Université de Montréal

Rôle de l'azote dans la structure et la fonction des communautés de cyanobactéries toxiques

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Rôle de l'azote dans la structure et la fonction des communautés de cyanobactéries toxiques

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Résumé

Dans cette étude de trois lacs sujets aux efflorescences de cyanobactéries, nous avons examiné la diversité des bactéries diazotrophes et des cyanobactéries toxiques. Nous avons tenté de définir les facteurs environnementaux influençant la composition des communautés phytoplanctoniques, la concentration ainsi que la composition des microcystines (MCs). Nous avons émis l'hypothèse que l'azote jouerait un rôle majeur dans le façonnement des communautés cyanobactériennes et influencerait la concentration et composition des MCs. Des concentrations de cette toxine ainsi que le gène *mcyE* codant pour l'enzyme microcystine synthétase ont été détectés à chaque échantillonnage dans tous les lacs. L'azote, particulièrement sous sa forme organique dissoute (AOD) ainsi que la température de l'eau étaient les facteurs environnementaux expliquant le mieux les concentrations des MCs, tandis que la biomasse de *Microcystis* spp. était globalement le meilleur prédicteur. Le gène *nifH* codant pour l'enzyme nitrogénase (fixation d'azote) a aussi été détecté dans chaque échantillon. Malgré les concentrations faibles en azote inorganique dissous (AID) et les densités importantes d'hétérocystes, aucun transcrits du gène n'a été détecté par réverse-transcription (RT-PCR), indiquant que la fixation d'azote n'avait pas lieu à des niveaux détectables au moment de l'échantillonnage. De plus, le pyroséquençage révèle que les séquences des gènes *nifH* et *mcyE* correspondaient à différents taxons, donc que les cyanobactéries n'avaient pas la capacité d'effectuer les deux fonctions simultanément.

Mots-clés : Cyanobactéries toxiques, lacs, azote, microcystines, fixation d'azote, diazotrophe, pyroséquençage, *nifH*, *mcyE*.

Abstract

In this study of three eutrophic lakes prone to cyanobacterial blooms, we examined the diversity of diazotrophic bacteria and toxic cyanobacteria. We evaluated the environmental factors effects on the community composition, the concentration and composition of the family of toxins microcystins (MCs). Since the assimilation of nitrogen and the synthesis of MCs in cyanobacteria are thought to be under the same control of the NtcA transcriptor, we hypothesised that nitrogen played a major role in shaping cyanobacterial communities and influenced indirectly the concentration and composition of MCs. The *mcyE* gene coding for the microcystin synthetase enzyme and MCs concentrations were detected at each sampling date in all lakes. Nitrogen, particularly under its organic dissolved form (DON) as well as water temperature were the environmental factors explaining the most variation in MC concentration although Microcystis spp. biomass was overall the best predictor. The *nifH* gene coding for the nitrogenase enzyme (N_2-fixation) was also detected at all times. Even though the concentrations of dissolved inorganic nitrogen were relatively low, and that heterocysts were present in high densities, no *nifH* transcripts were detected by RT-PCR, indicating that no N_2-fixation was going on at detectable levels at the time of sampling. Moreover, pyrosequencing revealed that sequences of the genes *nifH* and *mcyE* corresponded to different taxa, thus cyanobacteria did not have the capacity to perform both functions simultaneously.

Keywords : Harmful cyanobacteria, lake, nitrogen, microcystins, nitrogen fixation, diazotroph, pyrosequencing, *nifH*, *mcyE*.

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CCM : *Carbon Concentrating Mechanism*

Chl-a : Chlorophylle-a

CO_2 : Dioxide de Carbone

˚C : Degrés Celsius

K_m : Constante de Demi-Saturation

DIN : Azote Inorganique Dissous / *Dissolved Inorganic Nitrogen*

DON : Azote Organique Dissous / *Dissolved Organic Nitrogen*

ELISA : *Enzyme-linked immunosorbent assay*

HPLC-UV :*High Precision Liquid Chromatography with UV spectrum*

MC(s) : Microcystines / *Microcystins*

N : Azote / *Nitrogen*

N_2 : Diazote / *Dinitrogen*

NO_3^- : Nitrate / *Nitrate*

NO_2^- : Nitrite / *Nitrite*

NH_3 : Ammoniac / *Ammonia*

NH_4^+ : Ammonium / *Ammonium*

P : Phosphore / *Phosphorus*

PAR : Radiations Disponibles pour la Photosynthèse / *Photosynthetic Available Radiations*

