genus *Microcystis spp..* Finally, three sequences were 100% similar to *Snowella litorallis* in a cluster made only of *Snowella spp.* strains. Occurrences of the taxa are indicated on the DGGE gel pictures (Fig. 5).

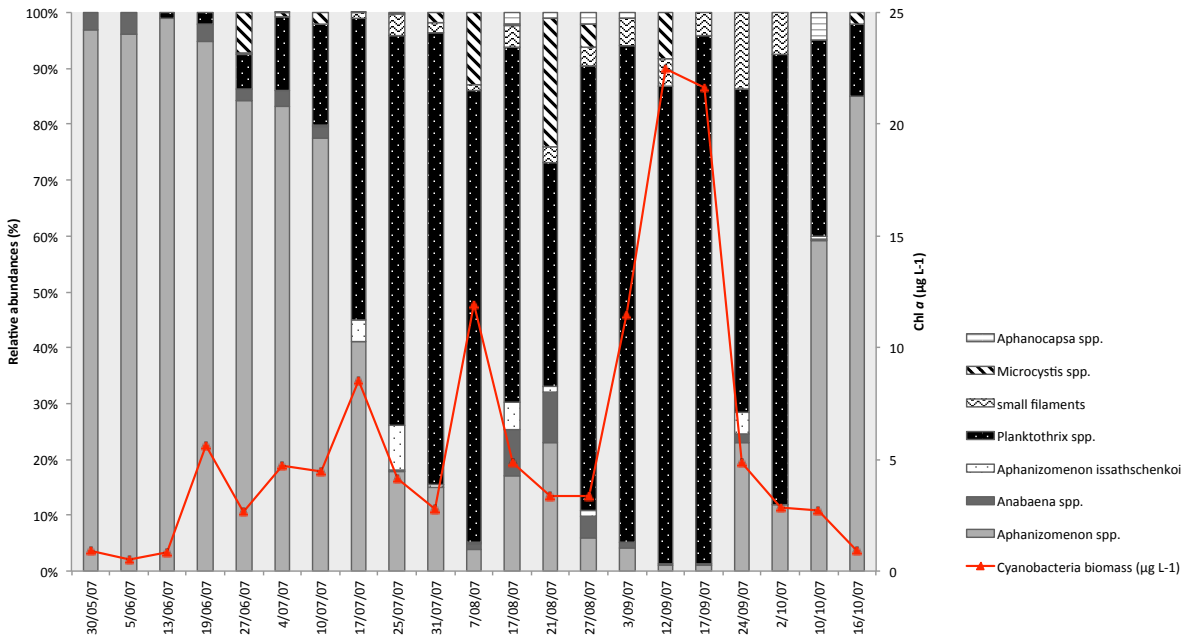



**Fig. 4**, Cyanobacterial composition by microscopic observations vs cyanobacterial biomass by HPLC analysis of carotenoids

**Table 5.** DGGE bands affiliations based on BLAST analyses. AN stands for Accession Number.

| DGGE | Sampling week number | Similarity | Most related strains | AN |
|---|---|---|---|---|
| Filamentous | | | | |
| | 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 | 99.5% | *Aphanizomenon flos-aquae* LMECYA 88 | EU078540 |
| | 29, 30, 31, 32, 33, 36, 37, 40 | 99.7 - 100% | Planktothrix agardhii LMECYA 153F | EU078516 |
| Unicelllular | | | | |
| | 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 | 99.2% | *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 |

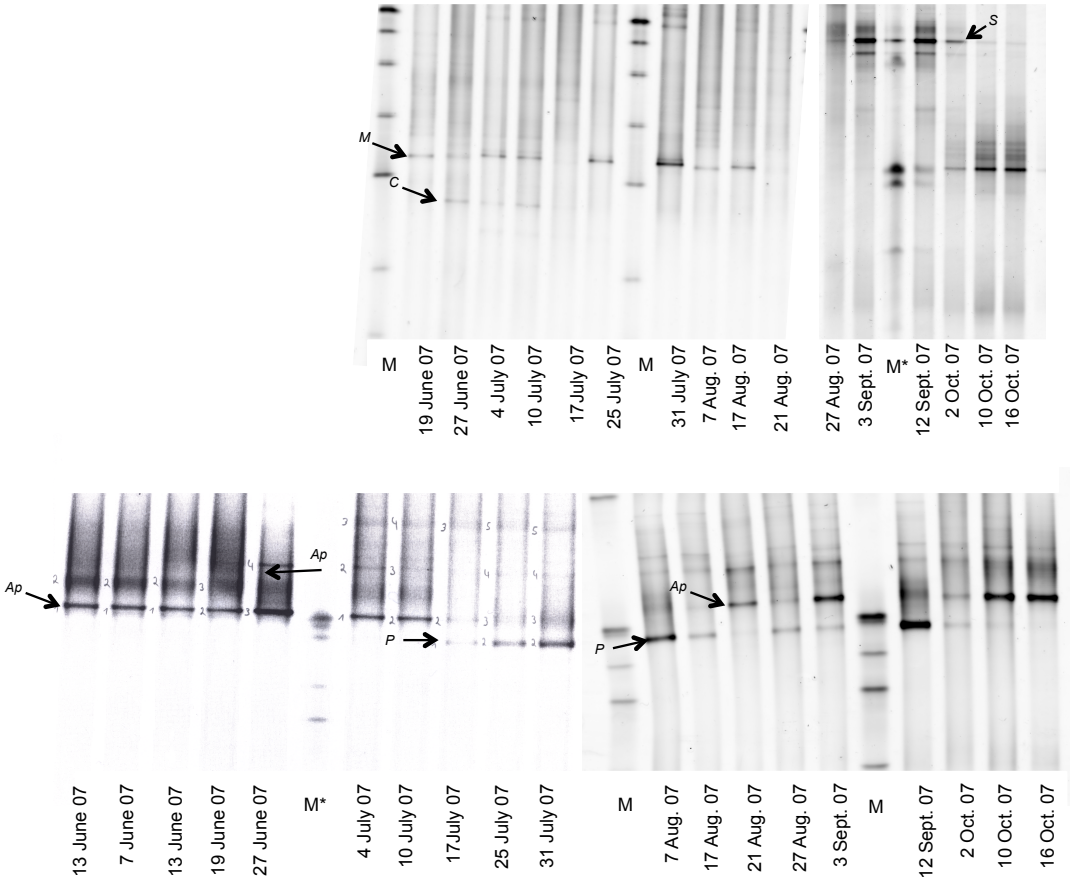



**Fig. 5**, 16S rDNA DGGE gel of Lake Falemprise 2007 survey. M: LAQUAN marker (home made DGGE marker); M*: modified LAQUAN marker; arrows indicate bands identification as follows: *Ap:* bands related to *Aphanizomenon spp.* sequences; *C:* bands related to *Cyanobium spp.* sequences; *M:* bands related to *Microcystis spp.*; *P:* bands related to *Planktothrix spp.*; *S:* bands related to *Snowella spp..*

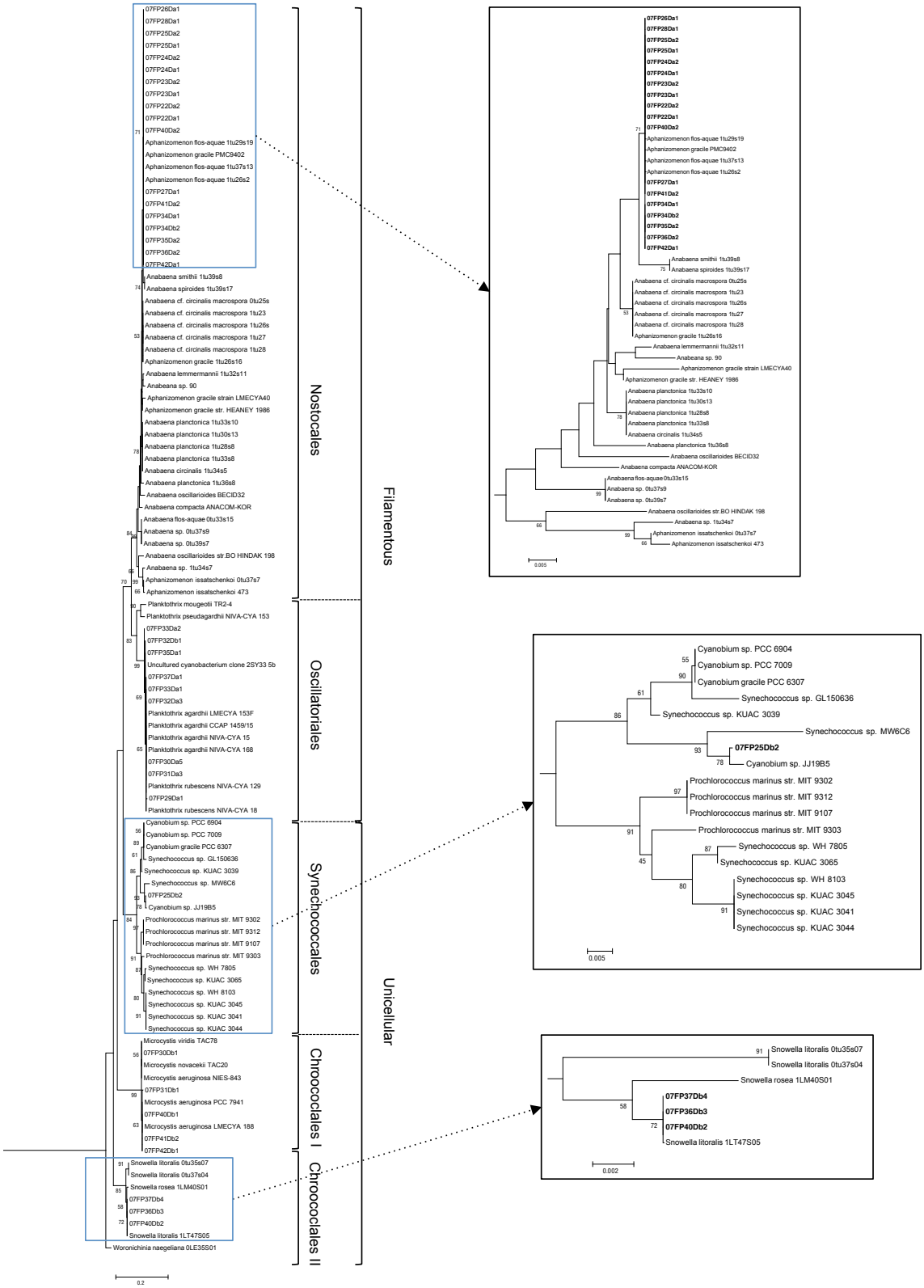



**Fig. 6**, The 16S rRNA distance tree of DGGE band sequences from Lake Falemprise. Total alignment length was 344 nt. Distances were computed using the Jukes and Cantor correction and a Neighbor-joining tree was built

with the software MEGA5 (Tamura *et al.,* 2011). Bootstrap replicate values are indicated at the nodes.

### 3.3. 4 Analysis of microcystins

Concentrations of microcystins were measured in the soluble phase (dissolved microcystins) and in the particulate phase (intracellular MC and MC attached to particles). From Wallonia and Brussels, 95 samples were sent to the University of Dundee for ELISA. All samples were positive for microcystins though wide variations occurred in the concentration values. The concentrations of microcystins were above the World Health Organization (WHO) drinking water guideline value (GV) of 1 µg MC-LR $L^{-1}$ for 52.63% of the samples, but only 6.3% of the samples had a value higher than the Dutch recreational water threshold level of 20 µg MC-LR $L^{-1}$.

Maximum MCs concentration of 57.5 µg $L^{-1}$ occurred in Lake Ri Jaune on the 18 August 2009. The ranges of were 0.12 to 6.11 µg $L^{-1}$ in Lake Falemprise (2007-2008, 2009), 0.95 to 3.11 µg $L^{-1}$ in Lake Féronval (2008, 2009), and 4.43 to 57.5 µg $L^{-1}$ in Lake Ri Jaune (2009). Results from additional samplings are listed table 5.

In Lake Falemprise, total MCs concentrations were below the WHO guideline value of 1 µg MC-LR $L^{-1}$ till the 27 June 2007 when concentrations started to increase to reach a maximum of 2.42 µg MC-LR equivalent $L^{-1}$ on 4 July 2007. Then, the total MCs dropped a second time below the WHO guideline value on the 17 July. A second peak reached 1.11 µg MC-LR equivalent $L^{-1}$ on 25 July. MCs concentrations remained under 1 µg MC-LR equivalent $L^{-1}$ until the end of the 2007 survey. In 2008, concentrations above 1 µg MC-LR equivalent $L^{-1}$ were observed between 17 July and 4 September. The maximum MCs total concentrations were observed on 14 August. MCs concentration measured for the additional samples are listed in the Table 6.

**Table 6.** Microcystin concentrations measured in additional samples from the Brussels and Walloon regions. MCs concentration is expressed as "µg MC-LR equivalent $L^{-1}$". n.d. : not determined. When waterbodies were sampled more than 2 times, we indicated a range of concentration.

| Lake | Year | Number of samples | Dominant genus/ genera or species | MCs concentration (µg MC-LR eq $L^{-1}$) |
|---|---|---|---|---|
| Lake Bambois | 2009 | 3 | *Anabaena spp./ Aphanizomenon spp.* | 1.15-2.63 |
| Cambre wood Pond | 2009 | 1 | n.d. | 0.74 |
| Clementine Square Pond | 2008 | 1 | n.d. | 0.93 |
| Ecaussine Quarry | 2007 | 1 | *Plankthotrix rubescens* | 7.982 |
| | 2008 | 1 | *Plankthotrix rubescens* | 13.7 |
| Lake Gouvy | 2007 | 1 | *Microcystis aeruginosa* | 0.584 |
| | 2009 | 1 | *Microcystis aeruginosa* | 16.6 |
| Ixelles Pond I | 2008 | 5 | *Aphanizomenon spp.; Microcystis spp.; Plankthotrix spp.* | 0.21-0.31 |
| Ixelles Pond II | 2008 | 2 | *Woronichinia spp.* | 0.96; 1.3 |
| Karreveld Castle Pond | 2008 | 1 | n.d. | 12.3 |
| King Baudoin Park Pond | 2008 | 1 | n.d. | 0.97 |
| Leybeek downstream Pond | 2008 | 1 | *Anabaena spp.* | 2.41 |
| Mellaerts klein Pond | 2009 | 1 | *Aphanizomenon spp.* | 0.81 |
| Neerdepede Pond II | 2008 | 1 | n.d. | 0.86 |
| Neerdepede Pond IV | 2008 | 2 | *Microcystis spp.* | 7.1; 13.4 |
| Prairie du cloître rouge Pond III | 2008 | 1 | n.d. | <0.1 |
| Lake Rénipont | 2009 | 1 | *Limnothrix spp.* | 2 |
| Virelles Pond | 2008 | 1 | *Anabaena spp.* | 1.45 |
| | 2009 | 2 | *Anabaena spp.* | 1.32; 1.96 |

In the Walloon region, there were wide variations in the percentage of dissolved MCs in Lake Falemprise, Lake Feronval, and Lake Ri Jaune (Fig 7). The dissolved microcystins concentrations ranged from 0.02 to 19.6 µg MC-

LR equivalent $L^{-1}$. Strikingly, some of the concentrations were higher for dissolved MCs than total MCs. It is likely due to the differences in quantification limits of different variants (Young et al., 2006) that may be present in the sample. Moreover, the absence of purification step during the extraction for total MCs measurement may explain this anomaly. Indeed, adsorption of MCs to remaining particulate matter may prevent antibodies recognition. Therefore, these samples where the dissolved MCs represented more than 100% were removed from the analysis. When total MCs were below the World Health Organization Guideline Value, dissolved MCs percentages ranged from 29.9% to 100% with an average of 76.1% ± 19.6%. Interestingly, dissolved MCs percentages were high when total concentrations were below 1 µg MC-LR equivalent $L^{-1}$, and low (below 28.71%) when MCs peaks were observed. This showed that most of the toxin was intracellular during the MCs peaks.

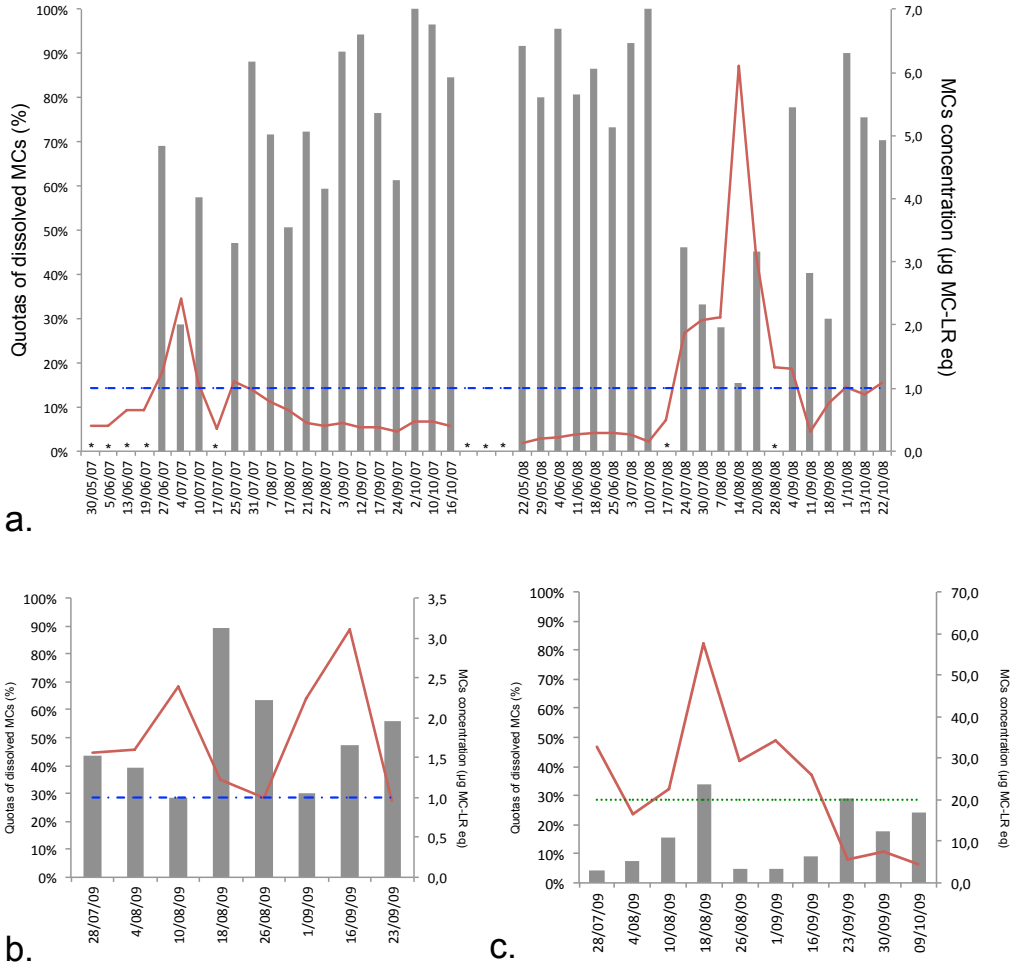



**Fig. 7**, MCs total concentrations and dissolved MCs percentage in Lake Falemprise (a), Lake Féronval (b), and Lake Ri Jaune (c). Histograms

represent dissolved MCs percentage, red curves represent the concentration of total MCs. Blue dash lines represent the WHO guideline (1997) value for drinking water (1 µg MC-LR $L^{-1}$), green dash line represents the recommended value of 20 µg MC-LR $L^{-1}$ for bathing restriction, as applied in the Netherlands. The '*' means that the dissolved MCs percentage was not determined.

### 3.3.5. Relationships between the environmental data and microcystins concentrations in Lake Falemprise

A Spearman rank order test was performed to characterize the relationships between MCs concentrations and environmental data. Negative correlations were found between total MCs and nitrogen (Table 7). Significant p-values below 0.01 were found for correlations between total MCs and total nitrogen, total MCs and nitrate, total MCs and nitrite, and total MCs and dissolved nitrogen. SRP was positively correlated with total MCs ($\rho$= 0.37 p-value=0.016). Surprisingly, a significant correlation of 0.41 (p-value= 0.006) was found between total MCs and diatom biomass. This result was comforted by the fact that a correlation of -0.59 (p-value= 0.006) occurred between total MCs concentration and silica ($SiO_2$). Finally, we found significant p-values for the correlation between total MCs and Euglenids biomass, and the measurement of wind speed for the day before sampling.

Dissolved MCs concentrations were correlated with total nitrogen ($\rho$= -0.38, p-value= 0.013) but not correlation was found with ammonium, nitrates, nitrites, or DIN. As shown for the total MCs concentration, significant p-values were observed for $\rho$ estimates for correlation between dissolved MCs and diatom biomass, dissolved MCs and silica, dissolved MCs and Euglenids biomass, and dissolved MCs and measurement of wind of the day before sampling (Table 7).

Significant p-values were observed for positive correlations between percentage of dissolved MCs and dissolved nitrogen, and total nitrogen concentrations. Also a significant negative correlation was found with Diatom biomass and was supported by a positive correlation with silica concentration. Finally, a significant negative correlation was found between the percentage of dissolved MCs and the average air temperature for the week.

**Table 7.** Relationship between percentage of dissolved MCs (%dissolved MCs) total MCs, dissolved MCs, and environmental variables assessed by Spearman correlation test. (ρ : Spearman correlation coefficient)

| Variables | % Dissolved MCs | | Total MCs | | Dissolved MCs | |
|---|---|---|---|---|---|---|
| | *ρ* | p-value | *ρ* | p-value | *ρ* | p-value |
| Diatom biomass | -0.38 | 0.026 | 0.41 | 0.006 | 0.39 | 0.011 |
| Dissolved nitrogen | 0.45 | 0.007 | -0.50 | 5x10-4 | | |
| Nitrate | 0.38 | 0.024 | -0.50 | 7x10-4 | | |
| Nitrite | 0.35 | 0.039 | -0.43 | 0.005 | | |
| Euglenozoa biomass | | | -0.31 | 0.043 | -0.34 | 0.026 |
| Outside week average T°C | -0.35 | 0.039 | | | | |
| Silica | 0.62 | 0.012 | -0.59 | 0.006 | -0.68 | 0.002 |
| Soluble reactive Phosphorus | | | 0.37 | 0.016 | | |
| Total nitrogen | 0.48 | 0.004 | -0.59 | 4x10-5 | -0.38 | 0.013 |
| Wind of the day before | | | 0.37 | 0.016 | 0.32 | 0.036 |

### 3.3.6. Exploratory study of MCs variants in Lake Ri Jaune

In Lake Ri Jaune, high concentrations of Chl *a* were recorded during a strong *Microcystis spp.* bloom formation. MCs concentrations reached a maximum of 57.5 µg $L^{-1}$. In order to characterize the co-occurrence of variants, we tested the approach used by Deleuze *et al.* (2011) on MC-LA, MC-LR, MC-RR, MC-YR, cyanopeptolin 1020, gonyautoxine 1, gonyautoxine 2, gonyautoxine 3, gonyautoxine 4 and microginin. M+2 patterns were only observed for microcystins variants. Ten samples were analyzed from Lake Ri Jaune. A total of seven peaks were observed between 900 and 1100 m/z. Differences of peak patterns were observed between the two tested matrices. Indeed, only five of the peaks were present with both 2,5-DHB and 1,5-DAN (Table 8). Strikingly, we observed higher intensities of peaks with 1,5-DAN. Also, higher intensities were observed for the 1024.6 m/z peak compared to others. At 995.6 m/z, MC-LR is supposed to produce a peak. However, it was not possible to clearly identify the present variant, the replacement of MDha by Dha amino acid suggested that it was not MC-LR. Further analysis will be done and discussed in the Phd thesis of Christelle Deleuze.

**Table 6.** Characterization of MC variants in Lake Ri Jaune. For both matrices (1,5-DAN and 2,5-DHB) '+' means presence of a peak, '-' means absence of peak, n.d. means not determined.

| m/z | MC variant | 1,5-DAN | 2,5-DHB | identified formula |
|---|---|---|---|---|
| 937.4 | n.d. | + | - | n.d. |
| 967.5 | [Asp3,Dha7]MC-LR | + | - | [Ala-Leu-Asp-Arg-Adda-Glu-Dha] |
| 981.5 | [Asp3]MC-LR | + | + | [Ala-Leu-MeAsp-Arg-Adda-Glu-Dha] |
| 995.6 | undefined | + | + | [Ala-X-(MeAsp or Asp)-Arg-Adda-Glu-Dha] |
| 1015.5 | [Dha7]-MC-FR | + | + | [Ala-Phe-MeAsp-Arg-Adda-Glu-Dha] |
| 1024.6 | [Dha7]MC-RR | + | + | [Ala-Arg-MeAsp-Arg-Adda-Glu-Dha] |
| 1031.4 | [Dha7]MC-YR | + | + | [Ala-Tyr-MeAsp-Arg-Adda-Glu-Dha] |

### 3.3.7. The *mcy* gene detection

The presence of *mcyA* and *mcyE* genes in the environmental samples suggests the presence of cyanobacteria, which possess the MCs biosynthesis operon. The primer design characteristics for both PCR (see Material and Methods) imply that at least the freshwater planktonic genera *Anabaena, Microcystis,* and *Planktothrix* can be detected. The distance between the 5' end of the *mcyA* gene and the 3' end of the *mcyE* gene covers about 30kb in *Microcystis* (Tillett et al., 2000). This would suggest that at least 30kb of the operon (with or without in/del) would be present, when both genes are present at the same time. In contrast, the detection of *mcyB* in this study only implies the presence MC biosynthesis genes of *Microcystis spp.* The total DNA was extracted from 162 samples. Regardless of the environmental conditions and sampling sites, the *mcyA* gene was detected in 77.8% of the samples, and the *mcyE* gene was detected in 80.3% of the samples. Potential MC-producing *Microcystis spp.* were detected in 77.8% of the samples after the first step of *mcyB* nested strategy, and in 95.1% of the samples after the second step. This result suggested that the concentrations of *Microcystis spp.* cells were under the detection limit of the first step of *mcyB* nested strategy, and that they were only detectable after 63 PCR cycles. Simultaneous detection of *mcyA, mcyB* (first reaction), and *mcyE* occurred only in 64.2% of samples (Fig. 8), plus 5.6% of samples after a second *mcyB* reaction. In 6.79% of the samples, *mcyA* and *mcyB* were detected while *mcyE* was absent. Meanwhile,

in 9.25% of the samples, *mcyB* and *mcyE* were found without *mcyA.* In those three cases mentioned above, the presence of *mcyB* is indicating that *Microcystis spp.* is at least one of the potential toxic genera. In the two samples from Ecaussines Quarry, *mcyA* and *mcyE* were found alone. This is in agreement with the microscopic observation where *Planktothrix rubescens* was observed but no *Microcystis spp.* colonies or cells.

Finally, *mcyB* was the only detected gene in 9.26% of the samples. This may suggests that the *mcyB* reactions have a lower detection limit than the other two genes. Nevertheless, PCR efficiencies and sensitivities were not compared.

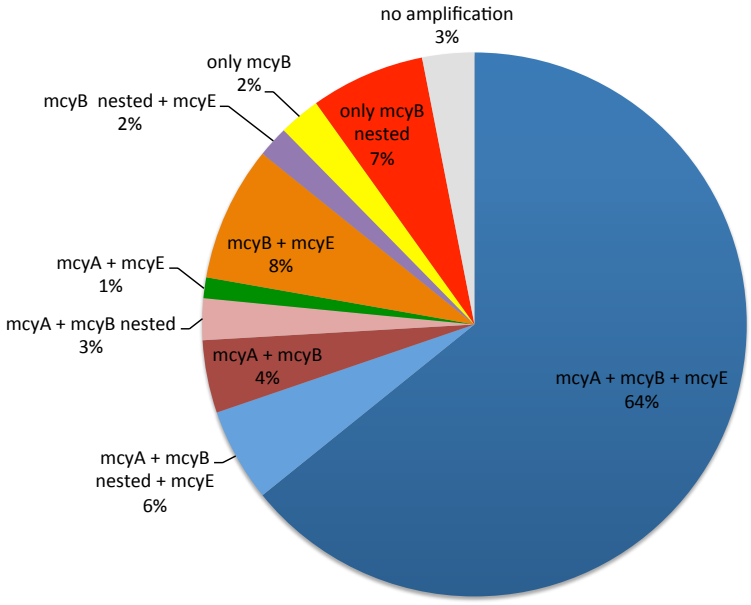



**Fig. 8**, Pie chart representing the occurrence of the three markers combined or alone in the sample set. '*mcyB'* means PCR were positive after the first step of the nested strategy. '*mcyB* nested' means PCR were positive after the second step of the nested strategy.

In 2007 at Lake Falemprise, the *mcyB* gene was always detected after the two successive PCR reactions, except in the sample of 24 September. The *mcyA* gene was only detected between 4 July and the 17 August. The *mcyE* gene was detected from 19 June to 27 August, and from 2$^{nd}$ to 16 November.

Regarding the PCA sample clusters there was no relationship between sample groups and gene detections. In 2008, there were no changes in gene

detections as all samples were positive for the *mcyA/B/E* detections except for the sample of 17 April.

### 3.3.8. Identifications of *mcyE* carriers by RFLP analysis

*In silico* analysis of the digestion of the *mcyE/ndaF* fragments with *AluI* for 27 strains from *Anabaena spp., Microcystis spp., Nostoc spp., Nodularia spp.* and *Planktothrix spp.* genera gave rise to six genus-specific patterns (Fig 9, a).

During this study, in Lake Falemprise, Lake Féronval, and Ixelles Ponds, we observed the formation of an unspecific fragment of about 400 bp. Wilmotte and colleagues (personal communication) previously analyzed this fragment, but its sequence was not related to any of the known *mcy* genes or NRPS sequences. To get rid of this unspecific fragment, a gradient PCR was performed and resulted in an increase of the annealing temperature to 60°C. The modified protocol was tested on DNA from strains that were isolated during a previous study (MIDICHIP project) before the start of this Phd. Due to the lack of DNA it has not been possible to perform a PCR on an artificial mixture of DNA. Four strains of *Microcystis spp.*, and one strain of *Planktothrix spp.* were successfully amplified and digestion patterns were corresponding to the *in silico* analysis patterns (Fig. 9, b).

RFLP of *mcyE* was carried out on 129 samples. Briefly, three genera were identified in our samples. The *Anabaena spp.* genotype was found in Lake Falemprise in 2008 and 2009, and Virelles Pond 2009. Both were associated with one or two *Microcystis spp.* genotypes (Fig. 9, c). The occurrence of at least one of the two different *mcyE* genotypes of *Microcystis spp.* was observed in all samples that were also positive for *mcyB* detection test (*i.e.* Lake Bambois (2009), Lake Falemprise (2007-2008), Lake Féronval (2008/2009), Lake Gouvy (2007/2009), Ixelles Ponds I/II (2007/2008), Neerpede Ponds II/IV (2008), Lake Rénipont (2009), Tercoigne Pond (2008) and Virelles Pond (2009)). In the quarry of Ecaussines (2007-2008), and Tercoigne Pond (2008), potential microcystin-producing *Planktothrix* were identified.

In Lake Falemprise and Ixelles Ponds, the RFLP analysis of 2007 and 2008 samples clearly showed a succession of the two different *mcyE* genotypes of *Microcystis spp..* So far, the alternation of *Microcystis spp. mcyE* genotypes was not corresponding to PCA classifications of samples or microcystins concentrations. Also, in few cases it was not always clear whether the two genotypes were actually present at the same time or that the high amount of product was not fully digested.

In Ri Jaune (2009), only one genotype (681bp/81bp) was characterized during the survey.

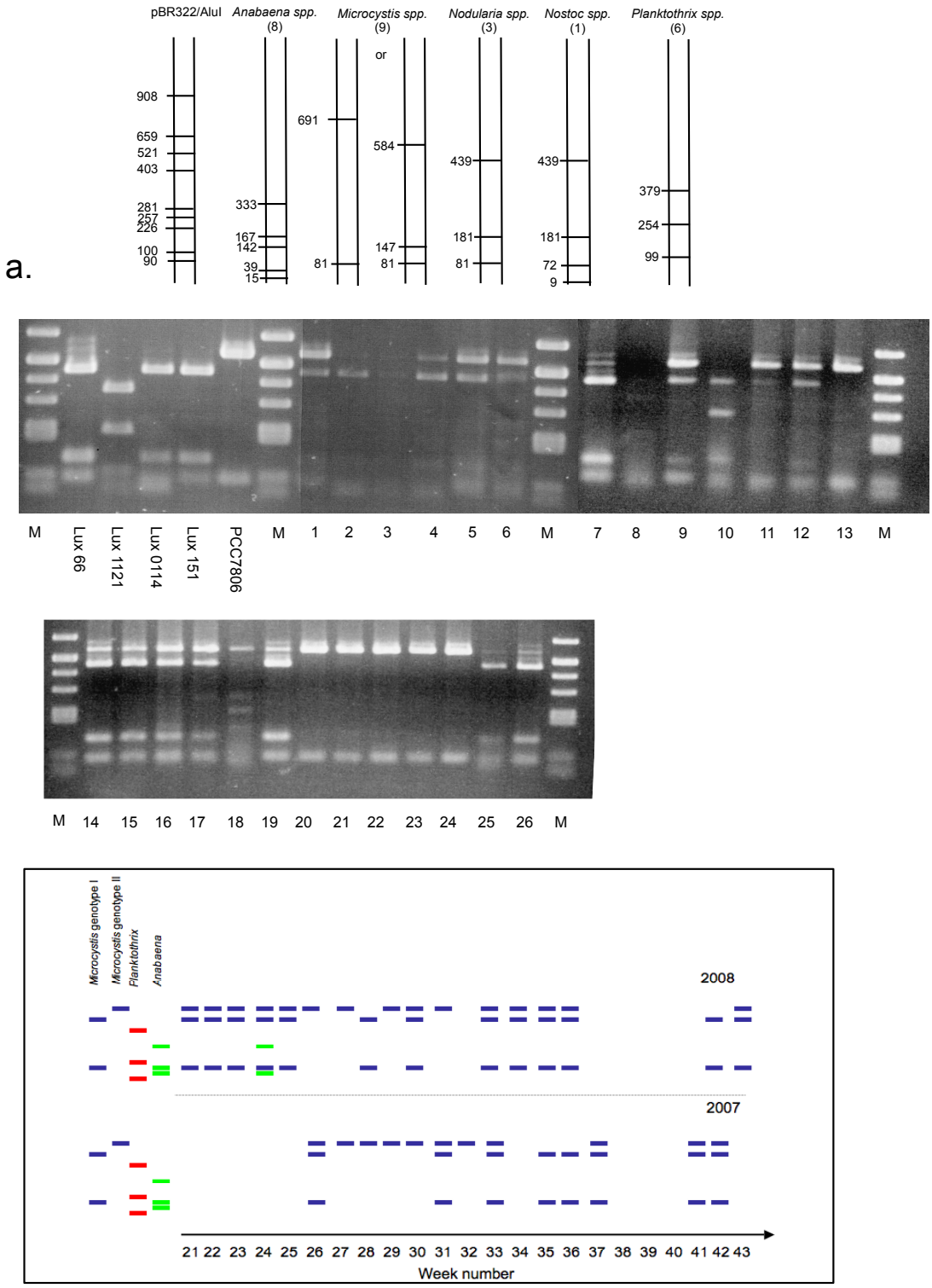



**Fig. 9**, **a,** Theoretical *mcyE/ndaF*-RFLP patterns of 27 strains of *Anabaena spp., Microcystis spp., Nodularia spp., Nostoc spp.,* and *Planktothrix spp.;* **b,** Strains and environmental samples *mcyE/ndaF* patterns. M: pBR322/AluI,1: Lake Falemprise 0708/07; 2, Lake Falemprise 27/08/07; 3, Lake Falemprise 03/09/07; 4, Lake Falemprise 12/09/07; 5, Lake Falemprise 10/10/07; 6, Lake Falemprise 16/10/07; 7: Ixelles Pond II 24/08/09, 8: Neerpede Pond II

14/08/09, 9: Neerpede Pond IV 14/08/09, 10: Tercoigne Pond 13/08/09, 11: Renipont Pond 1 , 12: Renipont Pond 2, 13: Lake Ri Jaune (verifier les dates sur labbook); **c,** illustration of *mcyE*-RFLP during Lake Falemprise monitoring 2007-2008.

### 3.3.9. Multiplex real time PCR assay: *mcyB* ratio (*mcyB/cpc*)

A total of 48 samples were analyzed. Unfortunately, the samples from Lake Ri Jaune showed a high variability in triplicate values. Indeed, except for the sample of 9 November 2009 (56.70% ± 5.26% *mcyB*), the standard deviation values ranged from 10.87% to 43.36%. As a result, we could not use these data in an analysis. From Lake Féronval samples, it was possible to evaluate the *mcyB* ratio for only to samples (1$^{st}$ October 2009: 55.25% ± 4.67%, and 9 November 2009: 3.77% ± 2.50%). We suggest that for those samples, the genomic DNA was degraded because of multiple freeze–thaw cycles, which were not endured by the other samples.

In 2007 at Lake Falemprise, the *mcyB* ratio varied from 0.04% to 29.14%. The maximum value of 29.14% ± 3.04% was obtained on 19 June just before the onset of the MCs concentration peak (Fig. 10). Unfortunately, we were not able to determine a value for the sample of 27 June. No relationships were observed between the *mcyB* ratio and total MCs or dissolved MCs.

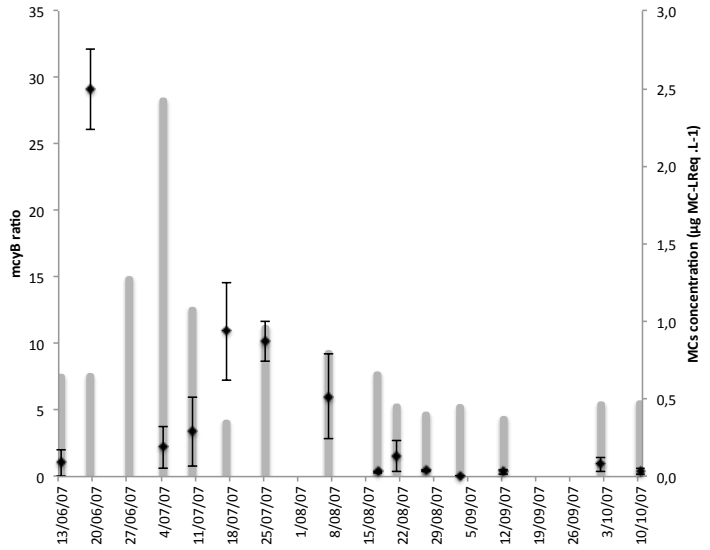



**Fig. 10**, *mcyB* ratio dynamics and MCs concentration dynamics in Lake Falemprise 2007

## 3.4. Discussion

### 3.4.1. Trophic status, cyanobacterial biomass and environmental conditions in the studied samples

Nutrients over-enrichments of waterbodies by anthropogenic activities led to the growth of cyanobacteria as harmful algal blooms (Paerl and Huisman, 2008). In the present study, both urban and rural waters were eutrophic or hypereutrophic during the summer.

In Lake Falemprise and Ixelles Pond I, where cyanobacterial communities were mostly composed by *A. flos-aquae* and *P. agardhii,* the total biomass of cyanobacteria was correlated with temperatures and TP concentration. In both waterbodies, SRP was rather low, often below 0.003 $mg.L^{-1}$. This suggests a phosphorus limitation (Reynolds, 2006). Although cyanobacterial biomass maxima were found during the summer, the concentrations were rather low compared to a previous bloom observed in 2002 (Verniers et al., 2005). A shift of the cyanobacterial community occurred in Lake Falemprise when there was a shift in nutrient limitation. *Planktothrix agardhii (*formerly *Oscillatoria agardhii*) favours less or weakly stratified waters, and is known to be independent from the TN:TP ratio (Oliver and

Ganf, 2000). Here, the thermocline seems to be unstable (J-P. Descy, personal Communication), and may occasionally be disrupted by meteorological events such as a windy episode, or may be established only during the day.

In Ixelles Pond II, *Woronichinia naegeliana* dominated the cyanobacterial community (data not shown, see Descy et al., 2011). This identification was confirmed by DGGE band sequence analysis (see chapter 2). SRP concentrations were in excess during most of the monitoring period. However, significant negative correlations between cyanobacteria biomass and dissolved nitrogen (ammonium, nitrates) implied that growth of *W. naegeliana* might rely on nitrogen availability. Cyanobacterial biomass maxima were observed in spring, summer, and fall. Besides, *W. naegeliana* colonies were observed while the pond was covered by ice (A. Peretyatko, personal communication). This indicates that *W. naegeliana* may be adapted to a large range of temperatures. Indeed, cyanobacterial biomass concentrations in the pond did not show any relationship with temperatures. For instance, *W. naegeliana* is quite widespread. So far, it can be found from Spitsbergen shallow ponds to Australian waterbodies (Van Donk *et al*., 2001; Day *et al.,* 1995), in polar, sub polar, temperate, and tropical regions (see Guiry and Guiry, 2014). It is also found in a wide range of trophic status from Finnish oligotrophic lakes to Belgian hypereutrophic ponds.

### 3.4.2. MCs contents, gene detection, *mcyE* carriers and DGGE

Highest MCs concentrations were measured in Lake Ri Jaune when *Microcystis spp.* was dominant. MALDI-ToF-ToF analysis revealed the co-occurrence of at least five MCs variants. Indeed, in addition to the classical MC-LR, we observed microcystins RR, YR, and microcystin LR with different degrees of demethylation (mono- or didemethylated), which were also the most common variants observed by Spoof and colleagues (2003). The [Dha7]-MC-RR variant seemed to be the most abundant. Besides, [Dha7]-MC-FR was observed for the first time in Belgian waterbodies. As 90 MC variants already were identified in the literature (Metcalf *et al.*, 2012), it is

likely that a quantification with methods directed to MC-LR could lead to an underestimation of the microcystins content in samples. In Flanders, over 37.5 $10^3$ µg MC-LReq $L^{-1}$ were recorded in *P. rubescens* dominated bloom (Descy *et al*., 2011). This result is in agreement with the previous study of German lakes where it was shown that *Planktothrix* strains were the most toxic (Fastner *et al.,* 1999). During the B-BLOOMS project (Wilmotte *et al.,* 2008), microcystins were detected and quantified by protein phosphatase inhibition assay (PP2A) and HPLC-DAD in blooms mostly dominated by *Microcystis spp.,* and for a few samples, where *Planktothrix spp.* was dominant. The estimation of MCs concentrations after conversion from µg/(g DW) to µg L-1 ranged from 0.35 to 25500 µg L-1, which is similar to our observations with ELISA.

No significant relationships were found between the occurrence of *mcy* genes and environmental conditions. However, our data showed that potential MC-producing cyanobacteria are constantly present in our waterbodies. The frequent detection of *mcyB* genes pointed out to the quasi omnipresence of potential toxigenic *Microcystis spp.*, even when MCs concentrations were low. As expected, *mcyB* was not detected in the Ecaussines Quarry; where only *P. rubescens* was observed by microscopy and identified by DGGE together with *Cyanobium spp.* (see chapter 2). In those samples, PCR with general primers for *mcyA* and *mcyE* were positive. The presence of *mcyE* gene carriers was verified in all samples for which MCs concentration exceeded the WHO GV except when dissolved MCs reached 1.2 µg MC-LReq $L^{-1}$ on 13 June 2007. By contrast, the *mcyA* gene was not detected in Lake Falemprise when the total MCs concentration exceeded the WHO GV for the first time in 2007. This may underline a different PCR efficiency for both reactions.

In Lake Falemprise, no *Microcystis spp.* related sequences were detected before 19 June 2007 by DGGE. However, the semi-nested PCR for the *mcyB* gene yielded positive results for samples that were taken before this date. Thus, potentially toxic *Microcystis spp.* indeed seem present in these samples. The absence of *mcyA* and *E* genes and the DGGE analysis may reflect the low abundance of *Microcystis spp.* during this period. According to PCR detection results, *mcyE*-RFLP patterns, and DGGE analysis, *Microcystis spp.* is potentially responsible for at least a part of the production of MCs while

the peak of MCs concentration was observed in 2007. Paradoxically, according to real time multiplex PCR data the percentage of toxigenic *Microcystis spp.* remained below 2.3% when the highest MCs concentration was observed.

Strikingly, *Planktothrix spp.* observed by microscopy and DGGE analysis did not seem to be toxigenic according to PCR-RFLP even though it was the dominant cyanobacteria. Low concentrations of MCs during the abundance peak of *Planktothrix spp.* (Fig) also suggested that it was a non MC-producing genotype. These two results may be explained by a mutation or deletion in the targeted region as in Christiansen *et al*. (2008), or by the absence of the *mcy* gene cluster.

*A. flos-aquae* was dominant when MCs concentrations were the highest, and during the N and P limitation. It may be a first clue for the production of MCs by *A. flos-aquae* (Fig. 11). None of the strains from the *A. flos-aquae* cluster were producing MCs. Though these strains belong to the *Dolichospermum* genus (Halinen *et al.*, 2007; Walkin *et al.,* 2009) that contains MC-producing *Anabeana.* strains, the 16S rRNA similarity threshold delimiting the genus is quite low (92%). It is likely that genomes significantly vary inside this genus. This could lead to the hypothesis that the potential *A. flos-aquae mcy* gene cluster may significantly differ from the *Anabaena* 90 *mcy* gene and this sequence different may prevent primer annealing. To date, there is no evidence in the literature that *A. flos-aquae* can produce MCs. Nevertheless, the hepatotoxic cylindrospermopsin was found in *A. flos-aquae* isolates from two German lakes (Preuβel *et al.*, 2006). Regarding the structure of the cylindrospermopsin, it is unlikely that anti MC-LR antibodies could cross react with it. Cylindrospermospin is a secondary metabolite encoded by a gene cluster that contained five polyketide synthetase modules (Mihali *et al.,* 2008). During this PhD thesis, we tested the M13/M14 primers (Schembri *et al.,* 2001), which led to the amplification of profiles with multiple bands including one fragment at the right size in samples from Lake Bambois, Lake Falemprise, Lake Féronval, and Virelles Pond. I tried to optimize the PCR but could not get rid of the multiple bands. Therefore, these results are not shown.

Here, the information on the dominant taxa was obtained with both microscopic and DGGE analyses. Nevertheless, less abundant co-occurring filamentous taxa (*ie Limnothrix spp., Pseudanabaena spp.)* were observed by microscopy but not detected by PCR-DGGE. It could be that they represented less than 1% of the cyanobacterial population, and that their DNA was not detectable by PCR. Moreover, it is known that the number of *rrn* operon differs between cyanobacterial genomes. Thus, multiple copies of the 16S rDNA from dominant taxa may have biased the representation of the true diversity. Also, mutations have been detected within multiple copies of the 16S rDNA of cyanobacteria (Engene and Gerwick, 2011).

Another explanation is that not all DGGE bands could be successfully re-amplified, and the DNA smear that surrounded some of the bands may be responsible for the sequence mixture that made it impossible to get a suitable sequence.

### 3.4.3. Relationships between MCs concentrations and environmental variables

In Lake Falemprise, we observed significant relationships between MCs concentrations (total and dissolved) and nutrients (DIN, SRP). This may be the reflection of the nutrient limitations of the MC-producer.

To our knowledge, it is the first time that a potential relationship between MCs concentration (total or dissolved MCs) and diatom biomass supported by a significant relationship between MCs concentration and SiO2 are reported. Plotting MCs (total and dissolved) and diatom biomass, showed that the highest values for diatom biomass were not always corresponding to total MCs concentration (data not shown). It is possible that the positive correlation between MCs concentrations (total and dissolved) and diatom biomass may be explained by the poisoning of diatom predators by toxins, by the inhibition of diatom competitors or a simple co-occurrence. In contrast, the negative correlation between the percentage of dissolved MCs and diatom biomass, suggested allelopathy of dissolved MCs fraction towards the diatom population. A similar observation was made with Euglenids biomass. Recently, allelopathic abilities of freshwater cyanobacteria have been well

documented (Leao *et al.,* 2009; Leao *et al.,* 2012). MC-LR was shown to have an infrequent allelopathic effect on macrophytes (LeBlanc *et al.,* 2005; Pflugmacher, 2002), and required an extremely high concentration to inhibit the growth of the mixotrophic haptophyte species *Prymnesium parvum* (James *et al.,* 2010). So far, inhibition of diatom or euglenozoa growth by MCs was not reported. Nevertheless, experiment on the biofilms species *Leptolynbya foveolarum* and *Nitzshia perminuta* pointed out to an inhibitory effect of the cyanobacterium on the diatom (van der Grinten *et al.,* 2005).

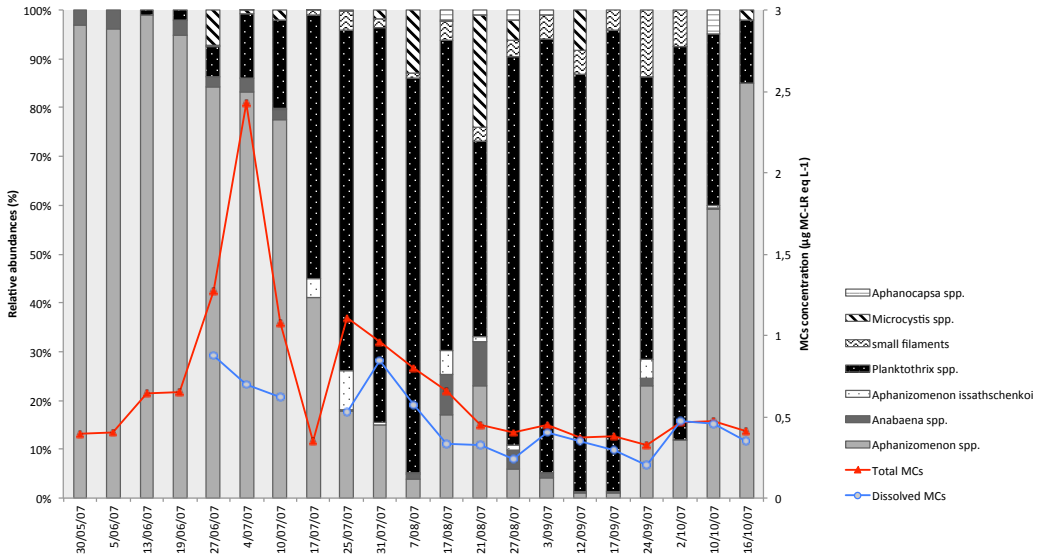



**Fig. 11**, Relative abundances of cyanobacterial genera versus MCs concentrations in Lake Falemprise (2007).

## 3.5. Conclusion

In this study, the presence of toxigenic cyanobacteria was observed in all studied waterbodies. In Lake Falemprise, toxigenic MC-producing *Microcystis sp.* were detectable in 41/42 samples. It was detected when cyanobacterial concentrations were low and it was impossible to detect *Microcystis spp.* on DGGE gels nor by microscopy. Thus, the *mcy* genes are good candidates for early-warning systems, to monitor freshwater bodies. The *mcyE* gene was always positive for samples with MCs concentrations above WHO GV. During this survey, the highest MCs concentrations were recorded in a *Microcystis spp.* dominated bloom in Lake Ri Jaune.

Counting of cyanobacteria and DGGE analysis in Lake Falemprise gave both the same dominant and additional genera. Indeed, we observed a richness of five filamentous cyanobacteria (*Anabaena spp., Aphanizomenon spp., Limnothrix spp., Planktothrix spp.,* and *Pseudanabaena spp.*), and four unicellular genera (*Aphanocapsa spp., Cyanobium spp., Microcystis spp.,* and *Snowella spp.*).

The *myE*-RFLP analysis allowed to identify the strains which possessed *mcyE* as well as mixtures of *mcyE* carriers in the environment. It was possible to characterize *Anabaena spp., Microcystis spp.,* and *Planktothrix spp.* as potential MCs-producing cyanobacteria. In Lake Falemprise, succession of two *Microcystis spp.* genotypes was observed but no relationship with environmental data or MCs concentrations could be found. In Lake Ri Jaune, only one genotype of *mcyE Microcystis spp.* carrier was observed even though MCs concentrations varied significantly. These results pointed the need for deeper characterization of *Microcystis spp.* genotypes in Walloon waterbodies.

In addition, no *Planktothrix mcyE*-genotype was characterized when high abundances of *Planktothrix spp.* were observed, and MC concentrations were quite low. Thus, *mcyE*-RFLP analysis seems to be an efficient way to identify MCs-producing cyanobacteria in the environment. Though *Microcystis spp.* genotypes were observed during the peaks of MCs concentrations, qPCR results did not show significant relationships with MCs concentrations. However, it was not possible to make any inference about the toxigenicity of *A. flos-aquae.*

Cyanobacterial biomass seemed to be related to the availability of nutrients in the three reference lakes. However, TP was not related to cyanobacterial biomass dominated by *Woronichinia spp..* Also, it was shown that the cyanobacterial biomass was significantly related to temperature in Ixelles Pond I and Lake Falemprise. In contrast, no significant relationship between cyanobacterial biomass and temperature was found in Ixelles Pond II, where *Woronichinia spp.* was the dominant cyanobacteria.

We reported for the first time a significant relationship between diatom/euglenozoa biomass and MCs concentrations. It is possible that this

result is the first evidence of an allelopathic effect of MCs on planktonic diatoms populations in the environment.

Finally, a newly described variant of MALDI-ToF-ToF for the characterization of Dha and MDha containing MCs variants was successfully applied on environmental samples. Five variants were characterized and the identification of a last one in still pending. These results underlined the need for the identification and quantification of multiple MCs variants to prevent human exposure to harmful cyanobacteria.

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# Chapter 4 – Genotypic variations of toxic and non-toxic strains of *M. aeruginosa* in the Westveldpark Pond (St Amandsberg, Belgium)

*Author's contributions:*

Water sampling and monitoring of the environmental variables were organized and performed by the partner of UGent. Countings of cyanobacterial cells were performed by Jeroen van Wichelen (UGent). UNamur team realized the pigments analysis and the partner at the University of Dundee realized the microcystins analyses.

The environmental DNA and *Microcystis* strains were sent to the University of Liège for the qPCR analysis.

The Phd applicant (Y. Lara) designed the SYBR green qPCR assay and performed the qPCR runs (SYBR green and Taqman). He realized all the statistical analyses and wrote the manuscript.

This chapter will be submitted as a research article in a peer reviewed journal.

# Chapter 4 – Genotypic variations of toxic and non-toxic strains of *M. aeruginosa* in the Westveldpark Pond (St Amandsberg, Belgium)

## 4.1. Introduction

*Microcystis aeruginosa* (Kützing) is a cosmopolitan unicellular colony-forming cyanobacterium, which is known to proliferate in eutrophic waterbodies worldwide, and to produce cyanotoxins. Among these cyanotoxins, microcystins are the most investigated. These hepatotoxic agents have been shown as a threat for animal and human health.

Colonies of *M. aeruginosa* are irregular in outline, lobate, and clathrate in older colonies. Cells are spherical, 4-6.5 µm wide, embedded in a transparent colonial mucilage (Komarek and Komarkova, 2002). Numerous studies have discussed the taxonomy of *Microcystis* using traditional morphological criteria (*i.e.* colony morphology, cell size, mucilage structure, pigment composition). The genus was first separated into 23 species (Geitler, 1932). Later, the strains lacking gas vesicles were excluded from the genus *Microcystis* (Stanier *et al.,* 1971). It is now assumed that the presence or absence of gas vesicles is the consequence of buoyancy strategies adopted by planktonic cyanobacteria when a nutrient limitation occurs at the water's surface (Sejnohova and Marsalek, 2012). The emergence of molecular techniques resulted in studies that pointed out the need to reassess species delineation inside the genus. The morphological characterization did not correlate with phylogenetic trees based on 16S rRNA sequences (Lepère *et al.,* 2000; Neilan *et al.,* 1997). In addition, the DNA-DNA hybridization values for strains assigned to five different morphospecies (*i.e. M. aeruginosa, M. ichthyoblabe, M. novacekii, M. viridis,* and *M. wesenbergii*) exceeded 70%. These results suggested that the five morphospecies should be considered as the same bacterial species (Otsuka *et al.,* 2001). However, the analyses of *cpcBA*-IGS and ITS-rRNA sequences revealed intra and interspecific variations within the genus (Neilan *et al.,* 1995; Otsuka *et al.,* 1999).

So far, microcystins production has been observed in *M. aeruginosa, M. botrys, M. flos-aquae*, *M. ichthyoblabe, M. novacekii, M. panniformis,* and

*M. wesenbergii* (Janse *et al.,* 2004, Via-Ordorika *et al.,* 2004). However, the last was regarded as a non MC-producing species by other authors (Xu *et al.,* 2008; Kurmayer *et al.,* 2002; Via Ordorika *et al.,* 2004).

It is impossible to distinguish toxigenic from non-toxigenic isolates based on the morphological criteria. However, toxic and non-toxic genotypes of *M. aeruginosa* have been described from the same bloom sample using molecular methods (Rohrlack *et al.*, 2001; Carrillo *et al.,* 2003). The direct consequence of this observation is that MCs concentration dynamics may be explained by the succession of toxic and non-toxic genotypes.

In the Netherlands, Kardinaal and colleagues (2007a) investigated the co-existence of several ITS-rRNA genotypes in three lakes with different topologies. In Lake t'Joppe, a deep stratified lake, the MCs concentration corresponded to the genotypic composition. This observation was less evident in Lake Volkerak and Lake Kinselmeer, two unstratified shallow lakes. More recently, Wichelen and colleagues (2010) studied the succession of two morphotypes, *i.e. M. aeruginosa* and *M. viridis* during a two-year survey (2007-2008) in the shallow unstratified Westveld Pond (Ghent, Belgium). During this multidisciplinary study, a total of thirty-four strains were isolated and affiliated to the two morphotypes. Twenty *M. aeruginosa* strains corresponded to three ITS-genotypes, ITS 2 (a non MC-producing genotype), ITS 3 (a MC-producing genotype), and ITS 4 (unknown toxicity). Finally, fourteen *M. viridis* strains corresponded to the ITS 1 genotype, and were recognized as MCs-producing strains. In both years, bloom events were dominated by *M. aeruginosa*. However, both morphotypes were producing microcystins and were associated to peaks of MCs production. Surprisingly, a strong and specific predation effect by amoeba directed toward *M. aeruginosa* was observed during the first year of the survey, and led to the hypothesis that biotic factors prevail over abiotic factors for the *M. aeruginosa* growth. Indeed, ITS-DGGE profiling did not allow establishing any relationship between *M. aeruginosa* ITS genotype dynamics and environmental parameters. So far, abiotic factors influencing the alternation of toxic and non-toxic genotypes are still quite enigmatic in the aquatic environments.

Competition between toxigenic and non-toxigenic strains has been investigated during laboratory experiments. Strikingly, non-toxigenic strains

exhibited a greater fitness while grown in monoculture under growth limiting conditions (Kardinaal *et al*., 2007b; Briand *et al.*, 2008; Leblanc Renaud *et al.,* 2011). In contrast, MC-producing strains were winning the competition when strains were mixed (Briand *et al.*, 2008; Leblanc Renaud *et al.,* 2011). This led to the conclusion that the benefits of producing microcystins might outweigh their energetic cost. However, the strains involved in these studies had different genotypes and contained genes involved in other metabolites biosynthesis or functions, which may influence the outcome of the competition. More recently, in a study that investigated the competition between the MC-producing *Microcystis* strain PCC7806 and its non MC-producing mutant, Briand *et al.* (2012) concluded in a significant difference in the fitness of the two strains, and the non MC-producing strain was favored during co-culture experiments. Authors stated that these differences may be due to the cost of microcystins production. Consequently, the results of these previous studies show the importance of genomic differences between the genotypes for their success in the environment. Indeed, it is already known that a wide oligopeptide diversity occurs among environmental colonies in the same sample (Welker *et al.,* 2004). Thus, it is possible that other metabolites than MCs may confer a greater fitness to certain genotypes than others. Thus, the genotyping of bloom cyanobacteria remains a very promising field of study. However, it is necessary to develop approaches that enable the monitoring of one genotype at a time in the environment.

In microbial ecology, the PCR-DGGE analysis is widely used to characterize the bacterial community structure. However, PCR biases due to multiple templates or nested strategy hinder the quantification of genotypes (Polz and Cavanaugh, 1998; Park and Crowley, 2010). The recent emergence of real time quantitative PCR technology has helped to understand the factors influencing the dynamics of bacterial genotypes in the environment. In order to estimate the proportion of MC-producing *Microcystis* genotypes in Lake Wannsee (Germany), Kurmayer and Kutzenberger (2003) developed *Taq* nuclease assays targeting the *mcyB* and *cpc* genes. They observed a proportion of *mcyB* gene ranging from 1 to 38%. Later, Briand and colleagues (2009) designed a multiplex assay using the same probes. In the same study, authors investigated spatial and temporal variations of *mcyB* proportion and

ITS genotypic composition in the Grangent Reservoir in 2007 (France). Wide variations of *mcyB* gene proportion, ranging from 6% to 93%, occurred in space and time. The percentage of *mcyB* genotypes was higher at the beginning and after the decline of the bloom. Lowest proportions were found when the bloom was at his maximum. In addition, a significant negative correlation was observed between the proportion of *mcyB* genotypes and the percentage of the dominant ITS-genotype. Using the same approach, Sabart and colleagues (2010) found less marked changes during a peak of *M. aeruginosa* in 2006 at the Grangent Reservoir. However, large variations in *mcyB* genotypes were observed in six different waterbodies. Interestingly, no correlation was observed between the proportion of *mcyB* genotypes and environmental factors or MC concentrations. It is possible that the *Microcystis* population carrying the *mcyB* locus includes in fact different subpopulations of genotypes, which may be influenced by the environmental conditions in a different way. This would partly explain the lack of correlation between the *mcyB* populations and the environmental parameters. Therefore, it would be interesting to see how a single MC-producing genotype evolves during time in the environment.

The main goal of this study was to investigate the influence of the environmental parameters on *M. aeruginosa* ITS-genotypes using real time quantitative PCR. This allowed to monitor whether or not the ITS-genotype dynamics correlated with *M. aeruginosa* morphotype dynamics, and whether or not ITS-genotypes correlated with MCs production *in situ*. In order to follow ITS 2 and ITS 3 in natural samples, two genotype-specific quantitative PCR assays were designed and tested on the two-year monitoring samples.

## 4.2. Materials & methods

### 4.2.1. Sampling site and collection

The sampled pond is located in Saint Amandsberg (Belgium) in Westveldpark (51°04′059″N and 3°46′427″E), in the neighborhood of Ghent. It is a highly eutrophic parkland pond of 2024 $m^2$ surface area and a 2 m maximum depth. Monitoring was performed as described in Van Wichelen *et al.* (2010): water

samples were taken between May 2007 and November 2008 on a weekly basis or twice per week during bloom conditions, and a single sample was taken during the winter season (December to March).

For microscopic observation and counting of *Microcystis spp.,* 250 mL water samples were fixed in Lugol's iodine, formaldehyde, and sodium thiosulfate. Identification and enumeration of cells and colonies were performed in one mL fixed samples using a Sedgewick– Rafter counting chamber (PYSER-SGI, USA) with a Leitz Diaplan light microscope.

For zooplankton characterization, five to 30 L of water samples were concentrated by filtration through a 70 mm mesh size plankton net. Then, samples were fixed in formaldehyde 5% (final concentration; v/v). Identification and enumeration were performed using a counting chamber and a Leica Wild M 10 stereomicroscope as described in Van Wichelen *et al*. (2010).

### 4.2.2. Physico-chemical parameters

For abiotic variables, water temperature, oxygen concentrations, pH, and conductivity were measured using a YSI multi-parameter probe (Yellow Springs, USA). For nutrient analysis, 0.2 mL $H_2SO_4$ at 5N were added to 50 mL of sampled water in order to measure total phosphorus (TP). Water was filtered through a previously dessicated Whatman (Kent, USA) GF/C filter until the filter got clogged in order to measure particulate C and N. $NO_2^-$ concentrations were determined using the sulfanilamide method. $NO_3^-$ concentrations were determined using an adapted cadmium reduction method. $NH_4^+$ concentrations were determined by the salicylate-dichlorocyanurate method. SRP ($PO_4$) concentrations were measured by the molybdate method. Particulate N and C were analyzed with a Carbo Erba NA 1500 elemental analyzer Thermo Scientific (CA, USA).

### 4.2.3. Chl a concentration and phytoplankton composition pigment analysis by HPLC

Water volumes, 15 and 200 ml, were filtered through (Whatman) GF/F, until filters clogged, then filters were conserved at -80°C until processed. Pigments were analyzed as described in Descy *et al*. (2000). Extraction was performed in 90% HPLC grade acetone. Phytoplankton cells were disrupted after two times of 15 minutes of ultrasonication separated by one overnight incubation at 4°C in the dark. HPLC analyses were performed with a Waters system comprising a Waters 996 PDA detector and a Waters 470 fluorescence detector using the gradient elution method (Wright *et al*., 1991). Commercial external standards (DHI, Denmark) were used for calibration.

As described in Descy *et al.* (2009), pigments used to represent the algal class abundances were: peridinin, fucoxanthin, neoxanthin, violoaxanthin, the sum of diadinoxanthin and diatoxanthin, alloxanthin, lutein, zeaxanthin, chlorophyll *b*, chlorophyll *a*, echinenone, ßε-carotene, ßψ-carotene and ßß-carotene. The biomasses of the algal classes, *i.e.* cyanobacteria, chlorophytes, chrysophytes, cryptophytes, diatoms, dinoflagellates, and euglenophytes were estimated in Chl *a* equivalent using a pigment ratio matrix CHEMTAX as in Sarmento and Descy (2008). The cyanobacteria biomass was calculated as the sum of the estimation of cyanobacteria type 1 based on zeaxanthin (ZEA) concentration and the estimation of cyanobacteria type 2 based on zeaxanthin (ZEA) and echinenone (ECH) concentrations.

### 4.2.4. Monitoring of *M. aeruginosa* ITS genotypes by a SYBR green qPCR assay

DNA was extracted as previously described in Van Wichelen *et al.* (2010). Ninety-seven samples were aliquoted to avoid the DNA degradation process by repeated freeze thaw cycles. In order to follow MC-producing and non MC-producing genotypes of *M. aeruginosa* in St Amandsberg, we designed a qPCR assay using the SYBR green technology. Two sets of primers targeted the ITS sequences of toxic and non-toxic strains. Primers were designed on the basis of all ITS sequences from van Wichelen *et al.* (2010), which were downloaded from GenBank, and aligned. Two primer sets were selected

because of their specificity to MC-producing strains of the ITS 3 genotype, and non MC-producing strains of the ITS 2 genotype (van Wichelen *et al.,* 2010), in a high GC-content region. The ITS 2 genotype was amplified using forward primer 5'-ATTCAGGTAGGAGACGAA-3' and reverse primer 5'-CTCTAACCACCTGAGCTA-3', and ITS 3 genotype was amplified using forward primer 5'-ATTCAGGTATGGGACGAA-3' and reverse primer 5'-CTCTAACCACCTGAGCTA-3'. Primer sequences were BLASTed against the GenBank DNA database to control their specificity.

Reactions were performed in 25 µL (final volume). It contained 2X ABsolute$^{TM}$ Blue QPCR SYBR® green fluorescein mix which included the proofreading Thermo-Start$^{TM}$ DNA polymerase, 150 nM of each primer, and 1 µL of template DNA. In order to optimize the annealing temperature of primers, we performed gradient temperature experiments for which the efficiency of reaction, $R^2$ of standard curves, and formation of primer-dimer was controlled. For this purpose, we used serial dilution triplicates that contained a gradient of six concentrations from approximately 50 to $5x10^{-4}$ ng.

For each run of samples, serial dilution triplicates containing approximately 50, 5, $5x10^{-1}$, $5x10^{-2}$ ng of the genomic DNA µg $.L^{-1}$ were prepared from *M. aeruginosa* W32 for the ITS 2 genotype and *M. aeruginosa* M31 for the ITS 3 genotype. PCR programs were performed as follows: for both reactions, samples were incubated at 95°C for 15 minutes. Then, 40 cycles were performed with a first step at 95°C for 15 seconds, a second step at 58°C for 30 seconds, and at 72°C for 30 seconds, then a final elongation step was performed at 72°C for 7 min. In order to control the formation of primer-dimer, a melting curve from 60°C to 90°C was undertaken. Samples were analyzed in duplicates. Runs were performed on a BioRad MiniOpticon real-time PCR detection system (Hercules, CA USA). Copy number for each sample was calculated as follow:

$$Copy\ number = \frac{2 \times 6.0221413 \times 10^{23} \times x}{660 \times genome\ size\ of\ NIES\ 843}$$

Where x represents the concentration measured.

To ensure the specificity of our reactions, negative controls were tested using strain DNA (approximately 50 ng) of different ITS genotypes. To investigate

the influence of mixture of DNA template, DNA from *M. aeruginosa* W32 (ITS 2) was mixed with DNA of *M. aeruginosa* M31 (ITS 3). Mixed samples were prepared in the following proportions: 40/20, 4/20, 0.4/20 (W32 ng/ M31 ng), and a last sample contained 120 ng of *M. aeruginosa* M31.

In order to compare each sample run, slopes and intercepts of each run were compared using a one way repeated measure ANOVA. For data analyses, only samples with Ct values above 10 and below 30 were treated.

### 4.2.5. Real time multiplex qPCR assay

In order to quantify the ratio of *mcyB* producers in *Microcystis* spp. populations, we used a multiplex real-time PCR strategy using TaqMan probes (Briand et, al. 2008), which was adapted to the BioRad MiniOpticon real-time PCR detection system (Hercules, CA, USA). The targeted genes were *mcyB* and *cpcBA* as described in Chapter 3. The genomic DNA of strain PCC7806 was used to perform the standard curves. For each run of samples, serial dilutions triplicates containing approximately 5 $x10^{1}$, 5, 5 $x10^{-1}$, 5 $x10^{-2}$ ng of the genomic DNA of PCC7806 were prepared.

The multiplex reaction was performed in 20 µL (final volume), it contained 2X KAPA PROBE FAST BioRad iCycler qPCR mix with fluorescein as reference dye, 100 nM of PC-probe, 300 nM for each of forward and reverse primer for the PC reaction, 250 nM of MCY probe, and 900nM for each of forward and reverse primer for the MCY reaction.

The *mcyB* ratio was determined using the ∂Ct approach, as described by Briand et al. (2009). The theoretical equation for the evaluation of *mcyB* ratio values was y= 3.32 x log(X) - 8.78, as defined by the original study. Samples were analyzed in triplicates.

### 4.2.6. MCs concentrations

The Scottish B-BLOOMS 2 partners at the University of Dundee carried out the microcystin analyses by ELISA. Polyclonal antibodies were conceived to recognize microcystin-LR (Metcalf et *al.*, 2000). In order to quantify the total amount of microcystins, 1 mL of water sample was boiled for 1 minute in a

water bath (Metcalf et *al*., 2001). The amount of soluble microcystins was estimated after 1 mL of raw sample was centrifuged at 10 000 rpm for 10 minutes by taking the supernatant for analysis. Toxin concentrations were expressed in µg toxin per L water (see Chapter 3 for further details).

### 4.2.7. Data analysis

Multiple linear correlations between MC concentrations, *M. aeruginosa* biomass and ITS genotypes abundances and environmental parameters (ammonium (mg .L-$^{1}$), conductivity (µS .cm$^{-1}$), dissolved oxygen concentration (mg .L$^{-1}$), dissolved nitrogen, light intensity (µE .m$^{-2}$ .s$^{-1}$), *M. viridis* biomass, nitrites (mg .L$^{-1}$), nitrates (mg .L$^{-1}$), outside humidity (%) (mean over seven days), outside temperature (°C), percentage of dissolved oxygen (%), particulate N and C (mg. L$^{-1}$), pH, photoperiod (hour), phytoplankton class biomasses (mg. L$^{-1}$), Secchi depth (m), silica (mg .L$^{-1}$), single cell biomass, SRP (mg .L$^{-1}$), sum of rain (mm), total *Microcystis* spp. biomass (µg Chl *a* eq. L$^{-1}$), TN (mg .L$^{-1}$) (not available for 2007 survey), TP (mg .L$^{-1}$), mean wind speed (m .s$^{-1}$) (three days, seven days), wind speed of the day before (m .s$^{-1}$), zooplankton abundances (ind .L$^{-1}$) were calculated using the Spearman rank correlation test. P-values were corrected by the Holm-Bonferroni method.

Principal component analysis (PCA) were carried out for 2007 and 2008 to group samples on the basis of environmental parameters and total cyanobacterial biomass. Two matrices were composed by 18 environmental parameters (ammonium (mg .L$^{-1}$), conductivity (µS .m$^{-2}$ .s$^{-1}$), DIN, light intensity (µE .m$^{-2}$ .s$^{-1}$), nitrates (mg .L$^{-1}$), nitrites (mg .L$^{-1}$), outside humidity (%), outside temperature (°C), percent of dissolved oxygen (%), pH, photoperiod (hour), Secchi depth (m), silica (mg .L$^{-1}$), SRP (mg .L$^{-1}$), sum of rain (mm), total cyanobacteria, TP (mg .L$^{-1}$), mean wind speed over three days (m .s$^{-1}$). In order to approximate normal distributions, datasets were square root transformed and standardized. Distributions were examined through quantile-quantile plots.

Linear regression analyses were performed to determine whether variations in ITS genotypes abundances (ITS 2/ ITS 3), percentage of *mcyB*,

and MCs concentrations (dissolved MCs, particulate MCs, and total MCs) could be explained by environmental variables in 2008. Data were first square root transformed and standardized as described for PCA. Explanatory (environmental parameters) and response (ITS genotype abundances, and MCs concentrations) variables were also square root transformed and standardized. The same 19 explanatory variables, *i.e.,* ammonium (mg $.L^{-1}$), conductivity (µS $.cm^{-1}$), dissolved oxygen concentration (mg $.L^{-1}$), light intensity (µE .m2 $.s^{1}$), nitrites (mg $.L^{-1}$), nitrates (mg $.L^{-1}$), percentage of dissolved oxygen (%), outside humidity (%), outside temperature (°C), pH, photoperiod (hour), Secchi depth (m), silica (mg $.L^{-1}$), SRP (mg $.L^{-1}$), sum of rain (mm), TP (mg $.L^{-1}$), three day mean wind speed (m $.s^{-1}$) were used for the fifth analysis. Six parameters (Chl *a*, cyanobacteria biomass, ITS genotype abundances, phytoplankton biomasses, and MCs concentrations) could be affected by the same environmental variables and thus were not considered as explanatory variables. Forward stepwise selections of parameters were performed using the 'stepwise' program of the R library FactoMineR in the R statistical package. Briefly, starting models had no parameter effects except intercept. Then, the most informative variables were selected one by one according to their Akaike's information criterion until the optimal model was found. Finally, we tested the significance of stepwise models using F statistics, and models were tested by two ways ANOVA.

## 4.3. Results

### 4.3.1. Environmental conditions

In 2007, the temperature of the epilimnion was not measured. The average air temperature for 7 days had a wide range of variation during the monitoring from 4.21 to 19.51°C. From the late spring to the end of the summer (1$^{st}$ May-21$^{st}$ September), the temperatures were above 13.1°C, with an average of 16.4°C. The highest temperatures (above 18°C) were recorded between 8 June-15 June and 17 July-23 July, with episodic peaks above 18°C all along the survey. The pH values of the epilimnion varied from 7.2 to 9.9. The conductivity slightly declined from 218 to 135 µS $cm^{-1}$ at the end of the survey.

Mean solar radiation was 313.04 µE $m^{-2}s^{-1}$. The photoperiod had an average length of 13.96 h and a maximum of 16.52 h on the 21$^{st}$ June. The estimated mean of the photic depth (Zeu) along the survey was 0.3 m. During the 2007 survey, the sum of rain for the period of the sample collection reached 194.4 mm, and the mean value was 3.74 mm. The wind speed was measured according to three parameters, the wind speed prior to the day of sampling (WS), mean wind speed 3 days prior to sampling (WS3), and mean wind speed 7 days prior to sampling (WS7). The calculated means for the 3 parameters were quite close, respectively, 3.01, 2.99, and 2.92 $m.s^{-1}$.

In 2008, the mean epilimnion temperature was 16.77°C whereas the mean air temperature over 7 days was 14.59°C. We observed a $\partial T(T^{epi} – T^{air})$ ranging from -1.59 to 5.09°C, and the difference in values of both curves were following the same trend (data not shown). Mean air temperature was 15.59°C. Between 4 May-24 September 2008, the air temperatures were above 10.8°C with a mean of 16.7°C. A temperature of above 18°C was recorded in the period 4 July-7July, and from 29 July to 11 August. The pH values of the epilimnion varied from 6.32 to 9.64. Like the previous year (2007), the conductivity slightly dropped during the study and a maximum value of 201 µS $cm^{-1}$ was found during winter. The weather was more rainy during the period of the 2008 survey, sum of rain for the whole period reached 804.7 mm, and the mean was 17.49 mm. The photoperiod, light intensity, sum of rain, pH, Secchi depth, and temperature data for the two period are presented in the figure 1.

### 4.3.2. Nutrients

Dissolved inorganic nitrogen (DIN) was essentially composed by ammonia (data not shown), which is the nitrogen source most readily uptaken by phytoplankton. In 2007, mean DIN concentration was 0.04 mg N $.L^{-1}$, it reached a maximum of 0.27 mg N $.L^{-1}$ on 18 June. In 2008, mean DIN was slightly higher with 0.07 mg N $.L^{-1}$ and a maximum concentration of 1.35 was reached during the winter in January.

Soluble reactive phosphorus (SRP) concentrations had a wide range of values in 2007 ranging from 0 to 0.127 mg $P–PO_4$ $.L^{-1}$ with noticeable peaks the 5 June, 18 June, 24 September and 31 October 2007. In 2008, the range of concentrations was rather low, ranging from 0 to 0.049 mg $P–PO_4$ $.L^{-1}$. The maximum concentration was reached on the 13 June but no significant correlation was found with any of the physical or chemical parameters. The total phosphorus (TP) was negatively correlated ($\rho$= -0.72) to the euphotic depths (Zeu). The concentration of dissolved oxygen was inversely correlated to the outside temperature ($\rho$= -0,56) with a significant p-value ($p > 2 \times 10^{-4}$), which is potentially due to the diffusion process, and positively correlated ($\rho$= 0,70, $p > 10^{-5}$) to the pH values of the epilimnion.

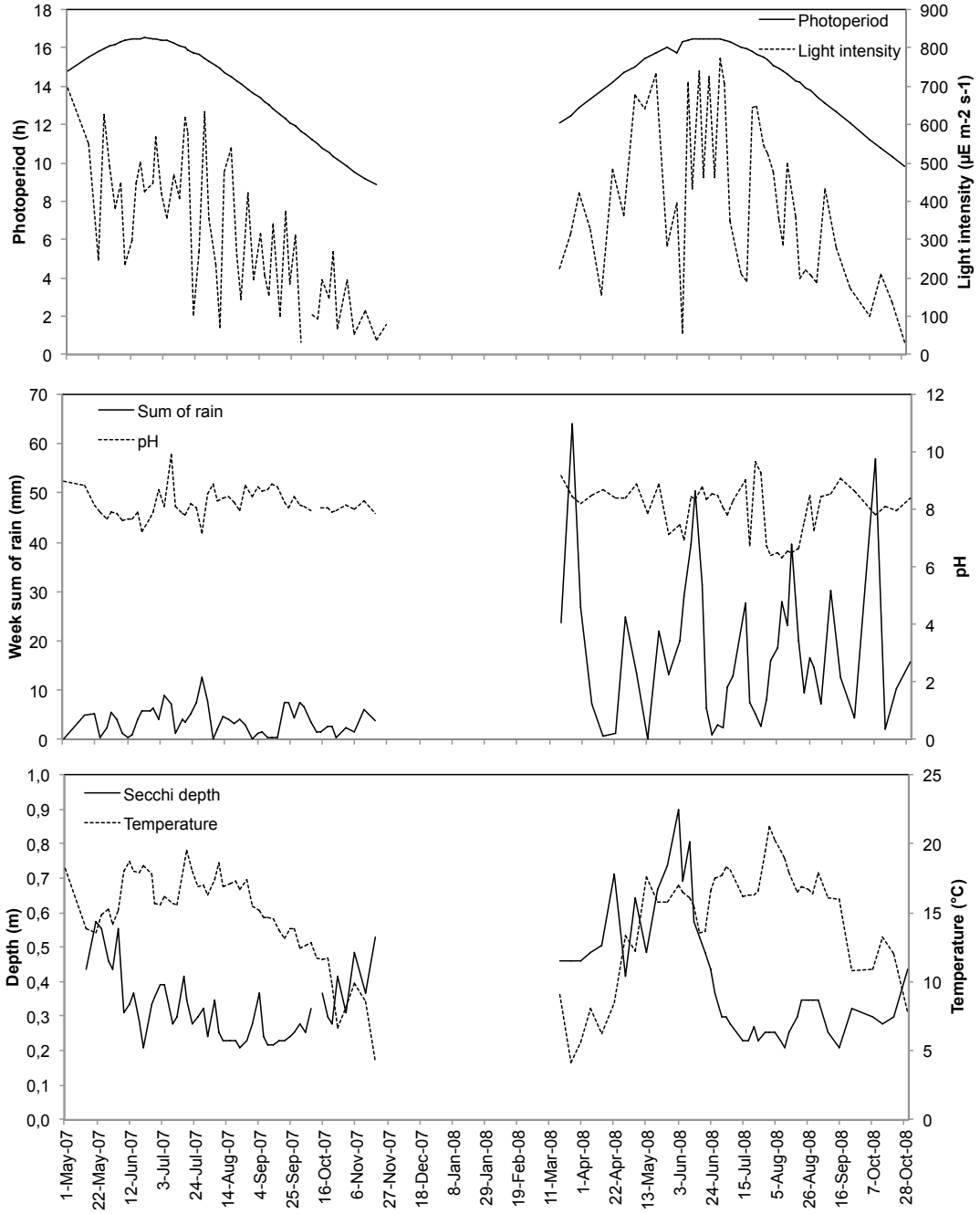



**Fig. 1**, Limnological and meteorological parameter dynamics in Westveldpark Pond (2007-2008).

### 4.3.3. Principal Component Analysis

PCA was performed using 17 environmental variables plus the cyanobacterial biomass. In 2007, 81.8% of the variance of the dataset was explained by six components (factors). The first and second factors explained 30.1% and 15.6% of the variance, respectively. Factor 1 was mainly explained by the

photoperiod, outside temperature, and light intensity with $cos^2$ values over 0.5. The correlations between each variable and loading score of the components are shown for the two main components in table 1. Briefly, PCAs according to factor 1 and 2 mainly represented the sample distribution according to season. In 2008, 80.7% of the variance was explained by six factors. The first two factors explained 27.16% and 19.51% of the variance, respectively. According to the $cos^2$ values, dissolved nitrogen, mostly ammonium, total phosphorus, and outside temperature were the variables that most explained factor 1. According to the correlation between each variable and loading score of components (table 1), the sample distribution was mostly explained by cyanobacterial dynamics and seasonality, including weather conditions such as the sum of rain and outside humidity.

**Table 1,** Correlations between each variable and the loading score of the components of PCA for 2007 and 2008. (Correlation values >0.5, and p-value <0.005)

| | 2007 | | | | 2008 | | | |
|---|---|---|---|---|---|---|---|---|
| | PC1 | | PC2 | | PC1 | | PC2 | |
| Variable | corr. | p-value | corr. | p-value | corr. | p-value | corr. | p-value |
| Ammonium | 0.65 | 8.1 x10-5 | | | 0.73 | 3.5 x10-7 | | |
| Conductivity | | | 0.55 | 1.1 x10-3 | 0.59 | 1.2 x10-4 | -0.51 | 1.1 x10-3 |
| Dissolved nitrogen | 0.70 | 9.5 x10-5 | | | 0.76 | 5.3 x10-8 | | |
| Light intensity | 0.71 | 6.3 x10-6 | | | | | -0.68 | 4.2 x10-6 |
| Nitrates | 0.5 | 3.9 x10-3 | | | 0.62 | 4 x10-5 | | |
| Nitrites | | | | | | | | |
| Outside temperature | 0.8 | 8.3 x10-8 | | | -0.74 | 1.1 x10-6 | | |
| Outside humidity | | | | | | | 0.79 | 4.8 x10-9 |
| Percentage of dissolved oxigen | | | | | | | | |
| Silica | 0.72 | 5.5 x 10-6 | | | | | | |
| Secchi depth | | | 0.64 | 1 x10-4 | 0.53 | 6.5 x10-4 | | |
| Soluble reactive phosphorus (SRP) | 0.58 | 5.8 x 10-4 | | | | | | |
| Sum of rain | | | | | | | 0.52 | 8.5 x10-4 |
| Photoperiod | 0.83 | 1.1 x 10-8 | | | | | -0.68 | 3.1 x10-6 |
| Total Cyanobacteria | 0.61 | 2.4 x 10-4 | -0.61 | 2.7 x10-4 | -0.71 | 1.8 x10-6 | | |
| Total Phophorus | | | -0.68 | 2.7 x10-5 | -0.78 | 1.2 x10-8 | | |

**Table 2,** Phytoplankton classes abundances. Values are expressed in µg Chl *a* equivalent per liter.

| Phytoplankton class | Year | Mean | Min | Max |
|---|---|---|---|---|
| | 2007 | | | |
| Chlorophytes | | 131.4 | 22.0 | 225.2 |
| Chrysophytes | | 29.8 | 0.0 | 74.6 |
| Cryptophytes | | 13.1 | 0.0 | 73.3 |
| Diatoms | | 1.63 | 0.0 | 21.9 |
| Dinoflagellates | | 0.1 | 0.0 | 1.5 |
| Euglenids | | 9.2 | 0.0 | 31.5 |
| Cyanobacteria | | 221.5 | 9.89 | 507.3 |
| | 2008 | | | |
| Chlorophytes | | 147.1 | 34.2 | 308.5 |
| Chrysophytes | | 9.3 | 0.0 | 40.7 |
| Cryptophytes | | 6.8 | 0.0 | 76.3 |
| Diatoms | | 2.5 | 0.0 | 80.2 |
| Dinoflagellates | | 0.3 | 0.0 | 2.2 |
| Euglenids | | 1.7 | 0.0 | 8.6 |
| Cyanobacteria | | 218.4 | 0.0 | 911.3 |

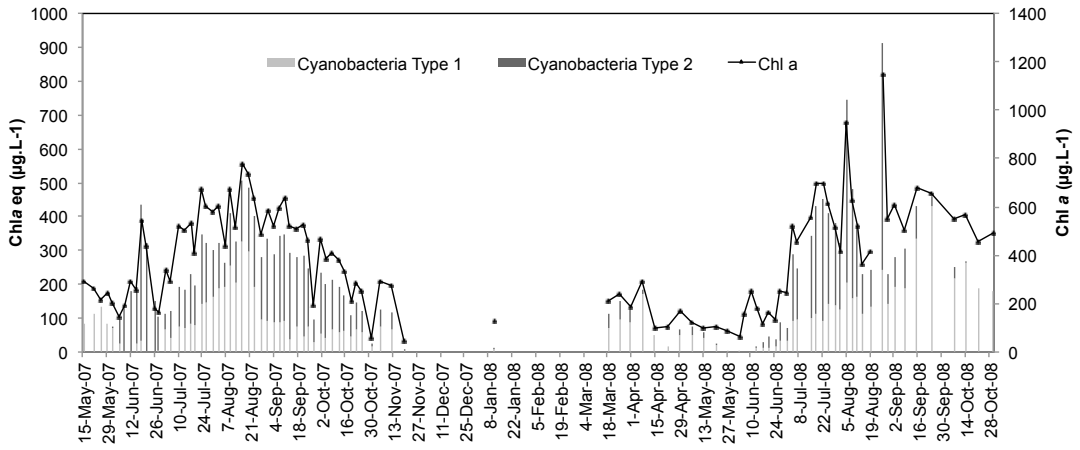



**Fig. 2,** Cyanobacteria type 1 and type 2 versus total Chl *a* dynamics.

4.3.4. Pigment analysis to determine, phytoplankton composition

Wide variations were observed in Chl *a* concentrations during monitoring. In 2007, the concentrations ranged from 45 to 780.7 µg .$L^{-1}$, and in 2008, from 65.3 to 1151.2 µg .L-1. Maxima were observed on 16 August 2007 and 26 August 2008. Bloom events occurred between the 8 and 26 June 2007, 10 July and 31 October 2007, and between the 4 July and 30 October 2008 (Fig. 2). Phytoplankton composition was dominated by cyanobacteria and chlorophytes in both years according to the pigment analyses. Cyanobacteria and chlorophyte concentrations ranged from 0 to 911.3 µg Chl *a* eq .$L^{-1}$ and 22 to 308.5 µg Chl *a* eq .$L^{-1}$, respectively (table 2). In 2007, cyanobacteria represented 22 to 82.7%, and chlorophytes represented 11.5 to 62.6% of the phytoplankton community. Community structure was also composed by chrysophytes, cryptophytes, and diatoms, which were representing from 0% to 15.2%, 24.9%, and 4.7%, respectively. Dinoflagellates and euglenids were present in a rather low proportion (from 0 to 5.4%).

In 2008, chlorophytes were generally more abundant than in 2007 (7.1 to 97.6%). Cyanobacteria made up to 79.2% of the phytoplankton community. Relative abundance maxima of chrysophytes, cryptophytes and diatoms were 22.9%, 10.3% and 18.8%, respectively. Dinoflagellates and euglenids were again rare during the 2008 monitoring.

Total cyanobacterial concentrations were divided in two types; cyanobacteria ‘type 1’ according to the zeaxanthin ratio, and cyanobacteria

'type 2' according to zeaxanthin and echinone ratios. Large variations occurred in the cyanobacteria dynamics of the 'type 1' and the 'type 2' during both years (Fig. 2). Briefly, proportions of both types varied in time. Type 1 was dominant when the first peak of Chl *a* occurred in 2007, whereas type 2 dominated during the rest of the 2007 period. In 2008, type 1 was most abundant in late summer after 15 July (Fig. 2).

*Microcystis spp*. dominated the cyanobacterial community in both years. As described by Van Wichelen *et al.* (2010), two morphotypes composed the *Microcystis spp.* population (*M. aeruginosa* and *M. viridis*). Briefly, in 2007, *M. aeruginosa* dominated until 26 June 2007. Then, *M. viridis* dominated the *Microcystis spp.* population until the end of the survey. In 2008, *M. aeruginosa* dominated during the entire survey (Fig. 3, a). In 2007, no significant correlation was found between both morphotypes and other variables. However, *M. aeruginosa* correlated significantly with temperatures (epilimnion: $\rho$= 0.66, p-value= 0.079; outside: $\rho$= 0.67, p-value= 0.052), cyanobacteria type 2 ($\rho$= 0.82, p-value=$10^{-4}$), Zeu ($\rho$= -0.73, p-value= 0.005), and TP ($\rho$= 0.67, p-value=0.07). *M. viridis* was correlated with photoperiod ($\rho$= 0.78, p-value= 3 x $10^{-4}$) and epilimnion temperature ($\rho$= 0.66, p-value= 0.09).

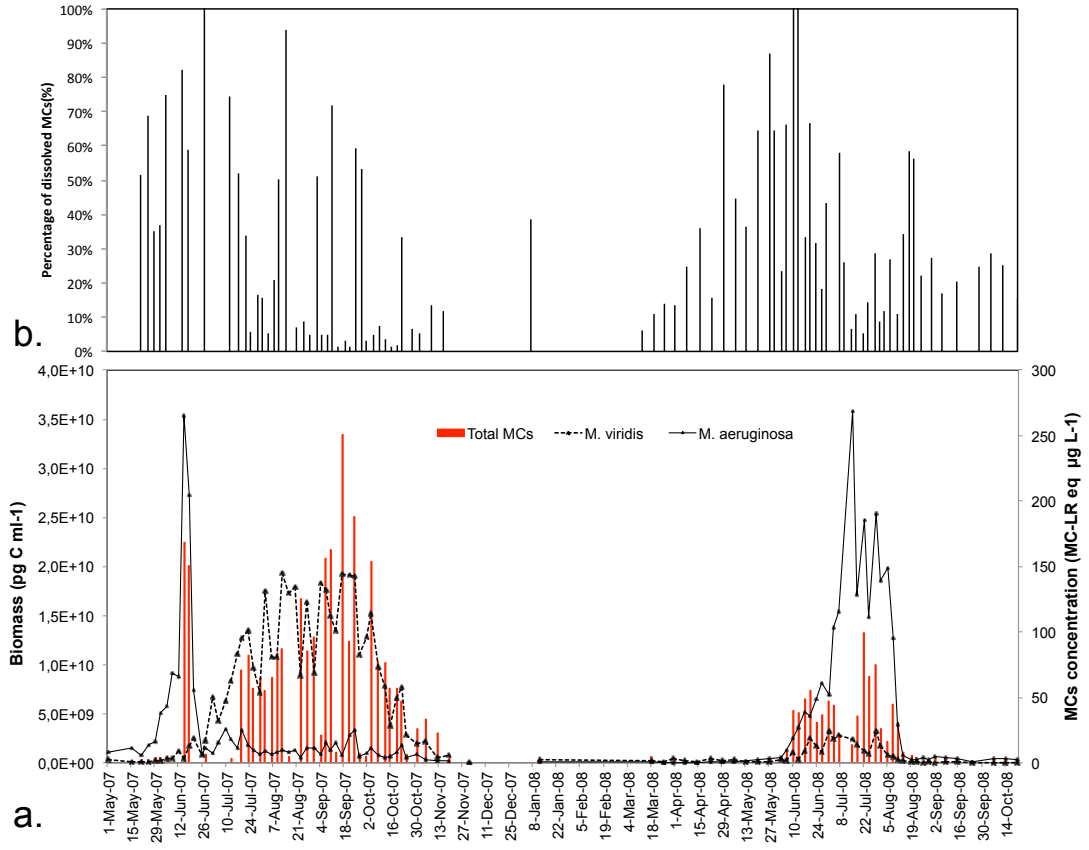



**Fig. 3a**. Dynamic of MCs concentrations in Westeldpark Pond (2007-2008). **b.** Dynamic of dissolved MCs fraction in Wesveldpark Pond (2007-2008).

### 4.3.5. MCs concentrations

Wide variations were observed in total MCs and dissolved MCs concentrations. Dissolved MCs ranged from 0.2 to 138.4 µg MC-LR eq $.L^{-1}$, which represented from 1.2 to 100 % of the total concentrations (Fig. 3, b). Total MCs concentrations ranged from 0.8 to 250.7 µg MC-LR eq $.L^{-1}$. MCs concentrations were above the World Health Organization guideline value for 96.8% of the samples.

In 2007, a first peak of MCs was observed between 12 June- 21$^{st}$ June while *M. aeruginosa* dominated the *Microcystis* spp. population (Fig. 3, a). During this event, the cyanobacteria type 2 proportion was higher than type 1. Later, the highest concentrations of *M. viridis* were observed when total MCs maxima were observed (Fig. 3, a). In 2007, no significant correlation was found between total MCs and other variables.

In 2008, the MCs maxima were lower than the maxima observed in 2007 while *M. viridis* was dominant. A significant correlation ρ= 0.83 (p-value $<10^{-4}$) was found between total MCs concentration and biomass of *M. aeruginosa,* and ρ= 0.79 (p-value= 0.0002) between total MCs concentrations and *M. viridis* biomass. Total MCs concentrations were also significantly correlated with the photoperiod (ρ=0.74, p-value= 0.0033), temperatures (epilimnion: ρ=0.71, p-value= 0.01; outside: ρ=0.69, p-value= 0.024), and single cells *Microcystis spp*. biomass (ρ=0.73, p-value= 0.0055).
No significant correlation was found between dissolved MCs and abiotic parameters during this monitoring in 2008.

According to the linear regression model for the MC concentrations (square root transformed), the photoperiod most significantly explained the total MCs concentrations for the samples in 2008. Ammonium, conductivity, dissolved oxygen, and total nitrogen were also significantly explaining MCs concentrations in the final model (table 3). The model explained over 70 % of the variance ($R^2$= 0.77, adjusted $R^2$= 0.71, F-statistic: 12.59 on 9 and 33 DF (degree of freedom), p-value: 2.253 $x10^{-8}$). Dissolved MCs were explained by photoperiod, ammonium, dissolved oxygen, and Secchi depth. The model explained half of the variance ($R^2$= 0.51, adjusted $R^2$= 0.45, F-statistic: 9.76 on 4, and 38 DF, p-value: 1.56 $x10^{-5}$). In addition, linear regression for particulate MCs was most significantly explained by total nitrogen, conductivity, light intensity, and outside humidity. The model explained over 70% of the variance ($R^2$= 0.72, adjusted $R^2$= 0.661, F-statistic: 12.14 on 7 and 33 DF, p-value: 1.47 $x10^{-7}$).

**Table 3,** Variables of the two linear model explaining the total MCs concentration and the dissolved MCs concentration dynamics in 2008. (SE indicates standard error, and *P* indicates p-value)

| Variable | Estimate | SE | *t* test value | *P* |
|---|---|---|---|---|
| *Total MCs* | | | | |
| Photoperiod | 0.71 | 0.13 | 5.86 | $3.3 x10^{-6}$ |
| *Dissolved MCs* | | | | |
| Photoperiod | 0.76 | 0.13 | 6.1 | $4.1 x10^{-7}$ |

### 4.3.6. Dynamics of toxic and non-toxic *M. aeruginosa* genotypes, and *mcyB* ratio

The specificity of the primers was first controlled. In order to compare the forward/reverse primer sets to the GenBank database, a BLAST analysis was performed. It showed that both forward primer sequences were specific to ITS sequences of *Microcystis spp*, whereas the reverse primer also shared 94.4% similarity (*i.e* one mismatch) with other bacterial genomes. The combination of the forward and the reverse sequences was only found in *Microcystis* spp. genome and not in any of the bacterial, archaeal, or green algal genomes available in the GenBank database.

A careful analysis of melting curves was performed for all environmental samples. No unspecific amplification was observed, which suggest that the reactions were exclusively specific to the ITS region in *Microcystis spp.*

In order to control the specificity of both PCR reactions for each genotype, DNA template from strain ITS 3 genotype was used as a negative control in the ITS 2 reactions, and DNA template from strain ITS 2 was used as negative control for the ITS 3 reactions. For both reactions, no amplification was observed for the negative controls.

An additional experiment was performed to study the influence of a mixture of DNA templates on the amplification of the ITS 2 genotype. Intra-assay coefficient of variation (CV) of the concentration of ITS 2 DNA strain was evaluated for four DNA mixtures (Fig. 4). The first mixture contained 40 ng of ITS 2 (target) template and 20 ng of ITS 3 template. The second mixture contained 4 ng of target template and 20 ng of ITS 3 template. The third mixture contained 0.4 ng of target template and 20 ng of ITS 3 template. An additional control samples that contained 0 ng of ITS 2 template and 120 ng of ITS 3 template was added to the analysis. The standard deviation of the concentration decreased with the concentration of the target, and the means of the observed concentrations were rather close to the measured concentrations. The CVs were low and ranged from 3.9 to 9.7 %. This led to the conclusion that the presence of ITS 3 template in the PCR mix was not influencing the amplification of the target template (ITS 2). Finally, in the

additional control sample, the ITS 3 template (120 ng) was amplified by ITS 2 assay. However, the DNA was detected only after 26 cycles, which corresponded to an estimated concentration equal to 0.011 ng (*i. e.* 3.96 $x10^{13}$ ITS 3 genome per liter). As all samples were diluted 4 fold and as it was unlikely that such concentration was present in the extracted DNA, it was assumed that such an unspecific amplification was not significant for the measurement of ITS 2 copy numbers in the environmental samples.

For both reaction (ITS 2 and ITS 3), the annealing temperatures were set to 58°C after a gradient temperature experiment (Fig. 5). No primer-dimer formation, or unspecific amplification was observed. Efficiency of both reactions ranged from 89.7% to 106%. Mean standard curve correlation coefficients were 0.996 for ITS 2 genotype and 0.995 for ITS 3 genotype. The parameters of each reaction are presented in the table 4.

**Table 4.** ITS genotype SYBR green qPCR assay: average parameters of the standard curves. S represents the slope average of standard curves, sd is the standard deviation, y-intercept represents the intercept average for each reaction, $R^2$ is the Pearson correlation coefficient, E represents the efficiency average of each reaction ($E = 10^{(-1/slope)}$).

| Target | S | sd | y-intercept | Sd | $R^2$ | sd | E | sd |
|---|---|---|---|---|---|---|---|---|
| ITS 2 | -3.418 | 0.141 | 36.36 | 0.145 | 0.996 | 0.003 | 96.42% | 5.67% |
| ITS 3 | -3.34 | 0.157 | 36.77 | 0.952 | 0.995 | 0.004 | 99.6% | 6.34% |

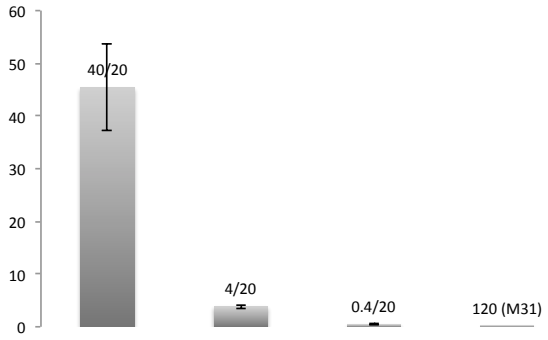



**Fig. 4**, Mixture of DNA template (ITS 2/ ITS 3) experiment. Histograms represent the mean concentration of ITS 2 template in the template mixtures (*i. e.* 40/20 (ng ITS 2/ ng ITS 3), 4/20 (ng ITS 2/ ng ITS 3), 0.4/20 (ng ITS 2/ ng ITS 3)), and 120 ng of the strain DNA M31 (ITS 3 genotype). Error bars indicate standard deviation.

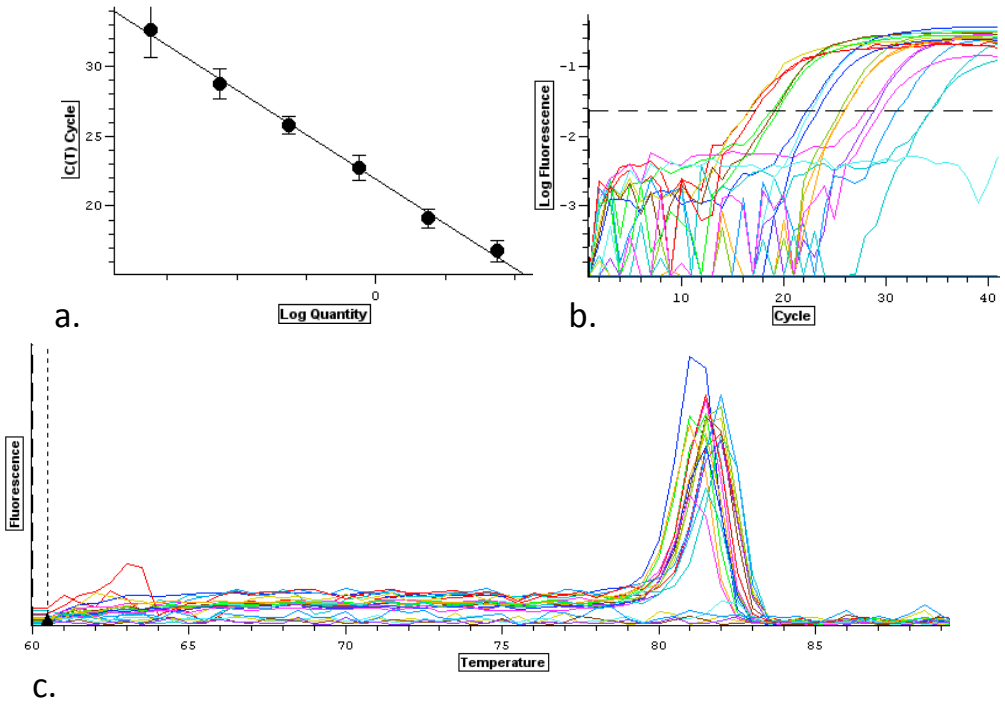



**Fig. 5**, Optimized standard curves of ITS 2 genotype amplification. a, standard curves; b, reads in logarithm of fluorescence for each dilution and controls; c, melting curves for dilutions and controls

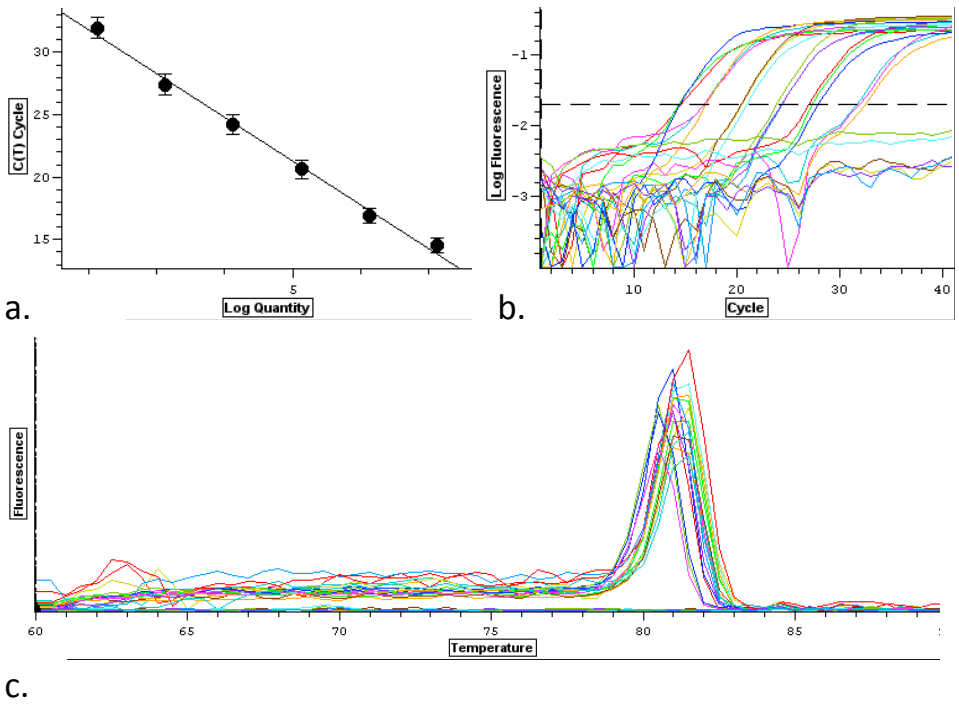



**Fig. 6**, Optimized standard curves for ITS 3 genotype amplification. a, standard curves; b, reads in logarithm of fluorescence for each dilution and controls; c, melting curves for dilutions and controls

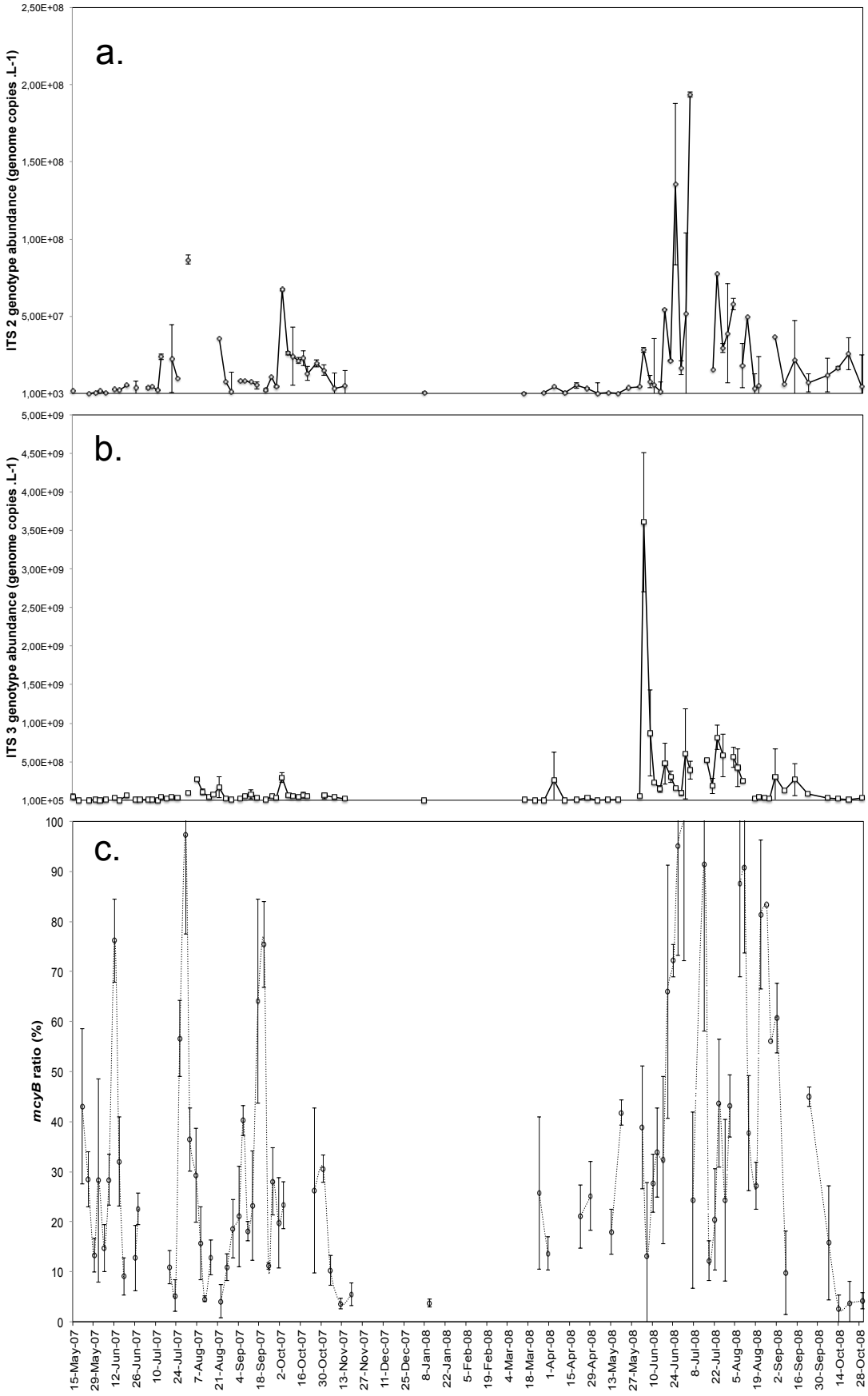
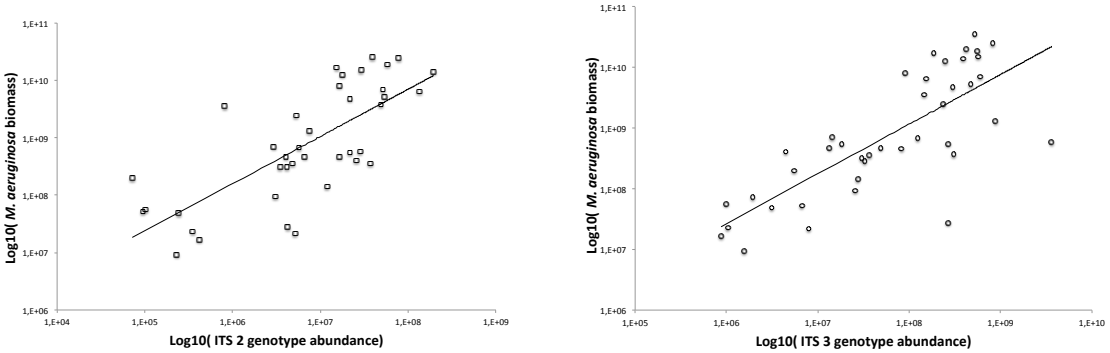

a.
ITS 2 genotype abundance (genome copies .L-1)
2,50E+08
2,00E+08
1,50E+08
1,00E+08
5,00E+07
1,00E+03
b.
ITS 3 genotype abundance (genome copies .L-1)
5,00E+09
4,50E+09
4,00E+09
3,50E+09
3,00E+09
2,50E+09
2,00E+09
1,50E+09
1,00E+09
5,00E+08
1,00E+05
c.
mcyB ratio (%)
100
90
80
70
60
50
40
30
20
10
0
15-May-07
29-May-07
12-Jun-07
26-Jun-07
10-Jul-07
24-Jul-07
7-Aug-07
21-Aug-07
4-Sep-07
18-Sep-07
2-Oct-07
16-Oct-07
30-Oct-07
13-Nov-07
27-Nov-07
11-Dec-07
25-Dec-07
8-Jan-08
22-Jan-08
5-Feb-08
19-Feb-08
4-Mar-08
18-Mar-08
1-Apr-08
15-Apr-08
29-Apr-08
13-May-08
27-May-08
10-Jun-08
24-Jun-08
8-Jul-08
22-Jul-08
5-Aug-08
19-Aug-08
2-Sep-08
16-Sep-08
30-Sep-08
14-Oct-08
28-Oct-08

**Fig. 7,** Genotypic dynamics in Westveldpark Pond. a., percentage of *mcyB* gene; b., abundance of ITS 2 genotype in genome copies per liter. c., abundance of ITS 3 in genome copies per liter.

As previously observed for Chl *a, Microcystis spp.* biomasses, total cyanobacteria biomass and cyanobacteria type 2 biomass, wide variations occurred in ITS genotype abundances during the two-year monitoring. However, no evident relationship between ITS abundances and environmental parameters or MCs concentrations was visible. The ITS 2 genotype abundances varied from 2.1 $x10^5$ to 8.67 $x10^7$ genome copy numbers in 2007 to 7.2 x $10^4$ to 2.94 x $10^8$ genome copy numbers in 2008. The abundances of ITS 3 varied from 3.76 x $10^5$ to 2.95 x $10^8$ genome copy number in 2007, and from 8.71 $x10^5$ to 3.61 x $10^9$ genome copy number in 2008. In 2008, the peak of the ITS 3 genotype happened at the beginning of the bloom period while the first MCs peak was observed.

In order to characterize potential relationships between the genotypes abundances, three correlation matrices were constructed using Spearman rank order test, for 2007, 2008, and for both years. No significant correlation was observed for 2007, whereas both genotype abundances (log) were significantly correlated with *M. aeruginosa* biomass (log) in 2008 (Fig 8), total cyanobacteria, and cyanobacteria type 2 (table 5). Besides, it is likely that significant correlations for the 2007-2008 matrix were mostly due to data from the year 2008.




a. b.

**Fig. 8**, **a.**, Relationship between the changes in the (log) abundances of non microcystin-producing genotype ITS 2 and (log) of *M. aeruginosa* biomass. **b.,**

Relationship between the changes in the (log) abundances of non microcystin-producing genotype ITS 3 and (log) of *M. aeruginosa* biomass

**Table 5**, Significant correlation coefficients between environmental variables and *M. aeruginosa* biomass, and ITS genotype abundances in 2008.

| | *M. aeruginosa* biomass | ITS 2 genotype | ITS 3 genotype |
|---|---|---|---|
| Cyanobacteria type 2 | 0.82 (****) | 0.57 (****) | 0.45 (****) |
| Dissolved MCs | 0.77 (****) | 0.58 (****) | |
| Euphotic zone depth (Zm) | -0.73 (****) | -0.64 (****) | -0.47 (****) |
| ITS 2 genotype abundance | 0.74 (****) | | 0.8 (****) |
| ITS 3 genotype abundance | 0.79 (****) | 0.8 (****) | |
| *M. aeruginosa* biomass | | 0.74 (****) | 0.79 (****) |
| Outside temperature | 0.58 (*) | | |
| Particulate MC-LR | 0.83 (****) | | 0.69 (**) |
| Single cell biomass | 0.76 (****) | 0.66 (**) | |
| Temperature of the epilimnion | 0.66 (*) | | |
| Total cyanobacteria biomass | 0.98 (****) | 0.75 (****) | 0.73 (****) |
| Total MCs | 0.83 (****) | | 0.68 (**) |

As the ITS genotype abundances (response variables) were discrete variables, two generalized linear model (GLM) were constructed. According to the GLM for the non-toxic ITS 2 genotype, the photoperiod, light intensity, and nitrate concentration explained its abundance. According to the GLM for the toxic ITS 3 genotype, the photoperiod was the most significant variable that explained its abundance in the final model (table 6).

As for ITS-genotypes, wide variations occurred in the percentage of *mcyB* genotype. The percentage of the *Microcystis* population carrying the *mcyB* gene varied between 3.6 and 97.2% in 2007, and between 2.6 and 100% in 2008. No significant correlation was found between the *mcyB* percentage and environmental parameters.

**Table 6,** Variables of the two generalized linear model explaining ITS 2 and ITS 3 abundances dynamics. (SE indicates standard error, and *P* indicates p-value)

| Variable | | Estimate | SE | *t* test value | *P* |
|---|---|---|---|---|---|
| | *ITS 2* | | | | |
| Photoperiod | | 0.78 | 0.14 | 5.60 | $3.2 \times 10^{-6}$ |
| Light intensity | | -0.54 | 0.14 | -3.92 | $4.3 \times 10^{-4}$ |
| | *ITS 3* | | | | |
| Photoperiod | | 0.97 | 0.23 | 4.15 | $2.4 \times 10^{-4}$ |

## 4.4. Discussion

This study is the first to describe simultaneously the dynamics of two co-existing ITS-genotypes (toxic and non-toxic), of *mcyB* genotypes proportion, and of dissolved and total MCs concentrations of a bloom-forming *M. aeruginosa* population during a two-year survey in a shallow urban pond. The main questions of this study were (1) whether the *M. aeruginosa* morphotypes correlated with ITS2 (non toxic) and ITS3 (toxic) genotypes dynamics, (2) whether MCs concentration was explained by ITS3, (3) whether the two ITS-genotypes were influenced by environmental factors or not, and if so, (4) whether these genotypes were responding differently to environment conditions. In addition, we attempted to characterize the main factors influencing the MCs concentrations (particulate and dissolved).

During this survey, the *M. aeruginosa* biomass was characterized by a sharp increase in June 2007 followed by a sudden decrease near the end of June 2007, which was explained by amoebae predation (Van Wichelen *et al.,* 2010). In 2008, the amoebae grazing still occurred but the protists' abundances were insufficient to affect the *M. aeruginosa* biomass.

Surprisingly, despite the fact that *M. viridis* was the dominant morphotype at the end of the bloom period in 2007, *M. aeruginosa* dominated the *Microcystis* population in 2008, as shown by microscopy observation. It is possible that the overwintering in the sediment and the abundance of precipitations favored *M. aeruginosa* in 2008.

### 4.4.1. Seasonal dynamics of toxic and non-toxic genotypes

The first year of the monitoring (2007), neither of the two *M. aeruginosa* genotypes nor the *mcyB* percentage were related to any of the environmental parameters. This may be a consequence of the strong amoeba predation, which in this case had more impact on the *M. aeruginosa* population than the abiotic factors.

The second year of the survey (2008), light intensity and photoperiod were shown to have a significant influence on the dynamics of both toxic and non-toxic genotypes, as shown by the generalized linear models. Toxic genotype abundances were slightly higher than non-toxic ones during both years. Our results were in agreement with previous laboratory experiments made by Leblanc Renaud *et al.* (2011). Furthermore, our results showed a significant difference in the response of the two distinct genotypes, ITS 2 and ITS 3, to the same environmental parameters.

In agreement with the observation made by Kardinaal *et al.* (2007a), the toxic genotype ITS 3 was more abundant at the onset of the bloom in 2008. This increase of abundance was matching with the first increase in MCs concentrations. However, no peaks of ITS 3 abundance were corresponding to the second peak of MCs concentration. This suggests that either ITS 3 genotypes were not the only responsible for the production of MCs during the bloom in 2008, or the overexpression of *mcy* genes may play an important role in the second part of the bloom season. However, a previous study on MCs genes expression showed a low influence of environmental parameters on MCs production, and disagrees with the later hypothesis (Kabernick *et al*., 2001).

Interestingly, we found a significant correlation between the extracellular MCs concentration and the non-toxic genotype. This finding suggests the existence of a potential commensalism interaction between the release of the toxic molecules and the growth of non-toxic cells. Recently, Briand *et al.* (2012) suggested the existence of cooperation between toxic and non-toxic strains. Here, we hypothesize that non-toxic cyanobacterial genotypes could cheat by using the extracellular MCs after the lysis of toxic cells, and this may benefit the growth of the non-toxic genotypes.

### 4.4.2. MCs dynamics

Clearly, the dynamics of MCs were resulting from the succession of both *M. aeruginosa* and *M. viridis* morphotypes. Moreover, as mentioned in the section above, more than one genotype was responsible for the production and dynamics of MCs during the two years monitoring, as observed elsewhere (see Kardinaal and Visser, 2005).

According to both correlation analysis and linear regression modeling, it seems that the photoperiod is the most related to the total concentration of MCs in the water column. This result suggests that photoperiod may directly affect the production of MCs. We hypothesize that the biosynthesis of MCs may be partly restricted to a night or day period, or that a change in production is occurring during the day/night cycle. This hypothesis is supported by the recent works of Penn *et al.* (2014) who found the evidence that the *mcy* genes were differently expressed throughout the day-night cycle. Indeed, the *mcy* transcripts doubled during the night period.

However, there is a possibility that the photoperiod may indirectly affect the MCs production throughout the *Microcystis* population dynamics. Indeed, we also found a strong relationship between the photoperiod and the *Microcystis aeruginosa* populations (both toxic and non-toxic genotypes), as well as between the photoperiod and the dissolved MCs.

### 4.4.3. Pigment ratios analyses

Carotenoid composition as a taxonomic marker for the phytoplankton class is used for more than a decade in freshwater environments (Descy *et al*., 2000). However, wide variations in pigment ratios have been found to occur in time and space (Descy *et al.,* 2009). There are also conflicts to assign abundances based on pigments among functional classes (Sarmento and Descy, 2008). In the case of cyanobacteria, the class “cyanobacteria type 1 (ZEA)” was not suitable to identify the population dominated by *Microcystis spp.* (Sarmento and Descy, 2008). Our results are in agreement with this observation. For the first time, we were able to find a significant correlation between the class “cyanobacteria type 2 (ZEA; ECH)” and the abundance of *Microcystis*

*aeruginosa* at both morphologic and genotypic levels. Though the "cyanobacteria T1 ZEA": Chl *a* ratio was previously found to be variable among cyanobacterial species (Descy *et al.,* 2009), our results suggest that the combination of pigments could be used to characterize the biomass of cyanobacteria at the genus or species level. Previous analyses have shown the heterogeneity of the carotenoid compositions of *Microcystis* isolates (Smit *et al.,* 1983). Besides, these authors showed a distinct clustering of isolates into seven groups based on their carotenoid composition. However, to our knowledge, there is no study that combines both the genetic characterization and carotenoids composition for a representative range of *Microcystis* strains.

## 4.5. Conclusion

In this study, the dynamics of MCs were explained by both *Microcystis sp.* morphotypes (*M. aeruginosa, M. viridis*). Also, the MCs production depended on the occurrence of multiple genotypes.

The *M. aeruginosa* population was influenced by the light intensity and photoperiod, and seemed to rise with precipitations during the second year of the survey.

For the first time, in this study, we were able to simultaneously monitor one toxic and one non-toxic genotype of *M. aeruginosa* using real time qPCR technology during a monitoring of 2 years. Both toxic and non-toxic genotypes appeared influenced by the photoperiod. In addition, the abundance of the toxic genotype (ITS 2) was influenced of light intensity.

Interestingly, we found a significant correlation between the extracellular MCs and the non-toxic genotypes, which suggests the existence of an indirect interaction between these populations.

Finally, the combination of pigments analysis and genetic characterization constitutes a promising detection tool for the proliferation of planktonic cyanobacteria.

## 4.6. References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# Chapter 5 – A cultivation-independent approach for the genetic and cyanotoxin characterization of colonial cyanobacteria

*Author contributions*

This chapter is divided in two parts. The first part is describing the work carried out with *Microcystis spp.* colonies, whereas the second part is describing the results obtained for *Woronichinia spp.* colonies.

Parts of the results were published as a methodological 'proof of concept' (Lara *et al.,* 2013). It included a genetic analysis (MLST, ITS-DGGE, *mcy* gene detection) combined with an anti-MC-LR ELISA of *Microcystis spp.* single colonies. Of *Woronichinia spp.* single colonies, a 16S rDNA analysis, *mcy* gene detection, and anti-MC-LR ELISA were performed.

The samplings of concentrated waters were carried out by Anatoly Peretyatko (VUB) in Tervuren and Brussel, by Jeroen van Wichelen (UGent) in St Amansberg, and by the Phd candidate (Y. Lara) in Falemprise. The ELISA assays were performed at the University of Dundee in the laboratory of Professor Geoffrey A. Codd. Also, additional single colony isolations for the determination of MCs contents were carried out at the University of Dundee.

# Chapter 5 – A cultivation-independent approach for the genetic and cyanotoxin characterization of colonial cyanobacteria

## 5.1. Introduction

Coccoid colonial cyanobacteria can be composed of a few to thousands of cells embedded in a common polysaccharide mucilage. They are abundant in freshwater habitats and can be responsible for massive proliferations containing toxins. *Microcystis* is the best-known coccoid colony-forming cyanobacterium producing blooms in nutrient-enriched standing freshwaters. It can produce microcystins (MCs), cyclic heptapeptides that are potent hepatotoxins and tumor promoters (Codd *et al.,* 2005). Over 90 structural MC variants have been characterized so far from a wide range of planktonic and benthic cyanobacterial genera (e.g. Sivonen & Jones, 1999, Metacalf & Codd, 2012). *Microcystis* is among the most commonly encountered MC-producing genera worldwide and therefore represents an adequate model organism for the study of MC production (Sivonen and Bröner, 2008). At the genetic level, the *mcy* gene cluster involved in MC biosynthesis consists among others of a combination of non-ribosomal peptide synthase (NRPS) and polyketide synthetase (PKS) genes. A recent multi locus sequence typing, carried out mainly on strains from Asian water bodies, resulted in at least eight different cryptic or panmictic lineages (Tanabe *et al*., 2009, Tanabe & Watanabe, 2011).

So far, resolution of the toxicological and corresponding genotypic characteristics within environmental populations of cyanobacteria has been limited by the technical need for strain isolation and cultivation. While this is possible for *Microcystis spp*., loss of the colony-forming habit almost invariably occurs (Visser *et al*., 2005). Moreover, a *Microcystis spp.* bloom population can include several genotypes that may produce MCs, and evolve in space and time (Kardinaal & Visser, 2005, Kardinaal *et al*., 2007). A further limitation of the strain isolation approach was shown by Fewer *et al.* (2009) in studies of a mixed *Anabaena/Nodularia* bloom in the Gulf of Finland, from

which only certain genotypes were selected during laboratory isolation and cultivation. In 2004, Janse *et al.* developed an individual colony approach in order to characterize ITS genotypes of *Microcystis spp.* individuals among a bloom population. One hundred and seven colonies were isolated. Colonies were divided into two parts, one part for PCR-DGGE targeting the ITS locus, the other for MALDI-TOF analysis. Colonies ended in 59 clusters, composed of non MC-producing, and of MC-producing clusters. For the ITS, multiple bands were detected for 28% of the amplified colonies, which suggested the aggregation of cells from different colonies. The MALDI-TOF analyses of the 107 colonies from Janse *et al.* (2004) were carried out in a different study (Via Ordorika *et al.* 2004), in addition to 215 other colonies of diverse morphospecies. The aim of this study was to relate the MC biosynthesis with the detection of *mcyA* and *mcyB* genes. It was shown that the combination of at least two *mcy* loci was reliable to assess the production of MCs by *Microcystis spp.* colonies. Up to eight MCs variants were detected in *M. aeruginosa* morphospecies, whereas all the *M. wesenbergii* morphospecies were found negative for MC.

Uncertainty exists regarding the production of MCs by a second planktonic cyanobacterial genus: *Woronichinia spp.* Although high concentrations of MCs were found in blooms dominated by *Woronichinia spp.*, species of other established MC-producing genera were also present as minor components (Willame *et al.*, 2005). *Woronichinia spp.* belongs to the Gomphosphaerioideae sub-family in the Merismopediaceae family. This colony-forming genus is characterized by the binary fission of cells in two perpendicular planes in successive generations and the location of cells at the end of mucilaginous stalks, which radiate from the center of the colonies (Komarek & Anagnostidis, 1999). *Woronichinia spp.* is a widely distributed bloom forming cyanobacterium. Hence, it has been reported in water bodies from Asia, America, Australia and Europe (Guiry and Guiry, 2014). *Woronichinia spp.* is frequent in European and Scandinavian lakes. It can dominate phytoplankton in oligotrophic, mesotrophic and eutrophic lakes and ponds. However, it seems to favor mesotrophic water bodies (Rajaniemi-Walkin *et al.,* 2006). Populations of *Woronichinia spp.* showed some resistance to extreme environmental conditions such as UV B exposure in a

microcosm experiment (Van Donk *et al.,* 2001), in sediment during early winter (Oberhostler *et al.,* 2006), or under an ice cover as observed in a Brussels pond during the BelSPO project B-BLOOMS2 (Anatoly Peretyatko, personal communication). Paradoxically, little is known about the genus *Woronichinia* since only two strains, 0LE35S01 and 1ES42S01, have been isolated (Rajaniemi-Wacklin *et al.,* 2006, Willame *et al*., 2006). Unfortunately, these strains could not be maintained in culture, although their 16S rRNA sequences were deposited in GenBank. Only one strain (1ES42S01) was tested for the presence of the *mcyE* gene and the result was negative (Willame *et al*., 2006).

Determination and characterization of MCs from small amounts of material is now possible through high resolution and powerful techniques such as enzyme-linked immuno-sorbent assays (ELISA) and matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS). Using samples from lake Wannsee (Berlin), Fastner *et al.* (2001) applied MALDI-TOF MS for the first time on intact single colonies of *Microcystis spp*. to study the diversity of the oligopeptides. They showed the presence of seven MCs that were co-occurring with other cyanobacterial metabolites in the same population of *Microcystis*. This approach provided qualitative information on the presence and diversity of the MC content at single colony level but did not provide quantitative data. However, an ELISA using antibodies against MC-LR, with good cross-reactivity to all other MC variants tested so far (Metcalf *et al.,* 2000), has been used to detect and quantify MCs in single filaments of *Planktothrix* spp. (Akcaalan *et al.,* 2006) and individual colonies of the *Microcystis* sp. strain EBRO (Young *et al.,* 2008). MC concentrations (MC-LR equivalents) ranged from 18.4 to 144.4 pg filament$^{-1}$ of *Planktothrix* and from 0.36 to 15.23 ng colony$^{-1}$ of the *Microcystis sp.* strain. Until now, micromanipulation of *Microcystis* colonies, directly isolated from the environment, has already been performed to characterize their MC production by MALDI-TOF MS analysis and their genotype by a maximum of two to three PCR reactions, as the DNA quantity was limited. In some cases, failure of amplification was explained by the lack of sufficient DNA (e.g. Janse *et al*., 2004, Via-Ordorika *et al*., 2004).

In the meantime, new approaches have been developed to study the genetic features of uncultured single cells or microbes. Enzymes like the DNA polymerase Phi29 allow the amplification of genomic data from single cells. It has therefore become feasible to use a new “culture-independent” method to study the complexity of microbial assemblages and functions. Phi29 is a rolling circle enzyme (Kvist *et al.,* 2007), which amplifies DNA by isothermal multiple strand displacement (MDA). DNA can thus be amplified about 5 billion times from a single bacterium (Raghunathan *et al.,* 2005). In cyanobacterial phylum, this whole genome amplification (WGA) technology has already helped characterize the metabolism of the uncultured unicellular nitrogen-fixing cyanobacterium UNCY-A (Tripp *et al.,* 2010) and the apratoxin pathway of Lyngbya bouillonii (Grindberg *et al.,* 2011). It was also possible to amplify and sequence whole genomes of marine cyanobacterial cells (*Prochloroccocus*) (Zhang *et al.,* 2006).

The purpose of the work presented in this chapter is (1) to improve the genotyping of environmental colonies of *Microcystis* by increasing the amount of genetic material for multiple PCRs in order to enable both their genetic characterization and biochemical analysis (in this case, MC concentration); and (2) to use this methodology on colony-forming cyanobacteria that are, to date, difficult or impossible to cultivate such as *Woronichinia,* and to detect whether secondary metabolite biosynthesis pathways are present in *Woronichinia spp.* genomes. We therefore proposed to apply the WGA technology (before performing multiple PCRs) together with an immunochemical assay for MCs, on single colonies from natural populations, with portions of the same colony in each case being used for both WGA and immunoassay.

## 5.2. Experimental procedures

### 5.2.1. Sampling

During the B-BLOOMS2 project (BELSPO) a Belgian network carried out sampling in the following Belgian waterbodies: urban ponds Ixelles I, Ixelles II (Brussels), Tervuren (Leuven), Westlvedpark (St Amansberg), and Lake

Falemprise (Silenrieux). Surface water was concentrated up to three times with a 50 µm pore size plankton net. Concentrated samples were stored at 4°C in the dark until processing (1 to 2 weeks) (Descy *et al.,* 2011).

### 5.2.2. Isolation of individual colony-forming *Microcystis spp.* and *Woronichinia spp.*

Twenty-five colonies of *Microcystis* were directly picked from fresh samples stored at 4°C from Lake Falemprise, Ixelles Pond I and Tervuren Pond. Seventeen *Woronichinia* colonies were isolated by the same procedure from samples from Ixelles Pond II and Tervuren Pond. Individual colonies were isolated using a 20 µL Whatman micropipette with sterile tips under a Leica S4E stereo microscope in sterile conditions. Each colony was washed three times by transfer through 100 µL droplets of sterile BG11 medium (Rippka *et al.,* 1979). Then, colonies were transferred one by one to sterile slides with wells and photographed at 100 and 400 X magnification using a Leica DM LB2 microscope equipped with a Canon Powershot S50 digital camera. The colony areas were evaluated using the AXIOVISION software from Zeiss. Then, the colonies were stored in 5 to 15 µl BG11 medium in 0.2 ml PCR tubes at –20°C until DNA amplification. Morphotypes were identified based on colony shape and cell arrangements according to the botanical morphological criteria of Komárek & Anagnostidis (1999).

### 5.2.3. Whole genome amplification

Multiple strand displacement amplification (MDA) was carried out with the Repli-g kit mini (QIAGEN). Colonies were boiled by incubation for 1 min at 99°C in a thermocycler. A volume of 0.5 µl of the disrupted colony suspension was used as template for the MDA reaction. Manufacturer's recommendations were followed as for amplification of genomic DNA from blood or cell material. Briefly, chemical lysis and DNA denaturation step were performed by addition of a solution that contains potassium hydroxide and DTT (83 mM), and incubated for 10 min on ice. Then, the denatured DNA was incubated at 30°C for 16h with DNA polymerase Phi29 and subsequently stored at -20°C

### 5.2.4. ELISA anti MC-LR

At the University of Dundee, aliquots from the remaining volume of the boiled colony suspensions were adjusted to 200 µl with MilliQ water. Then, 150 µl of the dilution were divided in triplicates for microcystin (MC) quantification using anti-MC-LR ELISA according to Young et al. (2008). The minimum detection limit of the ELISA was 0.02 ng MC-LR equivalent colony$^{-1}$.

### 5.2.5. PCR and sequencing

Proofreading polymerase Super *Taq* Plus (HT Biotechnology, Cambridge) (0.8 U/reactions) was used to perform PCR gene detection and sequencing. All reactions were performed in a 50 µL (total volume) reaction mixture containing 1 µl of MDA reaction product diluted 10-fold, 1 X Super *Taq* PCR buffer, with each deoxynucleoside triphosphate at a concentration of 0.2 µM, and 0.5 µM of each forward and reverse primer.

All PCRs were performed in a Bio-rad MJmini or a Bio-rad Icycler (Hercules, CA), and the amplicons were observed using 1.5% (w/v) agarose gel electrophoresis.

The PCR primer sets for 16S rRNA and ITS sequences were chosen for their specificity to anneal to cyanobacterial DNA. The PCR programs were performed as previously described in the literature (Table 1).

The presence of microcystin synthase gene clusters was investigated by the PCR detection of *mcyA, mcyB* and *mcyE* genes. Primers have been designed on the basis of sequences from different toxic genera, *Anabaena, Microcystis,* and *Planktothrix* for *mcyA* and *Anabaena, Microcystis, Nostoc, Nodularia,* and *Planktothrix* for *mcyE* (Hisbergues *et al.* 2003, Rantala *et al.* 2004, Jungblut and Neilan, 2006). A primer pair specific for *Microcystis* was used to detect the *mcyB* gene (Nonneman & Zimba, 2002).

To compare our environmental colonies to a dataset of *Microcystis* strains available from the GenBank database, we used three (*ftsZ, gltX,* and *recA*) of the seven markers used by Tanabe *et al.* (2007). The *ftsZ* locus was

chosen for its ability to cluster group G of Tanabe *et al.* (2009), whereas the two other markers *gltX* and *recA* were randomly chosen.

**Table 1.** Summary and references of PCR primers

| Target gene | Primer set | Reaction | Specificity | As described by |
|---|---|---|---|---|
| 16S-23S rRNA | 359F/23S30R | PCR/sequencing | Cyanobacteria | Taton *et al.,* 2003 |
| 16S rRNA | 979F | sequencing | universal bacteria | Hrouzek *et al.,* 2005 |
| 16S-ITS | CSIF(GC)/ULR | PCR-DGGE | Cyanobacteria | Janse *et al.,* 2003 |
| *rbcLX* | CW/CX | PCR | Cyanobacteria | Rudi *et al.,* 1998 |
| *rpoC1* | RF/RR | PCR | Cyanobacteria | Rantala et al., 2004 |
| *mcyA* | mcy-CdF/mcy-CdR | PCR | *Anabaena, Microcystis, Planktothrix* | Hisbergues *et al.,* 2003 |
| *mcyB* | mcyF1/mcyR2 | PCR | *Microcystis* | Nonneman and Zimba, 2002 |
| *mcyE* | mcyF2/mcyR4 | PCR/sequencing | *Anabaena, Microcystis, Planktothrix, Nodularia* | Rantala *et al.,* 2006 |
| *mcyE* | HEPF/HEPR | PCR | *Anabaena, Microcystis, Planktothrix, Nodularia* | Jungblut and Neilan, 2006 |
| *ftsZ* | ftsF/ftsR | PCR/sequencing | *Microcystis* | Tanabe *et al.,* 2007 |
| *gltX* | gltF/gltR | PCR/sequencing | *Microcystis* | Tanabe *et al.,* 2007 |
| *recA* | recF/recR | PCR/sequencing | *Microcystis* | Tanabe *et al.,* 2007 |
| A domain | MTF2/MTR | PCR | Unknown | Neilan *et al.,* 1999 |
| KS domain | DKF/DKR | PCR | universal bacteria | Moffit and Neilan, 2003 |

### 5.2.6. Cloning

Cloning of the colony T2's *mcyE* amplicons was performed using the CloneJET PCR cloning kit (Fermentas) following the manufacturer's recommendations. Two clones of the correct size (689 bp) were randomly chosen and sequenced in both directions using PJET1.2 Forward and PJET1.2 Reverse sequencing primers available with the kit.

### 5.2.7. PKS and NRPS clone libraries

PCR products with the adenylation domains from a NRPS operon, and the ketide synthase domains from PKS were cloned in pJET1.2/blunt Cloning Vector using the CloneJET PCR cloning kit (Fermentas) following the manufacturer's recommendations. Clones were first individually inoculated on LB plus ampicillin (100 µg/mL) agar plates, then, screened with direct PCR on cells. Positive clones were inoculated in fresh liquid LB media plus ampicillin (100 µg/mL). Plasmids were extracted using GeneJET$^{TM}$ Plasmid Miniprep Kit (Fermentas). The size of insert was checked by digestion with BglII. PKS clones with inserts of at least 700 bp and NRPS clones with inserts bigger than 900 bp were selected. Selected clones were sequenced using PJET1.2 Forward and PJET 1.2 Reverse sequencing primers.

### 5.2.8. DGGE fingerprinting

DGGE was performed as described in Janse *et al.* (2003). The ITS- PCR fragments were obtained for each single colony using the forward primer CSIF with a GC clamp and the reverse primer ULR. PCR products were loaded onto a 1.5 mm thick vertical denaturing gel of 6.5% (w/v) polyacrylamide (acrylamide-to-bisacrylamide ratio of 37.5:1) and a linear gradient of the denaturing urea and formamide, increasing from 25% or 30% at the top of the gel to 60% at the bottom. The run was performed at 60°C for 999 min at a constant voltage of 75 V. The gel was stained using 2 µl Gelstar dye (Biowhittaker Molecular Applications, USA) mixed in 15 ml sterile water. A second DGGE run was performed to analyze the presence of heteroduplex formation in the first run of DGGE. Excised bands were amplified with the forward primer CSIF with a GC clamp, and the reverse primer ULR, then, loaded on a gel as described above. Presence of heteroduplex is confirmed when the second profile is identical to the profile obtained for the colony on the first gel or when the profile is composed of two bands of equal intensities. Reproducibility of band patterns was verified by repeating analysis three times. Only sequences of good quality were used for phylogenetic analysis.

5.2.9. Sequences analyses

Analyses were carried out with the Geneious software (Drummond *et al.,* 2009). After the processing of chromatograms, all sequences were compared to the Genbank database using blastn and blastx for the protein-coding gene region.

For phylogenetic analyses, the sequences were aligned using the algorithm MUSCLE and manually corrected for each locus (Edgar, 2004). Distances and trees were computed using MEGA 5 software (Tamura *et al.,* 2011).

For the multilocus sequence typing (MLST), any unique sequence in the housekeeping gene dataset was considered as one sequence type (ST). The ST set was compared with the dataset of the sequences obtained by Tanabe *et al.* (2007). Haplotype analysis was performed using the software DnaSP version 5 (Librado & Rozas, 2009).

For both rooted distance trees (ITS/MLST), distances were computed using the Jukes and Cantor correction and trees were built with the Neighbor-joining algorithm (Fig. 4). Maximum-parsimony, Maximum likelihood and Neighbor-joining bootstrap replicates (1 000 replicates) were performed and values higher than 55% are indicated at the nodes. Maximum likelihood analysis were performed after the best DNA model was found using MEGA5. For both trees, we used *Synechocystis sp.* PCC 6803 sequences as outgroup. Total alignment length was 460 nt for the ITS alignment, and the total concatenated alignment of housekeeping genes contained 409 nt positions for the *ftsZ* gene, 341 nt for the *gltX* gene, and 446 nt for the *recA* gene. All positions containing gaps and missing data were eliminated. The bootstrap consensus tree inferred from 1000 replicates was taken to represent the evolutionary history of the taxa analyzed.

For the unrooted tree of the 179 ST, there were 1192 nucleotides in the final dataset (Fig. 5). The evolutionary distances were computed using the Maximum Composite Likelihood model and were expressed in the units of the number of base substitutions per site. Branches corresponding to partitions

reproduced in less than 50% bootstrap replicates were collapsed. All positions containing gaps and missing data were eliminated. The bootstrap consensus tree inferred from 1000 replicates was taken to represent the evolutionary history of the taxa analyzed.

For *Woronichinia spp.* 16S rDNA sequences (Fig. 6) and *rpoC1* (Fig. 9) sequences trees, there were 994 nucleotides in the final 16S rDNA dataset. Distances were computed using the Jukes and Cantor correction. Tree was built with the Neighbor-joining algorithm. Maximum parsimony, Maximum likelihood and Neighbor-joining bootstrap replicates (1000 replicates) were calculated and values over 50% are indicated at the concerned nodes. The *Anabaena* sp. 90 16S rRNA sequence was used as outgroup. All positions containing gaps and missing data were eliminated. The bootstrap consensus tree inferred from 1000 replicates was taken to represent the evolutionary history of the taxa analyzed.

For the PKS phylogenetic analysis (Fig. 10), a distance tree was constructed with amino acids sequences using the Neighbor joining method, after the distances were computed using a Poisson model. All positions containing gaps and missing data were eliminated. The bootstrap consensus tree inferred from 1000 replicates was taken to represent the evolutionary history of the taxa analyzed.

For the A-domain phylogenetic analysis (Fig. 11), a distance tree was constructed with DNA sequences using the Neighbor joining method, after the distances were computed using the Jukes and Cantor correction. All positions containing gaps and missing data were eliminated. The bootstrap consensus tree inferred from 1000 replicates was taken to represent the evolutionary history of the taxa analyzed.

Binding pocket signatures were characterized based upon the binding pocket residues positions in PheA, but are not discussed in this chapter.

## 5.3. Results and Discussion

### 5.3.1. Morphological characterization of *Microcystis spp.* colonies

Nineteen colonies were isolated from a *Microcystis spp.* dominated bloom in the Ixelles Pond I (Brussels); 4 were characterized as *M. ichtyoblabe,* 11 as *M. aeruginosa,* and 4 others which could not be identified. Three *M. aeruginosa* were isolated from a *Microcystis spp.* dominated bloom in Westveldpark Pond (St Amansberg). One *M. wesenbergii* and 2 unidentified colonies were isolated from the lake Falemprise (Silenrieux). Finally, one unidentified colony was isolated from a *Woronichinia spp.* dominated bloom in the Tervuren Pond (Leuven) (Table 2).

To characterize the genotypes corresponding to different morphotypes present in different ponds, we used 11 colonies from water bodies in Belgium (Fig. 1). The colonies were characterized by microphotography, ELISA for MCs and by using 5 to 9 genetic markers, except for the last 3 colonies, which were not photographed (Table 2). Six colonies were selected to represent the two morphotypes observed in Ixelles Pond I: 3 *M. aeruginosa*-like (X5, X38, X39), 2 *M. ichthyoblabe*-like (X8, X11), and a small colony without morphotype attribution (X40). The colonies F13 and T1 were respectively isolated from two other water bodies, Lake Falemprise and Tervuren Pond. It was not possible to distinguish whether the morphotype of F13 corresponded to *M. aeruginosa* or *M. viridis* (Fig. 1v). The vertical position taken by colony T1 in the liquid hindered its identification (Fig. 1u). Three other *M. aeruginosa*-like colonies (We1, We2, We3), which were not photographed, were isolated from Westveldpark Pond.

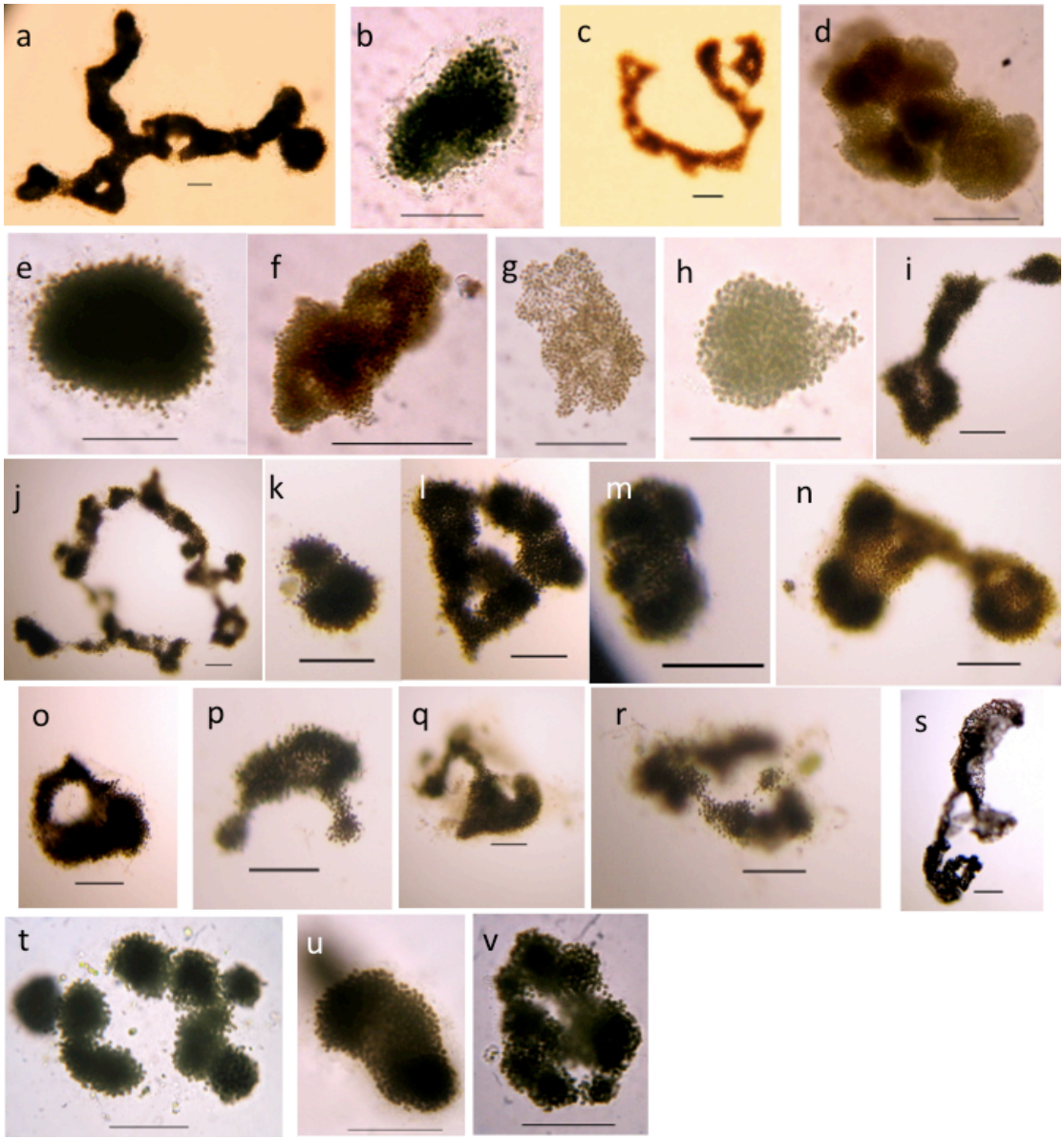



**Fig. 1**, *Microcystis*. Colonies taken from water bodies in Belgium (see Table 1): (a) X5; (b) X6; (c) X7; (d) X8; (e) X10; (f) X11; (g) X12; (h) X13; (i) X38; (j) X39; (k) X40; (l) X41; (m) X42; (n) X43; (o) X44; (p) X45; (q) X46; (r) X47; (s) F11; (t) F12; (u) F13, (v) T1. Scales bars = 100 µm.

**Table 2.** Summary of the results of the cyano-specific PCR reactions for *Microcystis spp.* colonies. Housekeeping gene detection was carried out by PCR targeting the ribosomal operon (16S-ITS), and the genes *ftsZ, gltX, recA, rpoc1, rbcLX.* The *mcy* gene detection was carried out by PCR targeting *mcyA*, *mcyB*, and *mcyE*. The results are indicated by '+' for the presence of a PCR band of suitable length and '–' for its absence. '*mcyE*' indicates that the primer set of Rantala *et al.* (2006) was used; '*mcyEb'* indicates that the primer set of Jungblut and Neilan (2006) was used. Lower case letters refer to the picture of the colonies in the figure 1. N/A: not available.

| Colony name | Sampling date | waterbody | resuspension volume (µl) | genus | morphospecies | 16SITS | *ftsZ* | *gltX* | *recA* | *rpoC1* | *rbcLX CWCX* | *mcyA* | *mcyB* | *mcyE* | *mcyE b* | picture |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| X5 | 24/08/07 | Ixelles Pond I | 10 | *Microcystis* | *aeruginosa* | + | - | - | + | - | - | + | + | + | N/A | a |
| X6 | 24/08/07 | Ixelles Pond I | 10 | *Microcystis* | N/A | - | - | + | - | - | - | - | - | - | N/A | b |
| X7 | 24/08/07 | Ixelles Pond I | 10 | *Microcystis* | *aeruginosa* | - | + | - | - | - | - | - | - | - | N/A | c |
| X8 | 24/08/07 | Ixelles Pond I | 7 | *Microcystis* | *ichtyoblabe* | + | + | + | + | + | - | - | - | - | N/A | d |
| X9 | 24/08/07 | Ixelles Pond I | 5 | *Microcystis* | *ichtyoblabe* | - | - | - | - | - | - | - | - | - | N/A | none |
| X10 | 24/08/07 | Ixelles Pond I | 6 | *Microcystis* | N/A | - | - | + | - | - | - | - | - | - | N/A | e |
| X11 | 24/08/07 | Ixelles Pond I | 7 | *Microcystis* | *ichtyoblabe* | + | + | + | + | + | - | - | - | - | N/A | f |
| X12 | 24/08/07 | Ixelles Pond I | 10 | *Microcystis* | *ichtyoblabe* | - | - | + | - | - | - | - | - | - | N/A | g |
| X13 | 24/08/07 | Ixelles Pond I | 10 | *Microcystis* | aeruginosa | - | - | + | - | - | - | - | - | - | N/A | h |
| X38 | 24/08/07 | Ixelles Pond I | 7.9 | *Microcystis* | *aeruginosa* | - | + | + | + | + | + | + | + | - | N/A | i |
| X39 | 24/08/07 | Ixelles Pond I | 7.9 | *Microcystis* | *aeruginosa* | + | + | + | + | + | - | + | + | + | N/A | j |
| X40 | 24/08/07 | Ixelles Pond I | 10 | *Microcystis* | N/A | + | + | + | + | + | + | + | + | + | N/A | k |
| X41 | 24/08/07 | Ixelles Pond I | 10 | *Microcystis* | *aeruginosa* | - | - | - | - | - | - | - | + | - | N/A | l |
| X42 | 24/08/07 | Ixelles Pond I | 7.5 | *Microcystis* | N/A | - | - | - | - | - | - | - | - | - | N/A | m |
| X43 | 24/08/07 | Ixelles Pond I | 8 | *Microcystis* | *aeruginosa* | - | + | + | - | + | - | - | - | + | N/A | n |
| X44 | 24/08/07 | Ixelles Pond I | 8 | *Microcystis* | *aeruginosa* | - | + | - | - | + | - | - | + | - | N/A | o |
| X45 | 24/08/07 | Ixelles Pond I | 7.5 | *Microcystis* | *aeruginosa* | + | - | - | - | - | - | - | - | - | N/A | p |
| X46 | 24/08/07 | Ixelles Pond I | 10 | *Microcystis* | *aeruginosa* | - | + | - | - | - | - | - | - | - | N/A | q |
| X47 | 24/08/07 | Ixelles Pond I | 10 | *Microcystis* | *aeruginosa* | + | + | + | - | N/A | N/A | - | + | + | N/A | r |
| We1 | 19/10/07 | Westveldpark | 6 | *Microcystis* | *aeruginosa* | + | + | + | + | + | + | - | - | - | N/A | none |
| We2 | 19/10/07 | Westveldpark | 5 | *Microcystis* | *aeruginosa* | + | + | + | + | + | + | + | + | + | N/A | none |
| We3 | 19/10/07 | Westveldpark | 8 | *Microcystis* | *aeruginosa* | + | + | + | + | + | + | + | + | + | N/A | none |
| F11 | 11/06/08 | Falemprise | 10 | *Microcystis* | *wesenbergii* | - | - | + | - | - | - | - | - | - | N/A | s |
| F12 | 11/06/08 | Falemprise | 7 | *Microcystis* | N/A | - | - | - | - | - | - | - | - | - | N/A | t |
| F13 | 11/06/08 | Falemprise | 9 | *Microcystis* | N/A | + | + | + | + | + | + | + | + | + | N/A | u |
| T1 | 29/10/07 | Tervuren | 15 | *Microcystis* | N/A | + | + | + | + | + | + | - | - | - | N/A | v |

5.3.2. MC concentrations and mcy genes in *Microcystis* colonies

Microcystins were detected in 7 of 11 colonies tested by ELISA (Table 3). The estimated toxin content per colony, based on cross-sectional area measurement (Young *et al.,* 2008), ranged from 70.15 to 854.9 pg. The cross-sectional area of *Microcystis* colonies was preferred as a more reliable indicator of colony volume than mathematical models, which tend to be specific for the populations from which they are drawn (Morrison, 2005). The MC concentrations per colony area ranged from 0.36 to 31.37 ng $mm^{-2}$. The highest concentration of MCs was found in colony F13, which was two times higher than previously found by Young *et al.* (2008) for colonies from the *M. aeruginosa* strain EBRO. MC concentrations (ng $mm^{-2}$) in *M. aeruginosa* (X5, X38, X39) and X40 from Ixelles pond vary by a 1.16 to 4.61 ratio. These variations are within the range which can be due to differences in gene expression, as was shown for *Microcystis* PCC7806 (Kaebernick *et al.,* 2000).

The *mcyA*, *mcyB*, and *mcyE* genes, three genetic loci involved in MC biosynthesis (Nonneman & Zimba, 2002, Hisbergues *et al.,* 2003, Rantala *et al.,* 2004) were detected for colonies X5, X39, X40, and F13, in which also significant MC concentrations were measured. None of these three loci were detected in colonies X8, X11 and T1, which were also negative in the ELISA. Thus, the detection of *mcy* genes corresponded to the ELISA results. The *mcyA* and *mcyB* genes were detected in colony X38, but not *mcyE* (nor the ITS locus). In this case, the WGA step may have been incomplete due to a low number of template cells, which could bias PCR efficiency (Rodrigue *et al.,* 2009). Indeed, the thick mucilage surrounding this colony could have impaired the colony disruption and release of cells into the suspension, so that less DNA template would be available for the MDA reaction.

Aside from this experiment, the partner laboratory in the University of Dundee isolated 34 colonies and measured their MC concentrations. These colonies were collected from diverse water bodies in Belgium. To compare MC concentrations within colonies, the concentration of MCs per $mm^2$ was calculated. This was done on four colonies originating from a strain isolated in 2007, as well as on the 7 MC-producing colonies, which are described at the start of this paragraph. The MC concentrations ranged from 0.24 to 149.7 ng $mm^{-2}$. No significant correlation was found between the colonies cross-sectional areas vs microcystins quantity (Fig. 2).

However, depending on the origin of the colonies, a linear relationship could be suggested, *e.g.* colonies from Ixelles Pond I or Westveldpark Pond. Unfortunately, the number of tested colonies per Pond was too small to be significant. Variations in MCs concentrations among *Microcystis spp.* strains (Ozawa *et al.,* 2005; Wilson *et al.,* 2006) may explain the lack of correlation between MCs quota and colony cross sectional-area. Indeed, it is likely that co-occurrence of different genotypes and/or morphotypes of colonies within the same environmental sample may play an important role in this relationship. In Hampstead Heath Boating Pond in London, Codd and colleagues (2005) found a significant correlation of 0.7 between the *M. aeruginosa* colony area and MCs concentrations in a sample composed of *M aeruginosa* and *M. flos-aquae.* No or barely detectable MCs were observed in *M. flos-aquae* colonies. Moreover, in laboratory, the analysis of culture *Microcystis* EBRO (Young *et al.,* 2008) confirmed the occurrence of a relationship between the colony area and the MCs concentrations. However, the age of the colonies influenced the significance of the correlation. Thus, significant correlations between colony area and MCs concentration were found for one week and two week-old colonies, whereas no significant correlation was found for three week-old colonies. Conclusively, in order to improve the prediction of the toxicity of *Microcystis spp.* dominated bloom; it would be necessary to take in account the morphology, the genotype, and the age of the colonies.

**Table 3.** Summary characterization of 11 colonies taken from water bodies in Belgium. Morphotypes were identified as *M. aeruginosa* (*aer*) and *M. ichthyoblabe* (*icht*). MC-LR equivalents per colony is the concentration of microcystin-LR measured by ELISA. Apparent colony areas were measured using the AXOVISION software from Zeiss. *mcy* gene detection was carried out by PCR targeting *mcyA*, *mcyB*, and *mcyE*. The results are indicated by '+' for the presence of a PCR band of suitable length and '–' for its absence. Sequence typing: for each locus, the Roman numerals indicate a unique sequence type (ST). N/A: not available; ND: not detected

| Colonies | Morphotype identification | MC-LR per colony | Apparent area | *mcy* genes detection | | | Sequence Typing | | | Concatenated sequences |
|---|---|---|---|---|---|---|---|---|---|---|
| | | (pg/colony) | (mm2) | *mcyA* | *mcyB* | *mcyE* | *ftsZ* | *gltX* | *recA* | |
| **Ixelles Pond I** | | | | | | | | | | |
| X5 | *aer* | 558.6 | 0.268 | + | + | + | N/A | N/A | I | **N/A** |
| X8 | *icht* | ND | 0.036 | - | - | - | II | III | IV | **IV** |
| X11 | *icht* | ND | 0.010 | - | - | - | III | II | III | **III** |
| X38 | *aer* | 442.3 | 0.046 | + | + | - | II | I | II | **II** |
| X39 | *aer* | 854.9 | 0.174 | + | + | + | I | I | I | **I** |
| X40 | N/A | 98.9 | 0.012 | + | + | + | I | I | I | **I** |
| **Lake Falemprise** | | | | | | | | | | |
| F13 | N/A | 658.7 | 0.021 | + | + | + | V | V | VI | **VI** |
| **Tervuren Pond** | | | | | | | | | | |
| T1 | N/A | ND | 0.018 | - | - | - | IV | IV | V | **V** |
| **Westveldpark Pond** | | | | | | | | | | |
| We1 | N/A | ND | N/A | - | - | - | VI | VI | VII | **VII** |
| We2 | N/A | 83.6 | N/A | + | + | + | VII | VII | VIII | **VIII** |
| We3 | N/A | 70.1 | N/A | + | + | + | VII | VII | VIII | **VIII** |

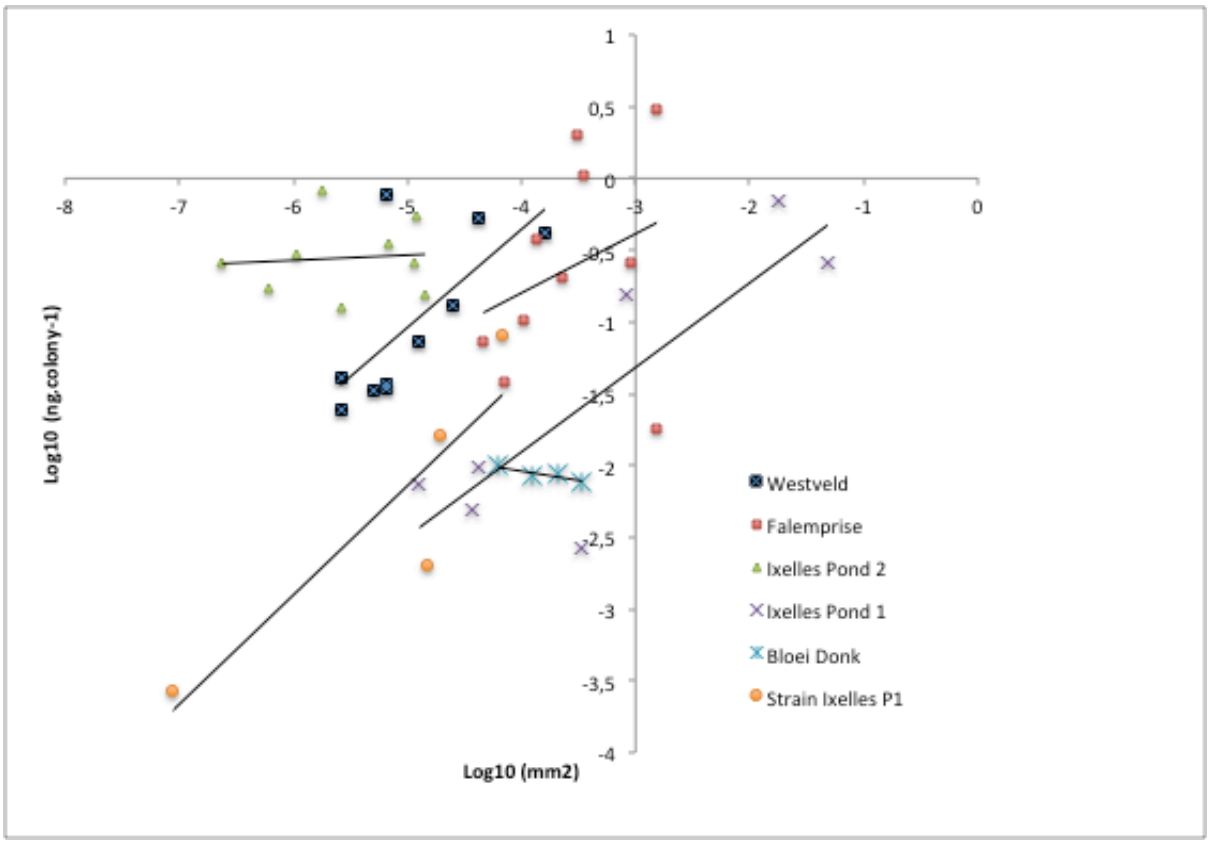
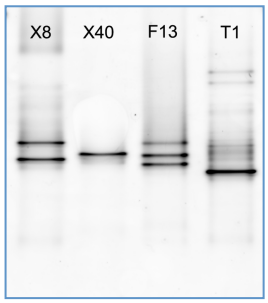



**Fig. 2**. Representation of colony area versus microcystin quota of 41 colonies directly taken from waterbodies and five colonies from a strain isolated from Ixelles Pond I. All data were log-transformed.

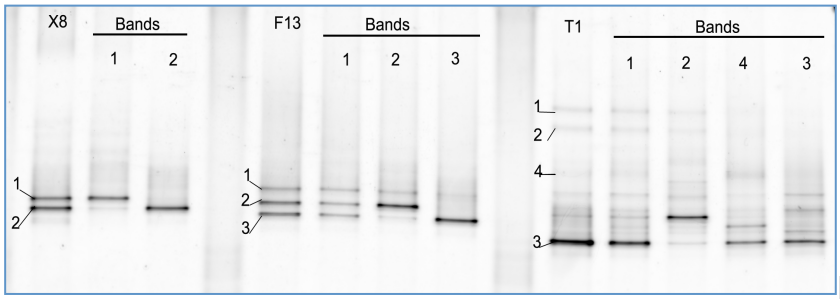
### 5.3.3. Genotyping of *Microcystis* colonies by ITS DGGE and multiple locus sequence analysis

It was possible to amplify the ITS sequences of colonies F13, T1, X8, X11, X39, X40, We1, We2, and We3 with a single PCR, whereas two successive PCRs were necessary for X5, which appeared identical to X39 and X40. However, amplification of the ITS from colony X38 was not successful. The number of bands (from 1 to 10) in the DGGE gel varied among the colonies. The presence of two bands of the same intensity was observed for colony X8. As described by Janse *et al.* (2004), this could be explained by the presence of two ribosomal operons with two different ITS sequences with the same copy number. Three bands were separated for colony F13, out of which two were of the same intensity and a one was less intense. The third band was a

heteroduplex that formed during PCR, with one strand of each of the two other amplicons (Fig. 3). A more complex profile was observed for colony T1. At least four bands with a 'smeared' region were detected. Three bands were re-amplified and loaded on a second DGGE. This resulted in profiles that indicated the presence of different ITS-genotypes. Since intensities were not equal, we hypothesize that colony T1 sheltered other *Microcystis* genotypes.



a



b

**Fig. 3a**. Gel picture of ITS-DGGE of the *Microcystis* colonies X8, X40, F13 and T1. **3b.** ITS-DGGE profile of bands re-amplified from the *Microcystis* colonies X8, F13 and T1. **X8 profile** indicates the presence of 2 ITS loci with different sequences but with equal copy numbers. **F13 profile** is composed of three bands where band n°1 is corresponding to a heteroduplex between a DNA strand corresponding to band n°2 and a complementary DNA strand corresponding to band n°3. **T1 profile** is composed by a dominant band corresponding to the main genotype of the colony and several bands that could be due to (i) heteroduplexes formed as describe above, (ii) migration artifacts where the same sequence migrates to different positions and/or (iii) different sequences present in one band as described in Sekiguchi *et al.* (2001).

Two ITS sequences were obtained for colonies F13, T1 and X8, as was the case for several strains of *Microcystis* in GenBank (e.g. NIES44, NIES98, NIES101, PCC7941). A careful comparison showed an insertion/deletion (indel) of up to five nucleotides, 17 nt after the $tRNA^{ILE}$ end, and 14 nt before

the box B region in both colonies and strains. Interestingly, colony F13 contained the only pair of ITS sequences that does not hold such indel occurrences. Phylogenetic analyses of partial sequences of ITS (460 nt) (Fig. 4a) showed that two sequences from the same colony, were closely related, except for colony F13, where the two sequences were far apart. Moreover, the full sequence of band n°2 showed only 99.4% identity with the ITS of NIES90, although the partial sequences used in the tree are identical. In addition, the ITS sequence of X39 was identical to X40 and the ITS of We2 was identical to We3.

We suggest that all these variations are evidence of intragenomic differences between the two ribosomal operons in *Microcystis* genomes, although the two copies in the genome of NIES843 were identical. However, intragenomic variations are known to occur in genomes from the cyanobacteria phylum (Engene *et al.,* 2011). Differences in ITS copies from the same *Microcystis spp.* genome may misrepresent the *Microcystis spp.* population structure present in a lake. Indeed, ITS richness may be overestimated. This may lead the to reconsideration of the use of the ITS locus for genotyping analysis of *Microcystis* in environmental populations.

The three (*ftsZ, gltX, recA*) gene loci were present in only one copy in the genome of *Microcystis* NIES843 (Kaneko *et al,.* 2007). Those three housekeeping genes were successfully amplified and sequenced for ten *Microcystis* colonies. It seems that each colony is a genetically homogeneous group of cells that share the same sequence type (ST). Indeed, in all chromatograms, neither ambiguities nor mixtures of sequences were observed after careful examination. This also suggests that cells from each colony have a clonal origin, in agreement with the ITS-DGGE results. All housekeeping gene sequences were highly similar (98.2 to 100%) to sequences obtained from the previous studies of *Microcystis* strains (Tanabe *et al.,* 2007, 2009, Tanabe & Watanabe, 2011). Description of the sequence types is given in Table 2. Analysis of the alignment of concatenated sequences (1196 bp) revealed eight ST for ten colonies. Moreover, *Microcystis* colonies X39 and X40 shared the same ST based on three loci. It seems likely that X40 was a sub-colony, with the same morphotype and genotype than X39. Colonies We2 and We3 also shared the same ST.

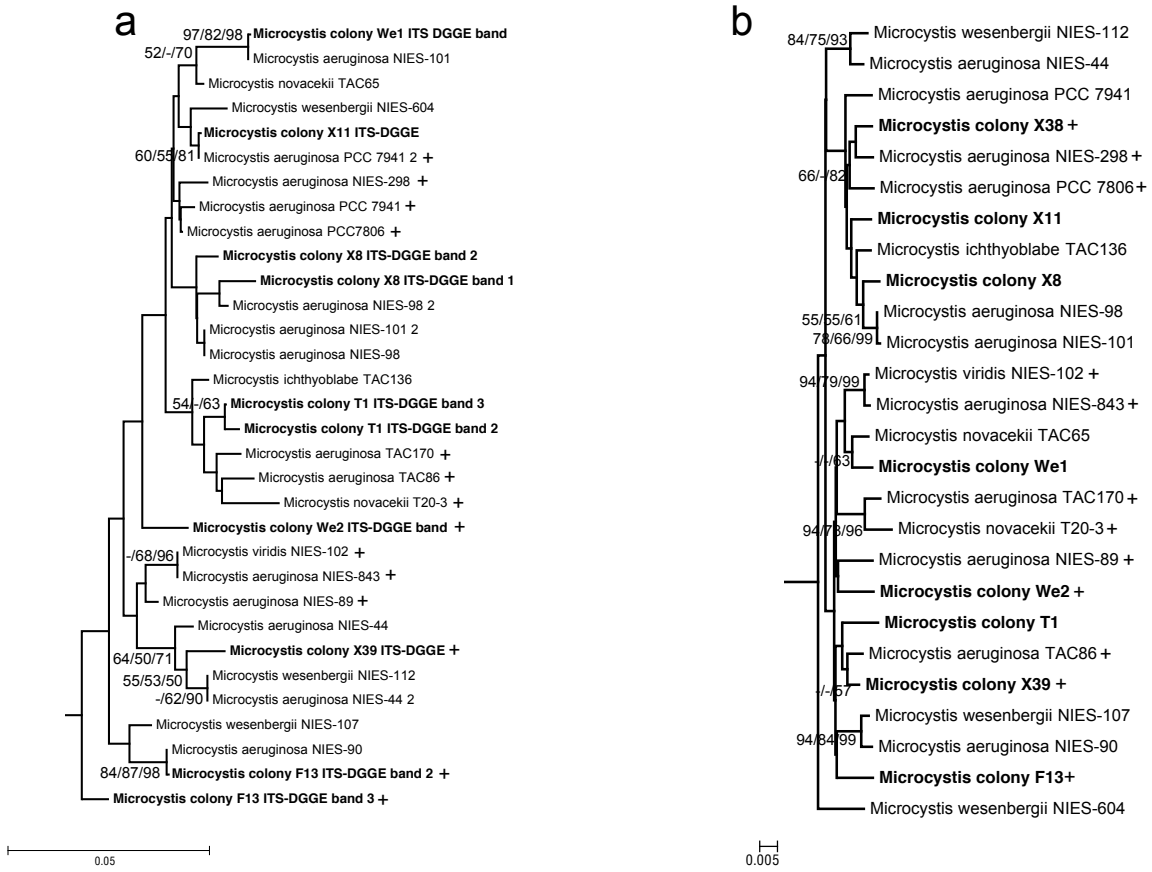



**Fig. 4a.** Distance tree of ITS sequences using the Neighbor–joining method. **4b.** Distance tree of concatenated sequences (*ftsZ/gltX/recA*) using the Neighbor–joining method. For both trees, we considered *Microcystis* colony X40 as the same as colony X39 and *Microcystis* colony We3 as the same as colony We2. ('+' indicates that MC measured was over the detection limit and at least two *mcy* genes were detected). Distance scale is expressed in units of the number of base substitutions per site.

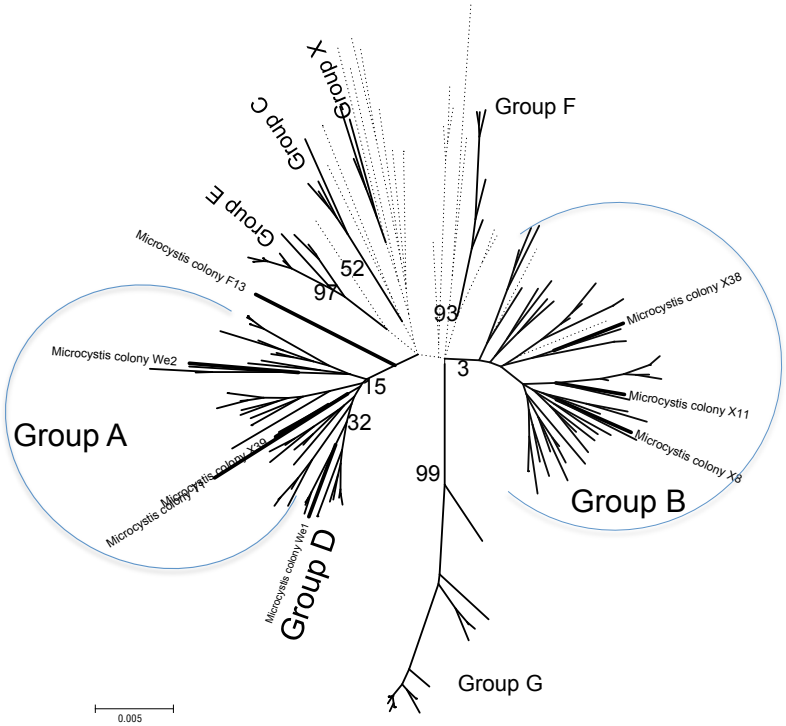



**Fig. 5**. Unrooted distance tree of 179 ST (groups names are given after Tanabe and Watanabe 2010) with the 11 Belgian colonies (bold branches) using the Neighbor-joining method.

Using the three loci, a haplotype analysis using the dataset of Tanabe *et al.* (2007, 2009) and Tanabe & Watanabe (2011) was carried out and 187 STs were obtained in total. The eight Belgian STs differed from the 179 Asian STs. The phylogenetic analysis of the 187 STs using the three concatenated loci (Fig. 5) resulted in the identification of the same groups (A to G and X) that were already described by Tanabe *et al.* (2009) using seven concatenated loci. The low numbers of discriminating nucleotides in our alignment, probably resulted in rather low bootstrap values for groups A, B, C, D, E, F and X. In contrast, group G was supported by a 99% bootstrap value, as in Tanabe *et al.* (2009). Seven of the Belgian STs belonged to groups A, B, and D. Colonies X39, X40 and T1 were clustered into the complex of MC-producing strains which forms group A. Colonies X8, X11 and X38 were positioned in group B, which is composed of MC-producing and non-producing STs. Colony We1 clustered with the non-producing strains of group D. In Tanabe *et al.* (2009), groups A and D were only composed by STs from Asian strains. Group B included strains from three continents of the Northern

hemisphere. This analysis results suggest that groups A and D are also potentially widespread in the Northern Hemisphere. The ST of colony F13 was not affiliated with any of the groups previously described (Fig. 5). F13 may thus be a representative of a novel *Microcystis spp.* lineage.

The comparison between the phylogeny based on the ITS and housekeeping gene sequences from the same strains (Fig. 4) suggests that both types of taxonomic markers may have undergone independent evolution. Indeed, topologies of both trees were not congruent. It could be explain by a difference in evolution rate between ITS and the three other loci.

### 5.3.4. *Woronichinia spp.,* taxonomic validation by 16S rDNA- ITS, and *rpoC1* analysis

The attempts to cultivate strains of *Woronichinia* from fresh environmental samples were unsuccessful. Seventeen *Woronichinia* colonies with a typical morphology (Komárek & Anagnostidis, 1999) were isolated from Tervuren Pond and Ixelles Pond II. The colonies consisted of an internal system of radially and more or less parallel oriented, unbranched thick stalks connected to the cells and usually having the same width as the cells. The colonies were dense and embedded in mucilage (Fig. 6).

Successful WGA were obtained for the seven colonies, and the amplification was checked by subsequent cyanobacteria-specific PCR reactions (data not shown). The 16S rRNA partial sequences of colonies T2, T5, T8, T9 and T11 were 983 nt long, and 513 nt long for W1. Similarities with the 16S rRNA sequence of *Woronichinia naegeliana* 0LE35S01 (AJ781043) were 99.7% for T8 and T9, 99.8% for T2, and 100% for T5, T11 and W1. In the phylogenetic analysis of the partial 16S rRNA gene sequences, the colonies form a sub-cluster with *Woronichinia naegeliana* 0LE35S01 with high bootstrap values for three methods (Fig. 7). This cluster is part of the sub-family of Gomphosphaerioideae with another highly supported cluster formed by *Snowella spp.* sequences as described in Rajaniemi-Walkin *et al.* (2006). In our alignment, the mean similarity among the 16S rRNA sequences belonging to the *Woronichinia spp.* clade was 99.75%, whereas it was 99.15%

for the sequences belonging to the *Snowella spp.* clade. In contrast, there was only 96.1% similarity within the group of sequences that represents the other Merismopediaceae *e.g.* members of Merismopedioideae sub family, *Synechocystis*-like strains and *Merismopedia*-like strains. As listed in Table 4, the computed mean similarity between *Woronichinia spp.* and its closest genus *Snowella spp.* was 95.75%. *Woronichinia spp.* and *Snowella spp.* are the only genera representative of the Gomphosphaerioideae that are present in Genbank. As all the sequences belonging to these two genera were included for the analysis, it is likely that any threshold at a genus level above 95.75% should not be taken into consideration. This threshold is corresponding to a distance of 0.044 substitutions per site as computed with the correction of Jukes and Cantor (1969). The distance between the genus *Woronichinia* and the other well-represented Chroococcales clades outside the Merismopediaceeae family is approximately two times its distance with the *Snowella spp.* sequences (Table 4).

Due to the lack of sequences from true *Merismopedia spp.* strains compared to *Synechocystis spp.* strains, it is unfortunately not possible to compute such threshold for the members of the Merismopedioideae sub-family. The Merismopediaceae family may give interesting insights for the study of 16S rRNA-based taxon delimitation for the Chroococcales order. Indeed, it is the most represented family in the literature and its taxonomical features are clearly defined and specific. Thus, it would be interesting to focus future 16S rDNA studies on the genus *Merismopedia spp.*.

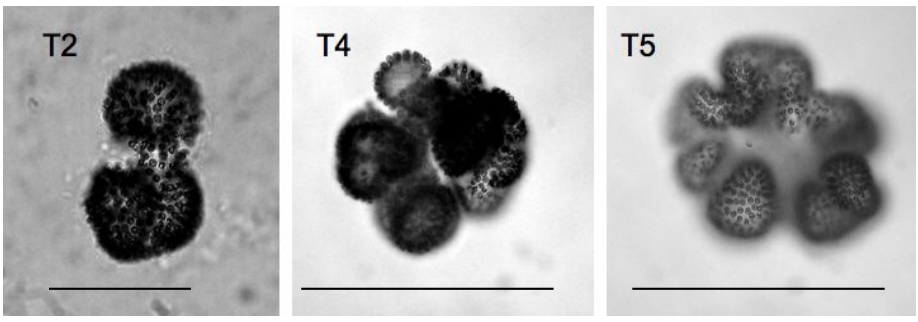



**Fig. 6,** *Woronichinia* colonies isolated from Tervuren Pond T2, T4, and T5. Scales indicate on pictures are: T2= 50 µm; T4 and T5= 100µm

**Table 4**. 16S rRNA sequences differences between *Woronichinia spp.* and the other Chroococcales genera. Nucleotide difference is expressed by the number of base differences per sequence from averaging over all sequence pairs between genera. Distance was computed with the correction of Jukes and Cantor (1969)

| | *Genus* | Nucleotide differences | Similarity (%) | Distance (substitutions/per site) |
|---|---|---|---|---|
| Merismopediaceeae | | | | |
| | *Snowella spp.* | 41,0 | 95.75 | 0,044 |
| | Merismopedioideae | 52,3 | 94.56 | 0,056 |
| Other Chroococales | | | | |
| | *Microcystis spp.* | 74,3 | 92.31 | 0,081 |
| | *Gloeocapsa spp.* | 80,1 | 91.70 | 0,088 |
| | *Cyanothece spp.* | 107,4 | 88.88 | 0,120 |
| | *Geminocystis* clade | 114,6 | 88.12 | 0,129 |

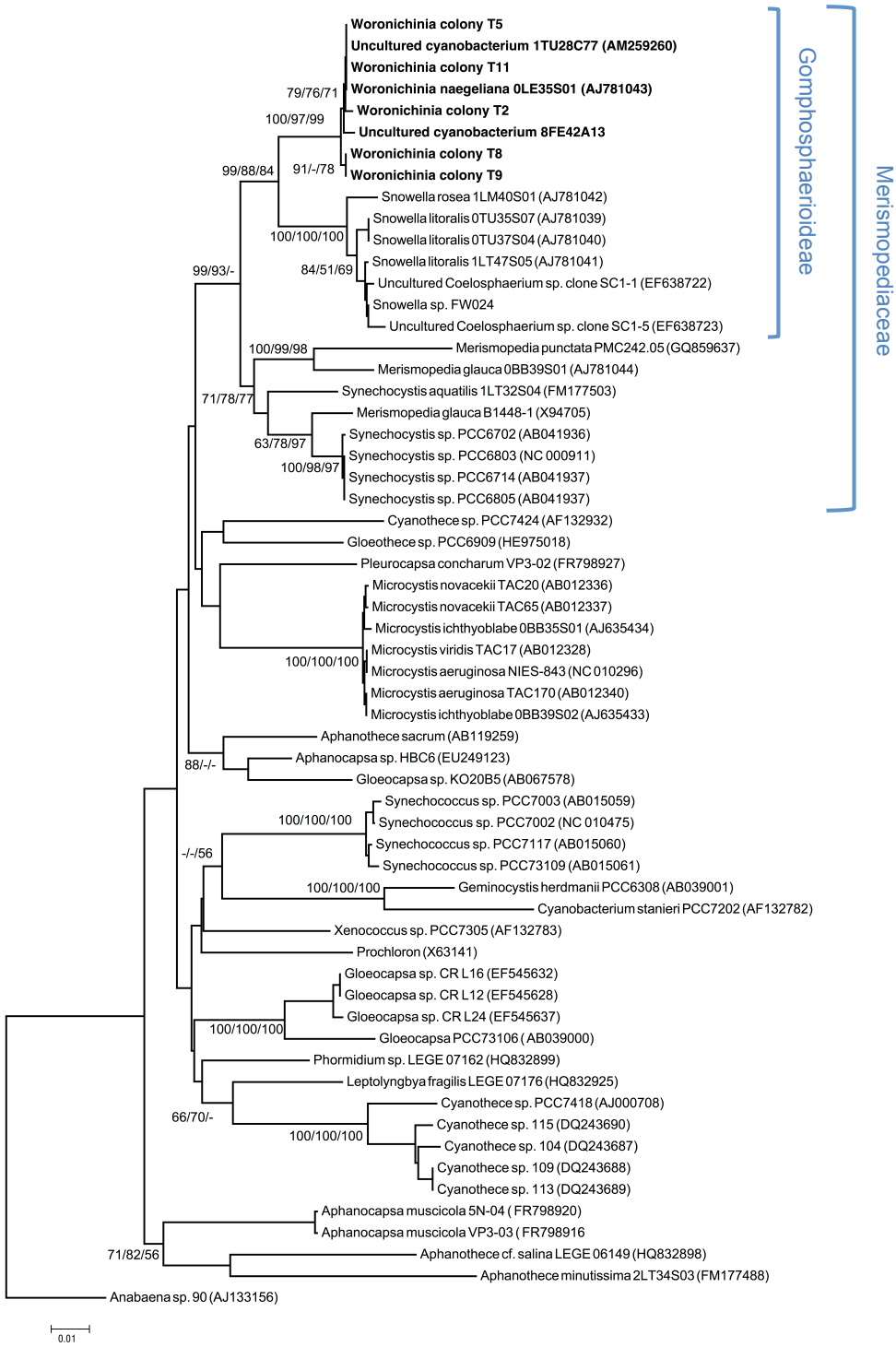



**Fig. 7**. *Woronichinia* 16S rRNA-based distance tree constructed using the Neighbor-joining method. Sequences obtained during this study are in bold

The sequenced ITS of *Woronichinia* colonies had an exact length of 405 nt. Sequences were differing by three to seven substitutions and similarities between the colonies ranged from 98.27 to 99.25%. All the sequences had the same highest BLASTN scores of 74% of similarity with the

uncultured cyanobacterium clone AL-03 16S ribosomal RNA gene (GQ401168) from a North Patagonian Andean lake.

The most closely related strain sequences were from *Synechocystis sp.* Sai002T2 (GU935368) and *Synechocystis sp.* PCC 6803 (BA000022). E-values ranged from 8e-30 to 1e-28 and similarities ranged from 74% to 77% with a rather low coverage ratio.

All new ITS sequences were successfully aligned with the ones from the clone AL-03, clone Emix1.11 isolated from Northern Baffin island in the Canadian Arctic (JX887886), clone 8FE42A13 isolated during this thesis at the lake Feronval, clones SC1-1 (EF638722) and SC1-5 (EF638723) isolated from a lake in New Zealand, the *Snowella spp.* strain FW024 isolated during this thesis and *Synechocystis sp.* PCC 6803 (BA000022) (Fig. 8). It was possible to identify the tRNA isoleucine and the conserved regions D1, D1', D2, D3, D4, Box A, Box B, and D5 as described by Iteman *et al.* (2000) for 11 strains from different cyanobacterial genera.

Different patterns in the D1-D1' helices of Merismopediaceae were found. Their size varied from 64 to 65 nt. The D1-D1' helices sequences of the 7 colonies and the clone 08FE42A13 were identical, whereas the secondary structure was very conserved with the clone AL-03. Intragenomic variations were found in ITS sequences for the *Snowella spp.* FW024 whereas only one ITS version was found in *Woronichinia* colonies as shown by their ITS-DGGE profiling (data not shown). Two different D1-D1' helix patterns were observed for *Snowella spp.* FW024. The first copy had the same structure as *Woronichinia spp.* whereas the second copy had an additional loop in the beginning of the stem structure, branched from the first loop. Clones Emix 1.11, SC1-1 and SC1-5 were similar to the second copy of FW024. A common terminal structure for the members of Merismopediaceae family was revealed by the observation of *Synechocystis spp.* PCC6803 D1-D1' helix. Indeed, all Merismopediaceae structures ended with a variable 7-base loop that followed a conserved 13-base (GAAAGYAAAUARY) loop.

Unlike the D1-D1' helices, V2 regions were quite variable in size, and structures within the Merismopediaceae family (Fig. 9). The V2 region patterns varied from no stem structure to two successive stem loop structures.

In the same way, BoxB structures were quite different for each of the taxa represented (data not shown).

The ITS is a molecular marker used in several studies to successfully distinguish intra- and inter-cyanobacterial species characteristics (Baurain *et al.,* 2002, Taton *et al.,* 2003). The ITS secondary structure has already been used to support the phylogenetic placement of *Aulosira bohemensis* (Lukeskova *et al.,* 2009). Therefore, we hypothesize that the secondary structure of *Woronichinia*'s ITS may be specific to this genus. Moreover, it seems that the homologies found between D1-D1' helices among the Merismopediaceae sequences may be specific to the entire family.

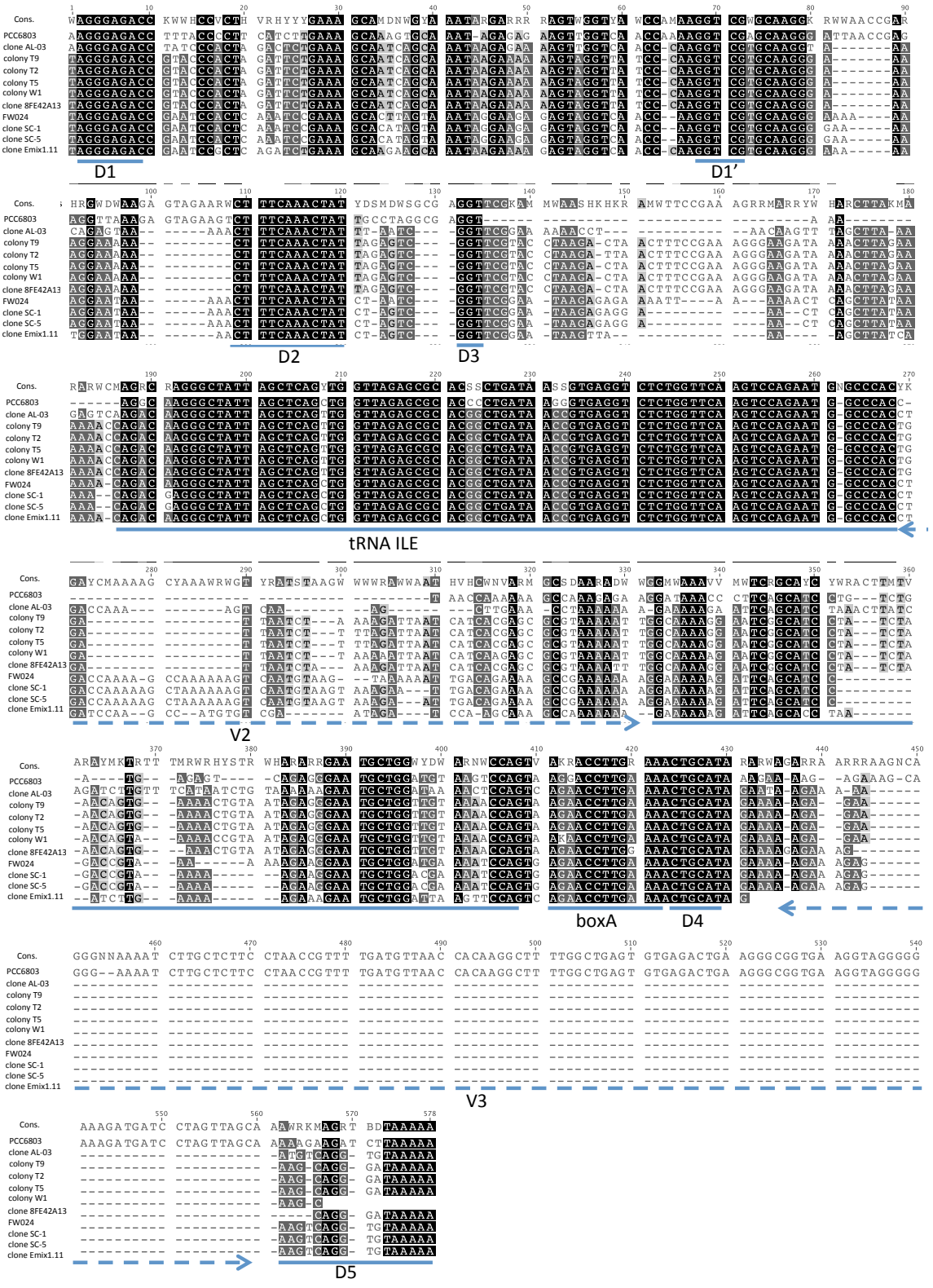
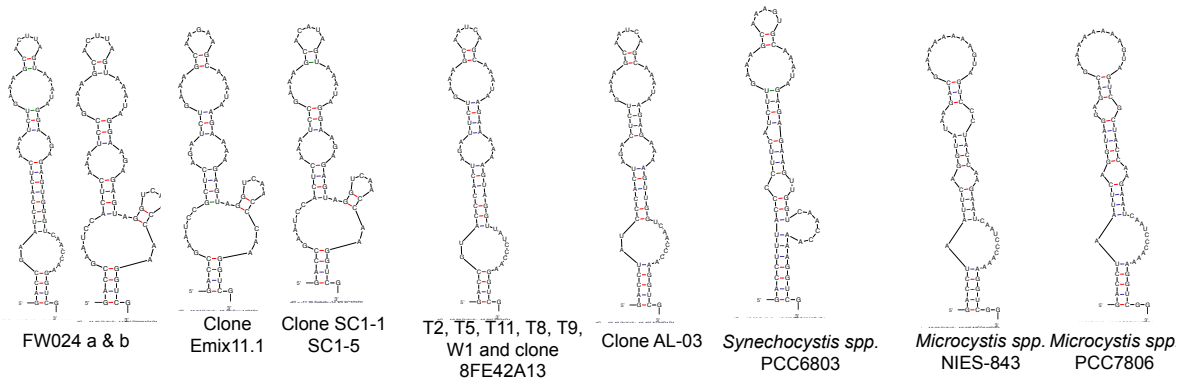
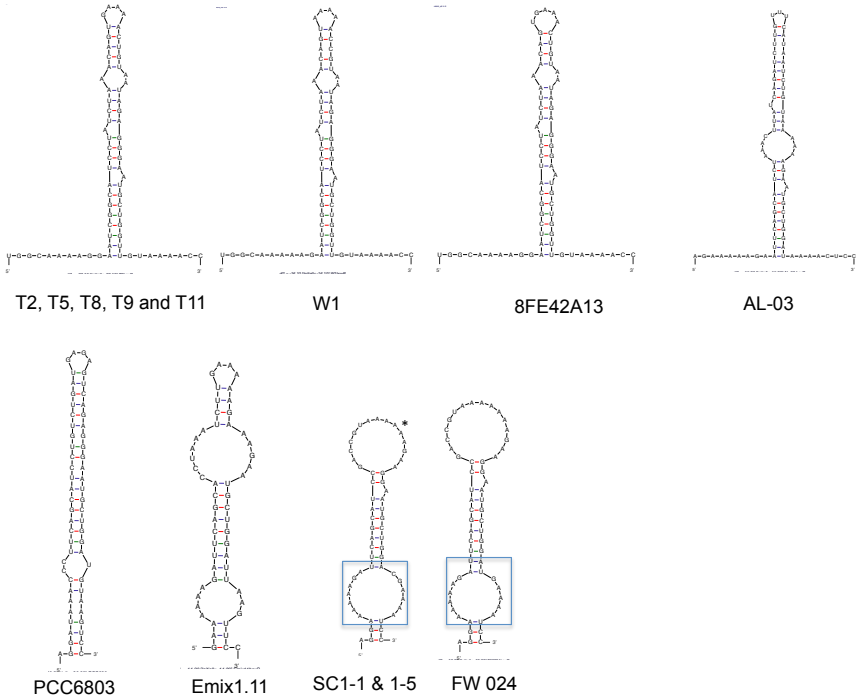



**Fig. 8**. ITS alignment of representative strains of the Merismopediaceae family. Conserved and variable regions were identified according to Iteman *et al.* (2000). A blue line indicates conserved regions, and variable regions are indicated with dashed arrows.




a.




b.

**Fig. 9.** a, Secondary structures of D1D1' helix in representative strains of the Merismopediaceae family plus two *Microcystis spp.* strains. The variable helices were defined by their position between the D1 and D1' conserved regions (see Fig. 7). b, Secondary structures of the V2 helix in representative strains of the Merismopediaceeae family. The variables helices were defined by their position between tRNA and BoxB.

The *rpoC1* gene was successfully amplified for six colonies (T2, T4, T5, T8, T9, and T11). The *rpoC1* gene encodes the gamma subunit of the RNA polymerase, which is thought to be absent from the RNA polymerases of other eubacteria (Xie *et al.,* 1989). Partial sequences (about 704 nt) were

obtained for analysis. The *rpoC1* sequences turned out to be less conserved within different genera than the 16S rRNA. Indeed, similarities with sequences from other genera were rather low compared to 16S rRNA values. Alignment with 29 cyanobacterial sequences from the previous study of Rantala *et al.* (2004), and the sequence of *Symbiobacterium thermophilum* IAM 14863 (AP006840) was easily accomplished. The *rpoC1* phylogeny (Fig. 10) showed the clustering of *Woronichinia'*s sequences with *Synechocystis spp.* PCC6803, and with unicellular cyanobacteria belonging to the Chroococcales order. Cultivated Chroococcales isolates are performing cellular division in more than one plane whereas Synechococcales strains divide only in one plane. Despite the lack of cyanobacterial sequences available in the GenBank database, strains were clustering according to their taxonomy except for special cases such as *Acaryochloris marina, Gloeobacter violaceus,* and *Thermosynechococcus elongatus,*

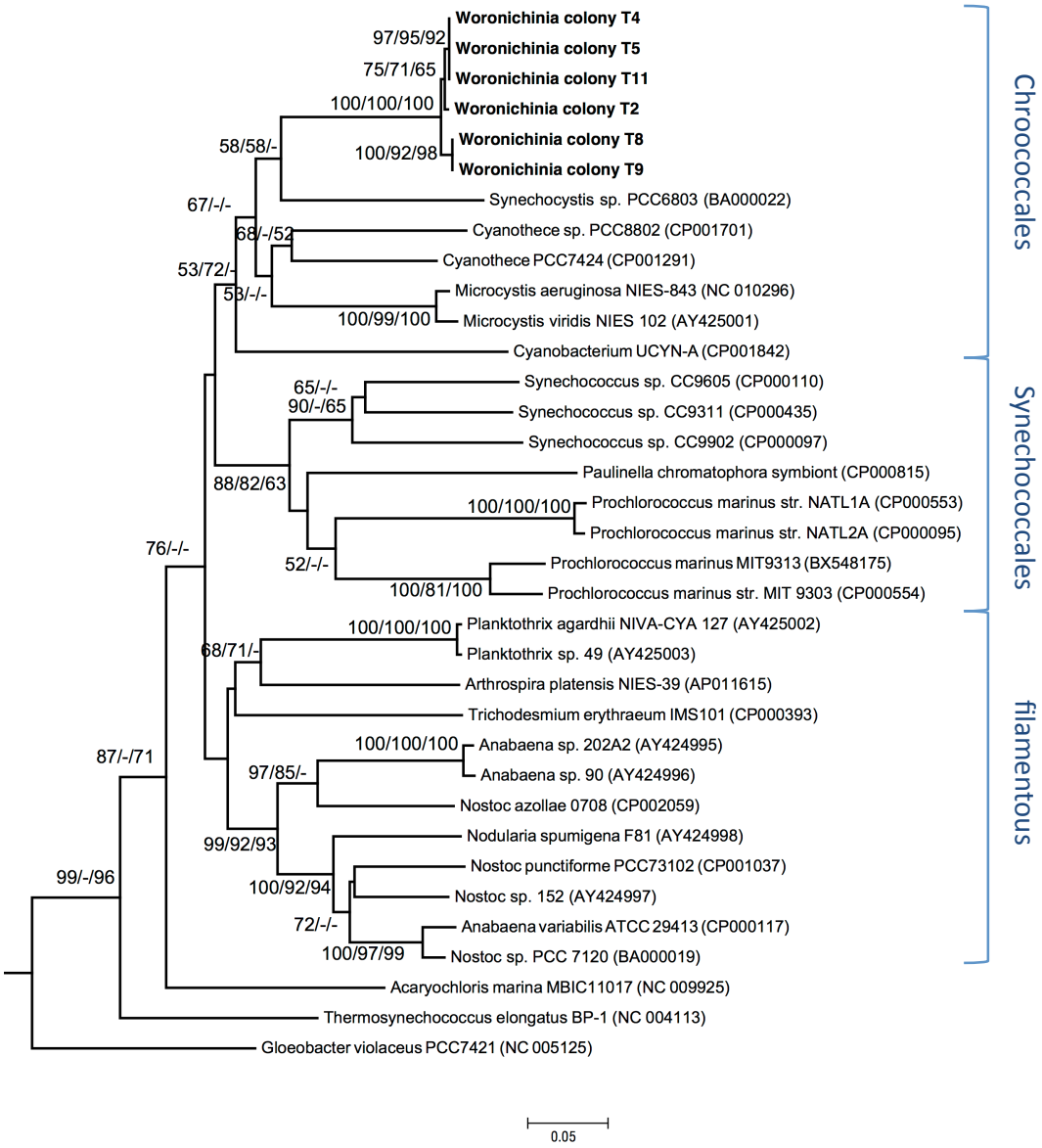



**Fig. 10**. Distance tree based on *rpoC1* partial sequences and constructed using the Neighbor-joining method. Sequences obtained during this study are in bold

### 5.3.5. ELISA reactions and mcy genes detections

The colleagues at the University of Dundee performed the ELISA anti MC-LR for the colonies T2, T5, T8, T9, and T11 but not for T4, as the resuspended volume evaporated. MCs were not detected in colonies T2 and T11. In colonies T5, T8, and T9, 0.021 to 0.051 ng of MC-LR equivalents per colony was measured, but the *mcyA* and *mcyE* PCR reactions were negative. Thus,

it is not possible to infer whether the MC(s) detected was (were) a product of the colony or whether it/they had been acquired from the environment (e.g. MC attached to the mucilage). An alternative explanation could be the presence of a *mcy* gene cluster for which the primer sequences used for the PCR are not conserved.

For colony T2, a PCR product was detected with one of the two primer sets for *mcyE* (Rantala *et al.,* 2004). The 689 bp amplicon was cloned and two inserts were sequenced, giving an identical sequence. Alignment and phylogenetic analysis of the sequenced fragments with all published *mcyE* sequences (Rantala *et al.,* 2004) showed no relation to known *mcy* gene sequences (data not shown). BLAST (BLASTP) analysis showed no relatedness to McyE but a 50% identity to a ketoacyl carrier protein synthase in *Microcystis* NIES-843 (Kaneko *et al.,* 2007). Beta-ketoacyl- ACP synthases are enzymes involved in polyketide and fatty acid synthesis. This suggests the presence of a ketoacyl synthase (KS) region. This KS region may be part of a NRPS-PKS or PKS complex that may be involved in the formation of one or more unknown secondary metabolites.

### 5.3.6. Characterization of type I KS domains in *Woronichinia* individual colonies

Putative PKS KS domains were successfully amplified for colonies T2, T4, T5, T8, T9, and T11. The PKS regions were amplified using the degenerated primer set DKF/DKR (Moffit and Neilan, 2003). The downstream and upstream primers are located in two PKS regions conserved in a range of eubacteria. Positive reactions gave a dominant band of about 700 bp. Moreover, each reaction gave a peculiar profile with lower additional bands.

Using the PCR products, clone libraries were successfully constructed for each of the colonies. Screening of the clone libraries was carried out by PCR using the same primer set as for the primary amplification. Clones were considered positive when the amplicon had a size higher than 659 bp.

Interestingly, we identified various numbers of silent mutations in the DNA sequences (0 to 5 per colony) as well as nonsense mutations (4 to 12 per colony). Also, an adenosine deletion was identified in a sequence from the

library of the colony T11 at the base 327, which resulted in a nonsense mutation. Nucleotide mutations amounted to 55,28 % of the total number of sequences.

Thirty clones were sequenced for the KS clone library of T2, and 17 DNA sequences were identical to each other whereas 13 were unique. Five of the fourteen translated sequences were identical, which shows that the mutations previously observed at the DNA level were silent. The nine other protein sequences were unique and at least differed by one AA. They were all related to a cloned type I KS from an uncultured bacterium (ADD65273) recovered from a diseased rhizosphere soil sample in China, with identities ranging from 64.3% to 64.8%.

Sequences of the T4 library (11 sequences), T5 library (11 sequences), T8 library (30 sequences), T9 library (28 sequences) and T11 (14 sequences) library were divided in two groups. The first group of translated sequences was identical (63.8% - 66.2%) to the AA sequence of the Chinese uncultured clone described just above. The second group of translated sequences was identical (96.7% - 97.7%) to the KS domain of the protein MicA (CAQ48259) from *P. rubescens* NIVA-CYA 98 (Rounge *et al.,* 2009). The *micA* gene is part of an organized operon that encodes the biosynthesis of a linear cyanopeptide called microginin. Recently, Bober and colleagues (2011) observed six microginin variants (microginin 478, microginin 757, microginin 51A, microginin 91E, microginin FR3, and microginin FR4) in a sample dominated by *W. naegeliana.* However, there was no real proof that it was the producer of the microginin. Even though the dominance by *Woronichinia* colonies was estimated to be over 99%, after the sample preparation *Microcystis flos-aquae* was found to represent up to 4% of the total bloom composition in the original sample. Therefore, a few remaining *Microcystis* colonies or cells may be present in the final sample. The colony size of *Microcystis spp.* may range from a few cells up to 1 mm, and a few colonies may be responsible for the microginin production in the Polish samples. However, our finding of the presence of microginin KS sequences in five *Woronichinia* genomes strengthens the data of Bober *et al.* (2011).

In order to analyze the genetic relationship of our KS sequences with the KS domains that belong to already elucidated operons, we have aligned

the translated sequences from our dataset with available cyanobacterial protein sequences of anatoxin, cryptophycin, curacin, cylindrospermopsin, jamaicamide, microcystin, microgninin, nodularin, nostophycin, myxothiazol and stigmatellin KS. We performed three different phylogenetic analyses (Fig. 11), which all revealed seven clusters of sequences. Six of these clusters were supported by bootstrap values higher than 50% for the three methods. The first KS modules of the McyD, and NdaD of *Anabaena*, *Microcystis*, *Planktothrix*, and *Nodularia* composed the cluster 'a'. The cluster 'b' is composed of the KS module from McyE and NdaF of *Anabaena*, *Microcystis*, *Planktothrix*, and *Nodularia.* The second KS modules of McyD and NdaD were composing the group 'c'. Clone sequences from this study were forming two clusters, 'e' and 'f'. The cluster 'e' was composed by the sequence of MicA of *Planktothrix rubescens* NIVA-CYA 98 together with clones belonging to T4, T5, T8, T9, and T11 libraries, with quite small distances between these sequences. In contrast, the cluster 'f' is grouping with rather long distances, the sequences of T2, T4, T5, T8, T9, and T11 libraries with a group of sequences of uncultured organisms (ADD65273, ADD65297), clones of Nostocales strains, and JamJ of *Lyngbya majuscula.* The cluster 'g' was previously described as strictly composed by a KS domain belonging to hybrid NRPS-PKS enzymes complex (Moffit and Neilan, 2003). At this time, the number of fully sequenced cyanobacterial PKS was limited to KS belonging to microcystin and nostopeptolide. Here, the hybrid NRPS-PKS cluster was confirmed by the relatedness of KS modules from barbamide, cryptophycin, cylindrospermopsin, jamaicamide, microcystin, nodularin, nostopeptolide, and nostophycin. Strikingly, all the KS modules of this cluster are situated downstream of a NRPS module or an adenylation module (e.g. NpnA). Interestingly, a sub-cluster is composed by JamM, JamP, and NosB, which are strictly PKS modules but are immediately downstream of a NRPS module. Unlike the KS domain that belongs to cluster 'g', the other KS from hybrid or mixed NRPS-PKS enzymes (such as McyD/NdaD, McyE, and MicA) are all located upstream of the NRPS modules.

The bootstrap values for the cluster 'd' were rather low, except for the NJ tree. KS modules from cryptophycin, curacin, and jamaicamide composed

this cluster. These three modules had the same module architecture and a similar gene neighbourhood.

Despite the fact that recombination was shown to occur between PKS modules in *Streptomyces* genes, It was proposed that the KS domains from the same clusters had a common ancestor (Jenke-Kodama *et al.,* 2006). Regarding the phylogeny and architecture of the KS modules described in the literature (Fig. 11), it is likely that the putative common ancestors had the same genes neighbourhoods and organisations. Although, there is an important lack of cyanobacterial PKS gene clusters available at the moment, it is likely that the KS phylogeny may help to predict the architecture of the protein to which it belongs.

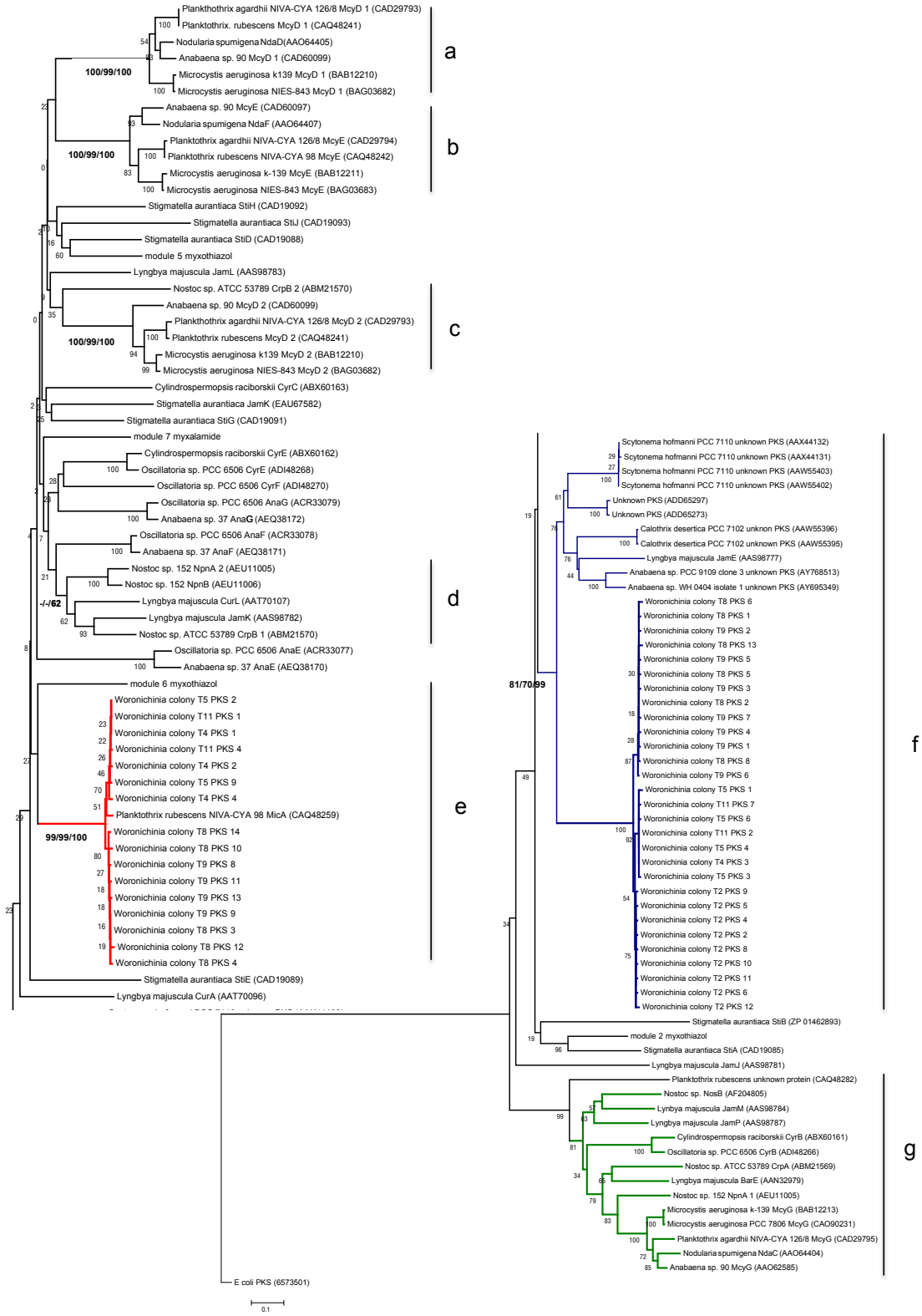



**Fig. 11**. Distance tree based on KS domain amino acid sequences and constructed using the Neighbor-joining method and the Poisson correction model.

### 5.3.7. Diversity of adenylation domains sequences in *Woronichinia* individual colonies

Using the degenerated primer pair MTF2/MTR (Neilan *et al.,* 1999) targeting the adenylation domain core I and core V motifs as defined by Marahiel *et al.* (1997), we were able to amplify PCR fragments above 908 bp. The sizes of bands slightly varied with colonies. The width of bands also varied, and double bands were distinguishable for two *Woronichinia* colonies (T8 and T9). Screenings of clone libraries were performed as described in the previous paragraph for PKS clone libraries. We sequenced a total of 117 partial sequences, which allowed detecting the A-domains present in the six *Woronichinia* colonies.

For the colony T2, it was possible to identify four groups of sequences on the basis of an alignment of 29 partial sequences. Clone 1, 2 and 3 represented the first group. Sequences were 1022 bp long and 96.6% similar with *ociB,* which is part of the cyanopeptolin encoding gene cluster of *P. rubescens* NIVA-CYA 98 (AM990463). Our sequences matched at the positions 55 329 to 56 350 which consist of the coding region of the second adenylation module of OciB. A second group sequences was composed of ten sequences, which were sharing 99.6% identity together. Seven sequences were identical and three differed by one base. Complete sequences (clone 4, 5, and 6) were 977 nt long and 70.1% identical to A-domains of plasmids of *Cyanothece* PCC7424 (CP001292) and *Cyanothece* PCC7822 (CP002199). Both blast hits were part of uncharacterized truncated NRPS clusters. A third group consisted of clone 7, 8 and 9. Sequences (1037 nt) were sharing from 84.9 to 85.4% identity with the *mcn* like gene cluster encoding for cyanopeptolin biosynthesis in *Microcystis sp.* PCC7806 (Nishizawa *et al.,* 2011). The fourth group was composed of clone 10 and 11 (1061 nt). The clone 10 was 92.2% similar to the first A-domain module encoded in *ociB* in *P. agardhii* NIVA-CYA 116, whereas clone 11 was 92.9% similar to the same sequence.

Thirty-three clones from the NRPS A-domain of the clone library of the colony T4 were partially sequenced and aligned. We identified 11 groups of sequences. Clones 1, 7, 9, 10 and 11 were representing five groups of

sequences, which were enigmatic. Indeed, it was not possible to find any relationship with cyanobacterial sequences or any evidence that they were chimera. The clone 2 represented a group of sequences that was sharing 95.9% similarity with the second A-domain of the cyanopeptolin gene *ociB* in *Planktothrix sp.* NIVA-CYA 205 (EU109504). The clone 3 represented a group of sequences, which were sharing 92.6% similarity with the first A-domain module encoded by the cyanopeptolin gene *ociB* in *P. agardhii* NIVA-CYA 116 (DQ837301). The clones 4 and 8 represented groups of sequences that shared 89.6%, and 90.2% similarity with cyanopeptolin third A-domain module of the gene *ociA* in *P. rubescens* NIVA-CYA 98 (AM990463), respectively. The clones 5 and 6 were respectively related to the third A-domain module of *ociB* in *P.* rubescens NIVA-CYA 98 (AM990463) with rather high (98.1%) and rather low (80.7%) similarity scores.

Thirty-one clones were partially sequenced to screen the *Woronichinia* T8 A-domain library. Five groups of A-domain sequences were characterized. The first group was represented by nine fully sequenced clones, which shared 99.2% identical positions. Those sequences (clones 1 to 8, and 10) were partially related to the sequence coding for the third A-domain module of *ociB* gene in *P. rubescens* NIVA-CYA 98 with a similarity score of 85.1%. Clone 9, which shared 91.7% of similarity with the A-domain present in *ociC* in *P. rubescens* NIVA-CYA 98, represented a second type of sequences. Clones 11, 12, and 13, which shared 99.1% identical sites, represented a third type of sequences. Those sequences were sharing 69.6% similarity with an A-domain encoded by the plasmid of *Cyanothece sp.* PCC7424. Clone 14 represented the fourth group, which showed a high similarity (96.7%) with the fifth A-domain module of *ociB* in *P. rubescens* NIVA-CYA 98. Finally, clone 15 was 98.5% similar to the sequence of the second A-domain module of *ociA* in *P. rubescens* NIVA-CYA 98.

For *Woronichinia* T9, 28 sequences were partially sequenced and aligned. We obtained nine complete sequences, which were describing eight. The clone 1 showed 86.6% similarity with the third A-domain module of *ociA* in *P. rubescens* NIVA-CYA 98 with. Clone 2 showed 84.9% similarity with the third A-domain of *ociB* in *P. rubescens* NIVA-CYA 98*,* whereas clone 3 shared 96.8% similarity for with the second A-domain module of *ociB* of NIVA-CYA

98. Clone 4 shared 98.7% similarity with the second A-domain of *ociA* of NIVA-CYA 98. Clone 5 and 6 differed from 2 nt from each other. They were both similar 99.3% with the second A-domain of *ociA* in *P. rubescens* NIVA-CYA 98. Clone 7 showed 92% similarity with *ociC* A-domain, and in *P. agardhii* NIVA-CYA 116. Clone 8 showed 93.4% similarity with *ociA* third A-domain module in *P. rubescens* NIVA-CYA 98. Finally, clone 9 showed over 98.7% similarity to the third A-domain module *ociB* in *P. rubescens* NIVA-CYA 98. Ten clones of colony T5 and T11 were randomly sequenced. No new blast hits were observed. Three groups of sequences were recovered from the colony T5 A-domain library. First group of sequences (clone 2, and 5) was similar (84.3%-84.5%) to the third module of *ociB* in NIVA-CYA 98. The second group (clone 1, 6, 7, 8, and 9) was similar (89.2%) to the third module of *ociA* in NIVA-CYA 98. Finally, clone 3 and 4 were 89.5% and 89.8% similar to the third module of *ociB* in NIVA-CYA 98, respectively. All sequences from colony T11 were sharing 98.5% identity, and similar (84.3%) to the third module of *ociB* in NIVA-CYA 98.

In the phylogeny of the NRPS A-domain DNA sequences, many genes described in the literature clustered with the sequences identified in this study (Fig. 12). This includes the cyanopeptolin gene clusters from three different genera (Nishizawa *et al.,* 2011, Rounge *et al.,* 2007, Rouhiainen *et al.,* 2000), the microginin gene cluster *micC* (Rounge *et al.,* 2009), the nostopeptolide A gene cluster (Hoffmann *et al.,* 2003), and the nostophycin gene cluster (Fewer *et al.,* 2011). Thus, it appears that we did not find loci with very distinct sequences from the ones already known.

The A-domain phylogeny (Fig 12) based upon DNA sequences allowed to cluster sequences according to the substrate-binding pocket specificity, as proposed by Marahiel and colleagues (1997). As shown for cyanobacterial sequences from strains of diverse genera, branches were diverging in the phylogenetic trees according to the amino acid specific activation (Barrios-Llerena, et al., 2007). Also, as shown previously by others, aromatic acid residues activation modules were significantly clustering together (cluster I) (Tooming-Klunderud *et al.,* 2007). Also, A-domain sequences specific to leucine were found together with A-domain sequences specific to proline (cluster V). Also, the two A-domain specific for the selection of glycine in *npnB*

and *nosC* formed the cluster IV. Finally, A-domain specific to Threonine formed a tight cluster (cluster VIII). In the present study, new clades were formed, A-domain sequences specific for the selection of serine and arginine clustered together with the A-domain specific to glutamine (cluster VI), all of these amino acids have side chains electrically and positively charged or uncharged. Despite the fact that some of the A-domain specific to unusual amino acids homotyrosine clustered with the one for the selection of 2-Aminoheptanoic acid (Ahp) (cluster III), we also found a cluster formed only by A-domain for the selection of homotyrosine (cluster II). We observed that A-domain for the selection of amino acids with hydrophobic side chains such as isoleucine and valine were clustering together (cluster VII) (Fig. 12).

Briefly, sequences recovered from our study were found in cluster I, cluster III, cluster VI, cluster VII, and cluster VIII (Fig. 12). Also, phylogeny of translated sequences dataset gave the same clustering (data not shown). Due to the lack of characterization of the NRPS gene clusters in GenBank, many clones from different *Woronichinia* colonies generated tightly clustering clades. However, some of the sequences were significantly and closely related to *ociA* and *ociB* A-domain modules from *P. rubescens* NIVA-CYA 98, with similarity values over 96.1%. This suggests that *Woronichinia spp.* may potentially possess the cyanopeptolin biosynthetic gene cluster in its genome or that the *Woronichinia* colonies may shelter cyanobacteria cells which possess these genes. As seen with the PKS phylogeny, some of the clone sequences were related to a microginin cluster too. This may be a supporting evidence for Bober et al. (2011), who found cyanopeptolin and microginin variants in water samples dominated by *Woronichinia spp..* Moreover, recently Gademann *et al.* (2010) showed that the protease inhibitor activity of cyanopeptolin 1020 was as toxic as some well-known microcystins for the crustacean *Thamnocephalus platyurus*. This suggests that *Woronichinia spp.* may be considered as potentially hazardous for exposure.

Interestingly, clone sequences from colony T2 and T8 were not closely related to any of the modules that are described in the literature. Nevertheless, those sequences belonged to cluster I, which contains A-domain sequences specific to aromatic amino acids. Thus, it is possible that these sequences are evidence of an undiscovered NRPS gene cluster in

*Woronichinia spp.* genomes, or that are A-domain sequences specific to an aromatic amino acids, which has not been described in the literature yet.

Despite the fact that we could not conclude about the production of microcystins by *Woronichinia spp.,* our data showed that NRPS and PKS sequences were present in this genus, and thus appear more widespread in the cyanobacterial phylum than shown before. Indeed, it was the first discovery of such sequences inside the Merismopedioiceae family.

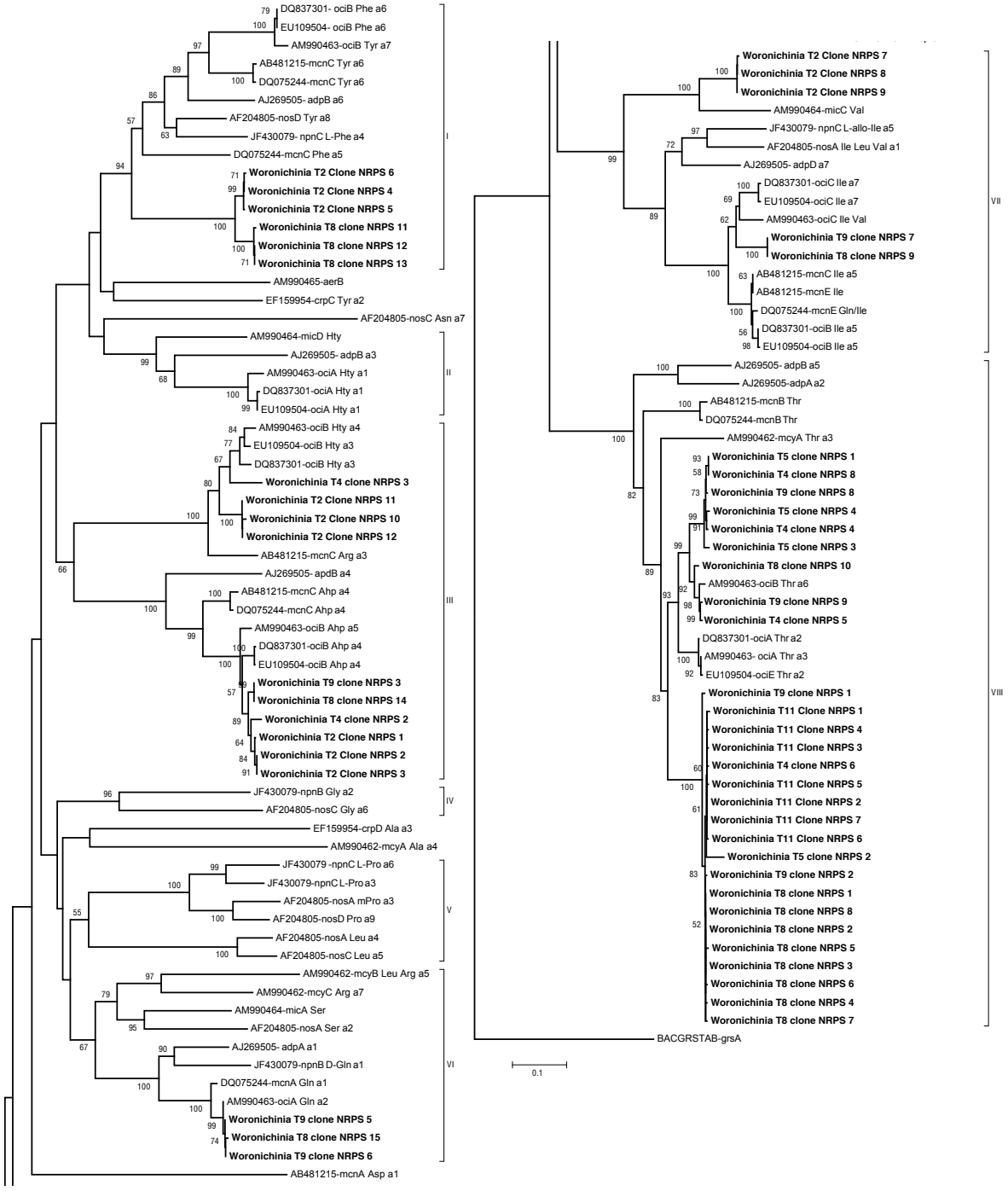



**Fig. 12.** Distance tree based on partial A-domain sequences, built using the Neighbor-joining method with a Jukes and Cantor correction.

## 5.4. Conclusion

Two methods, WGA and ELISA for MC immunoassay, were successfully applied for the concurrent amplification of MC synthase genes and the quantification of MCs in single colonies of 2 cyanobacterial bloom-forming genera, *Microcystis spp.* and *Woronichinia spp.*. This approach allowed the detection and quantification of MCs in single environmental colonies of *Microcystis spp.*, plus the characterization of their genotypes on the basis of the presence or absence of 3 *mcy* genes (*mcyA*, *mcyB*, and *mcyE*) and the sequences of 3 housekeeping gene loci (*ftsZ*, *gltX*, and *recA*) in the same colonies. The STs of the colonies from Belgian water bodies were compared to the large analysis of strains from Asian waters carried out by Tanabe & Watanabe (2011). It resulted in the discovery of a potential new cryptic lineage, represented by colony F13.

In addition, this approach yielded gene sequences from *Woronichinia spp.*, a cyanobacterium that is difficult to isolate and maintain in laboratory culture. The 16S rRNA analysis confirmed the colony identification based on morphology. We have obtained for the first time new genetic data for this genus, such as the *rpoC1* gene sequences and the sequences and secondary structures of the ITS. The first discovery of NRPS and PKS DNA sequences in *Woronichinia* colonies highlights the need for further study of this widely occurring genus, to better assess its ability to produce MCs and/or related metabolites.

Advances in microbial ecology can be hindered by problems with strain isolation and cultivation. In the last 10 years, this problem has been solved to some extent by metagenomic approaches (e.g. Venter et al. 2004). However, the need to identify and characterize microbes, which are responsible for the production of antibiotics, toxins and other bioactive compounds in natural and anthropogenic environments, remains high. It is also mandatory to define the role that uncultivated organisms play in their environment. The approach presented here provides a high-resolution analysis from individual colonies of cyanobacteria taken directly from the environment.

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# Chapter 6 - Conclusions

# Chapter 6 - Conclusions

## 6.1. Research objectives

The main aim of this thesis was to use, develop and optimize different molecular tools to bring insight into the genotypic diversity of MC-producing (toxic) and non MC-producing (non toxic) cyanobacteria in Belgian waterbodies, to identify the main MC-producers present in the Walloon region, and to elucidate the main factors influencing the dynamics of co-existing toxic and non toxic genotypes from the same taxa. In this chapter, the main results obtained during this PhD research are summarized and evaluated.

## 6.2. Culture-dependent and –independent approaches

In the chapter 2, we characterized the diversity of cyanobacteria in Belgian shallow lakes and ponds by culture-dependent and –independent approaches. We observed an important richness of cyanobacterial taxa in the samples from 11 Belgian surface waters. Even though the two methodological approaches could not be applied to the same samples, they appeared to be complementary.

Low temperature of incubation (20°C) during isolation of strains seemed to favor 'accompanying' species that were most of the time not detectable in the environmental samples. PCR detection and ELISA results illustrated the potential toxicity of these 'unseen' strains, highlighting the ecological importance of this hidden diversity. Indeed, the use of universal primers for the detection of *mcy* genes and NRPS-A domain showed that *Cyanobium* isolates may potentially possess the *mcy* genes, and that *Snowella* potentially possess a cluster of NRPS genes known to be involve in the biosynthesis of several cyanotoxins. In addition, a high concentration (48.2 MC-LReq µg/L) was found in a strain of *Cyanobium.*

On the other hand, we could not isolate gas vacuolated bloom-forming taxa such as *Aphanizomenon, Planktothrix,* and *Woronichinia.* This is problematic because they are often associated with toxic blooms, and are

often encountered in Belgian freshwaters. However, in the case of *Aphanizomenon* and *Woronichinia,* the potential of microcystins production is not yet proven. In order to bypass the limitation of culture dependent method, we developed a culture independent approach that allows both MCs quantification and genetic characterization of colony forming cyanobacteria (Chapter 5). This approach was successfully applied to the *Microcystis* spp. and *Woronichinia* spp. genera.

In the present study, DGGE analysis (Chapter 2 and 3) allowed to characterize the presence of taxa that may produce MCs. In the Chapter 3, DGGE results were in agreement with microscopy observation. In Lake Falemprise, the protagonists were characterized by both methods. Then, *mcyE*-carriers were successfully characterized by RFLP. As a result, *Microcystis mcyE*-carriers appeared dominant in our samples. This finding was supported by the *Microcystis* specific *mcyB* PCR detection assay (Chapter 3).

## 6.3. *Microcystis* genotypic dynamics

For the first time, we were able to simultaneously monitor one toxic and one non-toxic genotype of *M. aeruginosa* using real time qPCR technology during a 2 years monitoring. Real time qPCR allowed to characterize the variations that could not be shown by the previous ITS-DGGE survey done by our BBLOOMS 2 partner from UGent. Our results showed that both toxic and non-toxic genotypes appeared influenced by the photoperiod. In addition, light intensity influenced the variations of the abundance of the toxic genotype. However, there is no proof that these genotypic advantages are mandatory for the success of *Microcytis spp.* in natural habitats.

Interestingly, we also found a significant correlation between the extracellular MCs and the non-toxic genotypes, which suggests the existence of an indirect interaction between these populations. This finding could help understanding the role of MCs.

## 6.4. Genetic characterization of *Microcystis* and *Woronichinia spp.* colonies

Sequences of 3 different housekeeping genes (*ftsZ*, *gltX*, and *recA*), of 3 mcy genes, and the Internal Transcribed Spacer (ITS) were analyzed for 11 colonies of Microcystis. MCs were detected and quantified by ELISA in 7 of the 11 Microcystis colonies tested, in agreement with the detection of mcy genes. Sequence types (ST) based on the concatenated sequences of housekeeping genes from cyanobacterial colonies from Belgian water bodies appeared to be endemic when compared to those of strains described in the literature.

*Woronichinia spp.*, is a cyanobacterium that is difficult to isolate and maintain in laboratory cultures. In Chapter 5, we amplified DNA from environmental colonies of *Woronichinia spp. via* whole genome amplification. The amplification products were used as template for multiple PCRs. After a morphological identification of the colonies, the 16S rRNA gene sequences were compared with sequences from the GenBank database for confirmation of the colonies' identity. The first *Woronichinia* ITS and *rpoC1* gene sequences were characterized during this study and are now publicly available. Amplification and cloning of NRPS-A and PKS domains revealed the first sequences potentially related to the cyanopeptolin gene cluster, the microginin gene cluster and another unknown NRPS and PKS gene clusters. These are the first sequences involved in the biosynthesis of secondary metabolites available for the genus *Woronichinia* spp.

## 6.5. Evaluation of molecular techniques as a complementary tool in the risk assessments

The use of molecular tools allows a fast detection of potentially toxic cyanobacteria before the development of a bloom or an increase in the MCs concentrations. In addition, it is possible to identify the MCs-producing organisms with taxa-specific PCR assays or a RFLP approach. These techniques require a small amount of cells, and therefore can be used for the early detection of harmful cyanobacteria.

WGA based characterization represents a cheap and fast alternative as it yields important amounts of DNA that can be subsequently used for the detection of MC-producing colony forming cyanobacteria.

# Chapter 7 – Future research

# Chapter 7- Future research

At the end of this thesis, I identify several research areas that would deserve further studies.

## 7.1. Study of the genus *Woronichinia*

A genomic study of the genus *Woronichinia* would allow to deepen the knowledge regarding the role of this genus in the production of cyanotoxins. This would involve:

- a metagenomic approach to characterize the entire communities
- a single colony approach targeting *Woronichinia*'s genomes

## 7.2. *Cyanobium* genotype diversity and toxicity

During this study, three clusters of *Cyanobium* were characterized. Up to now, only two freshwater *Cyanobium* genomes were sequenced, and PKS sequences were identified in both genomes (Shih *et al.,* 2012). In addition, we potentially detected *mcy* genes in *Cyanobium*. A study that combines both genomic and ecological approaches will provide a better understanding of the roles and interactions of picocyanobacteria in freshwater environment.

## 7.3. Nostocales toxicity

So far, there is a lack of data concerning the toxigenic potential of planktonic Nostocales genotypes in Belgian waterbodies. However, anabaenopeptolides cylindrospermopsin, and microcystins, gene clusters were found in *Anabaena sp.* (Rouhiainen *et al.,* 2007; Mihali et al., 2008; Rouhiainen et al., 2000). In addition, cylindrospermosin was reported in *Aphanizomenon flos-aquae* isolates (Preuβel *et al.*, 2006), and saxitoxin in *Anabeana sp.* (Mihali *et al.,* 2008). Therefore, more effort should be devoted to the study of Nostocales in Belgian waterbodies. Regarding the large dataset accumulated on Lake Falemprise and Lake Feronval, a polyphasic approach may be developed to

determine the toxigenic potential of members of Nostocales order present in both lakes. Cultivation approach should be developed to isolate Nostocales and characterize their genotypes using different genetic markers, and determine their secondary metabolite production by mass spectrometry.

During this PhD, a few single colonies of *A. flos-aquae* and one *Anabaena spp.* were isolated. Only the genome of *Anabaena spp.* could be amplified (data not shown). Thus, single colony and filament isolation may be optimized for the amplification of *A. flos-aquae* and *Anabaena spp.*. Raman microspectroscopy should be coupled with WGA or PCR to test the presence of microcystins (Halvorson *et al.,* 2011). Raman microspectroscopy is a non-destructive method, which will leave all the cellular material available for the genetic analysis.

## 7.4. Perennial blooms of *Planktothrix*

Several perennial blooms of *Planktothrix rubescens* and *Planktothrix agardhii* are occurring in quarries in the region of Ecaussinnes. One of these was sampled two times during this PhD thesis. MCs were measured in both samples and *mcyE*-RFLP indicates the toxigenicity of *Planktothrix* cells. Further investigation of these blooms could bring interesting results for the dynamics of this genus and its potential toxicity.

# Curriculum vitae

## CURRICULUM VITAE

**Name**: Mr Yannick Lara

**Nationality**: French

**Birth**: 1 June 1981

**Email**: yannicklara@gmail.com

**Address**: Avenue du Luxembourg, 61
4000 Liège
BELGIUM

**Status**: Not married

**Actual position**: PhD student

## Research interest

My broad research interests include bacterial diversity assessment, molecular taxonomy, bacterial species delineation, evolution, secondary metabolites and their role in the environment, functional diversity, and bacterial genotypic dynamics.

## Professional background

| | |
|---|---|
| 2008-present | **PhD Student at the University of Liège –** Genetic diversity and dynamics of planktonic cyanobacteria in Belgian fresh waterbodies. |
| 2007 (Jun-Dec) | **Research assistant at the University of Liege** – BBLOOMS2 BelsPo project |
| 2006 (*six months*) | **Master Training** - Community Restriction Fragment Length Polymorphism (C-RFLP) analysis compared with Denaturing Gradient Gel Electrophoresis (DGGE) *Laboratoire d'Océanographie de Villefranche sur Mer (LOV)* |
| 2005 *(six months)* | **Master Training -** Lytic and temperate viruses in Bacteria and Archaea in the Mediterranean Sea, *Laboratoire d'Océanographie de Villefranche sur Mer (LOV)* |
| 2004 (*six months*) | **Master Training -** Modification of IHN virus genes order, for immersion bath vaccination, *Laboratoire de Virologie moléculaire des poissons, Institut National de Recherche Agronome (INRA)* |
| 2003 (one month) | **Technician** *Centre National de Séquençage, GENOSCOPE, France* |

04/2002-08/2003 **Technician** *Centre National de Séquençage, GENOSCOPE, France*

## Education

2005- 2006 Master 2nd of Biological Oceanology and Marine Environment, Pelagic environment and ecology- *Université Pierre et Marie Curie (Paris 6)*

2004-2005 Master 1st degree of Biological Oceanology and Marine Environment-*Université Pierre et Marie Curie (Paris 6)*

2003-2004 Master 1st degree in Sciences of life, Cellular biology and physiology, followed as free auditor- *Université d'Evry Val d'Essonne, France*

2002-2003 Degree in Sciences of Life**,** Cellular biology and physiology- *Université d'Evry Val d'Essonne, France*

2000-2002 UDTA2Y in Sciences of Life- *Université d'Evry Val d'Essonne, France*

## Teaching experiences

Courses

2010, 2012 Assistant during the algology practical course in 2010 and 2012 at the University of Liège

Master student supervision

Maxime Wauthy Master Training for the grade of Master II in 'biologie des organismes et écologie (BOE) – Title of thesis: «Diversité et toxicité potentielle des cyanobactéries dans des plans d'eau wallons » (Great distinction) Université Catholique de Louvain

Adeline Deward Master training for the grade of Master II in 'biochimie et biologie moléculaire et cellulaire' (BBMC)- Title of thesis «Taxonomie moléculaire de souches d'Oscillatoriacées antarctiques » (Distinction). University of Liège

## Funding

BELSPO – Research assistant contract

FRIA (FRS-FNRS) – Phd fellowship

## Language skills

Language: French (mother tongue),
English (fluent),
German (basic knowledge)

## Further skills

| | |
|---|---|
| Field work | Off shore sampling, field work with *CTD*, nets, Niskin |
| Laboratory techniques | bacterial cultivation methods, Algal cultivation method, ultrafiltration, epifluorescence microscopy, flow cytometry |
| Molecular biology | CARD-FISH, Q-PCR, PCR Mutagenesis, Cloning, PFGE, DGGE |
| Computer | Mac OSX, MS Windows, Linux Ubuntu, HTML, Javascript, Unix |
| Bioinformatics | bioedit, geneious, mauve, mega, mothur mummer |

## Outreach and education

| | |
|---|---|
| 2010 | Participation as speaker in the 8th edition of 'Doc Café' a public conference on the topic of organic agriculture. . Réjouisciences Ulg organized this event. |
| 2010 | Article in the Bulletin n°426 of the ASBL 'Sciences et culture': http://www.sci-cult.ulg.ac.be/Couvertures/Couverture426.pdf |
| 2009 | Article on the outreach website of the University of Liège 'Reflexions': http://reflexions.ulg.ac.be/cms/c_16490/fr/algues-bleu-vert-ou-rouges-couleurs-toxiques?cid=j_15783&part=1 |
| 2008 | Participation to the "Printemps des Sciences 2008" for the 8th edition of the event on the topic of Climate change and Biodiversity. Réjouisciences Ulg organized this event. |

## Other experiences

| | |
|---|---|
| *2004, 2005, and 2006* | Surf and aquatic activities summer camps coordinator (children from 5 to 11 years old) |
| *2004* | Host in exposition hall, "La Compagnie" agency |
| *2003-2004* | General Secretary Assistant of Evry University sports *2002, 2003* Data capturer, Evry University sports association |
| *Summer 2000, 2001* | Logistic technician, "Colirail" and "Essort" agency |

## Publications & Conference Contributions

**Publications**

1. Arguelles-Arias A, Ongena M, Halimi B, **Lara Y**, Brans A, Joris B, and P Fickers (2009) Bacillus amyloliquefaciens GA1 as a source of potent

antibiotics and other secondary metabolites for biocontrol of plant pathogens. Microb Cell Fact 8:6

2. Berlemont R, Spee O, Delsaute M, Lara Y, Schuldes J, Simon C, Power P, Daniel R, and M. Galleni (2013) New Organic Solvent Tolerant Esterase (RBest1) Isolated by Metagenomics: Insights into the Lipase/Esterase Classification. Rev. Argent. Microbiol. 45 :1
3. **Lara Y**, Lambion A, Menzel D, Codd G A, and A. Wilmotte (2013) A cultivation-independent approach for the genetic and cyanotoxin characterization of colonial cyanobacteria. Aquat. Micro Ecol. 69 :35-143
4. **Lara Y**, Laughinhouse H D, Van Wichelen J, Vyverman W, Codd G A, and A Wilmotte. Genotypic dynamic inside a monospecific bloom of Microcystis, in preparation
5. **Lara Y**, Lambion A, and A. Wilmotte Discovery of NRPS and PKS coding sequences in individual environmental colonies of the cyanobacterium *Woronichinia*. (FEMS microbiology or Applied and Environmental Microbiology)**,** in preparation
6. Laughinghouse H D, Adey W H, Johnson G, Young R, **Lara Y**, and K M Müller Evolution of the northern rockweed *Fucus distichus* in a regime of glacial cycling: implications for benthic algal phylogenetics (PlosOne). **in** preparation
7. De Carvalho Maalouf P, **Lara Y**, Strunecky O, Wilmotte A, and HD Laughinhouse IV Phylogenetic confusion in *Leptolyngbya antarctica* genus:, in preparation
8. Mares J, **Lara Y**, Hauer T, Kastovsky J, Johansen J R, and A Wilmotte Ribosomal RNA Analysis of Uncultivable Cyanobacteria of the Genus *Stigonema* by Single Cell Technique (Applied Environmental Microbiology), in preparation

**Reports**

1. Descy J P, Pirlot S, Verniers G, **Lara Y**, Wilmotte A, Vyverman W, Vanormelingen P, Van Wichelen J, Van Gremberghe I, Triest L, Peretyatko A, Everbecq E, and G 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)
2. Descy J P, Pirlot S, Verniers G, Viroux L, **Lara Y**, Wilmotte A, Vyverman W, Vanormelingen P, Van Wichelen J, Van Gremberghe I, Triest L, Peretyatko A, Everbecq E, and G A Codd. **Cyanobacterial *blooms: toxicity, diversity, modelling and management "B-Blooms 2".*** Final Report Brussels: Belgian Science Policy 2011 – 84 p. (Research Programme Science for a Sustainable Development)

**Conference talks**

1. **Lara Y**, Lambion A, Codd G, Descy J P, Vyverman W, Triest L and A Wilmotte, « B-BLOOMS2 : Contribution à l'étude des cyanobactéries toxiques dans les eaux de surfaces belges ». Les journées du GIS (Groupe d'Intérêt Scientifique) Cyanobactéries, Sète, France, 19 - 21 janvier 2009
2. **Lara Y**, Lambion A, Menzel D, Codd G A, Peretyatko A, Triest L, and A Wilmotte. Single colony approach applied to Microcystis and Woronichinia "What single colonies can tell us?" Young Botanists' Forum 2010 Cryptogamy in Belgium 19 November 2010– National Botanic Garden, Meise

**Poster presentations**

1. **Lara Y**, Boutte C, Peretyatko A and A Wilmotte, A new approach to analyze genotypes of colony-forming cyanobacteria from environmental samples. Proceeding of the 7th European Workshop on the Molecular Biology of Cyanobacteria, Ceske Budejovice, Czech Republic, August 31- September 4, 2008, p 111
2. **Lara Y**, and A Wilmotte, Analyse bioinformatique de différents marqueurs génétiques pour la caractérisation taxonomique et génotypique des algues bleues. Résumé des communications EcoVeg5, Cinquième Colloque d'Ecologie des communauté végétales, Gembloux, Belgique, 8 - 10 avril 2008, Section Posters
3. Wilmotte A, **Lara Y**, Lambion A, Peretyatko A, Triest L, and G A Codd. Whole Genome Amplification of environmental colonies of the cyanobacterium Woronichinia. Proceeding of the 13th International symposium on phototrophic prokaryotes. 9-14th August 2009, Page 89
4. **Lara Y**, Lambion A, Peretyatko A, Vanormelingen P, Leporcq B, Codd G A, and A Wilmotte. Molecular and toxicity characterization of environmental single colony from the genus Microcystis and Woronichinia. Proceeding of the 14th International symposium on toxicity assessment, Paul Verlaine University, Metz, France 29th August - 4th September 2009, Page 23
5. **Lara Y**, Lambion A, Codd G A, and A Wilmotte. Potential secondary metabolites biosynthesis operons in environmental colonies of Woronichinia. Proceedings of 8th International Conference on Toxic Cyanobacteria, Istanbul, Turkey, from 29th August - 4th of September 2010. ID 206
6. **Lara Y**, van Gremberghe I, Van Wichelen J, Vymerman W, Deleuze C, De Pauw E, Codd G A, and A Wilmotte. Molecular survey of toxic and non-toxic Microcystis genotypes succession during a bloom in Westveld Park pond (Belgium). Proceedings of 8th International Conference on Toxic Cyanobacteria, Istanbul, Turkey, from 29th August - 4th of September 2010. ID 207