P_i : Phosphore Inorganique / *Inorganic Phosphorus*

SRP : Phosphore Réactif Soluble / *Soluble Reactive Phosphorus*

TN : Azote Total / *Total Nitrogen*

TP : Phosphore Total / *Total Phosphorus*

WRT : Temps de résidence de l'eau / *Water Residence Time*

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Chapitre 1 : Introduction générale

1.1. La problématique des cyanobactéries

Mondialement, les efflorescences (*blooms,* fleurs d'eau) de cyanobactéries sont de plus en plus fréquentes en raison de l'eutrophisation accélérée des cours d'eau (Elser 1999, Kaebernick et Neilan 2001). L'augmentation de ce phénomène est en partie due aux changements climatiques et aux activités anthropiques (relatif à l'activité humaine) telles que le développement résidentiel et l'agriculture (Paerl et Huisman 2008). Une efflorescence algale est une accumulation de biomasse importante qui peut s'observer à l'œil nu. De fortes biomasses algales provoquent souvent une diminution importante de l'oxygène dans la colonne d'eau due à la respiration (Paerl et Huisman 2009) et à la consommation d'oxygène par la décomposition. Les accumulations de cyanobactéries, en surface, près des rives ou à l'intérieur de la colonne d'eau, affectent les services rendus par l'écosystème (p. ex. baignade, tourisme, source d'eau potable). Elles sont visuellement peu attrayantes, et dégagent parfois des odeurs nauséabondes. Plus particulièrement, les fortes abondances des cyanobactéries inquiètent, car certaines souches produisent des composés toxiques (cyanotoxines) qui causent des risques de santé aux mammifères, incluant les humains (Carmichael, 1994). Les microcystines (MCs) constituent la famille de cyanotoxines la plus fréquemment produite et mesurée en eaux douces (Carmichael, 1994; Chorus and Bartram, 1999) et sont synthétisés par un grand nombre de taxons (p. ex. *Microcystis*, *Planktothrix*, *Anabaena*; Cronberg and Annadotter 2006). Chez les humains, une exposition à court terme aux MCs peut provoquer des troubles gastro-intestinaux et des dermatites, tandis qu'une exposition prolongée favoriserait l'apparition de certains cancers,

dont celui du foie et le cancer colorectal (Davis et al. 2009). Plusieurs cas d'intoxication et de mortalité animale ou humaine ont été recensés mondialement dans les dernières décennies (Carmichael 1994, Vasconcelos et al. 1996, Kaebernick et Neilan 2001).

Au Québec, la problématique des efflorescences de cyanobactéries est devenue une priorité de santé publique depuis que plusieurs plans d'eau, dont des réserves d'eau potable, sont régulièrement contaminés par des cyanotoxines. La région de l'Estrie est particulièrement touchée par les efflorescences de cyanobactéries (Giani et al., 2005; Rolland et al., 2005). Le développement résidentiel ainsi que les activités agricoles à l'intérieur des bassins versants des lacs de la région contribuent aux apports en matière organique et en nutriments, favorisant davantage la croissance du phytoplancton dans ces cours d'eau. De plus, plusieurs lacs de la région sont peu profonds et bien brassés, ce qui permet la remise en suspension des nutriments dans la colonne d'eau. Finalement, le climat tempéré du sud du Québec favorise la formation d'efflorescences de cyanobactéries qui ont généralement un optimal de croissance à température élevée (Paerl and Huisman, 2008). Les concentrations de MCs dans certains lacs et réservoirs de la région dépassent régulièrement la limite fixée à 1.0 $\mu g \cdot L^{-1}$ (équivalent MC-LR) par l'Organisation Mondiale de la Santé (Chorus and Bartram, 1999).

1.2. Facteurs influençant la dominance des cyanobactéries

Les cyanobactéries possèdent plusieurs adaptations qui leur confèrent un avantage compétitif relativement aux changements induits par les activités anthropiques, faisant en sorte qu'elles sont en augmentation dans plusieurs cours d'eau. Elles s'adaptent très bien à

différents milieux grâce à certains traits métaboliques particuliers (p. ex. pigments spéciaux, vacuoles gazeuses, hétérocystes) qui les avantagent face aux autres micro-algues. Quelques-unes de ces caractéristiques sont décrites ci-après.

Les cyanobactéries ont généralement un meilleur optimal de croissance par rapport aux autres algues à une température plus élevée (Robarts and Zohary, 1987; Davis et al., 2009; Kosten et al., 2012). Cela explique bien le fait que dans nos régions tempérées, les efflorescences de cyanobactéries sont surtout observées pendant la saison estivale. Les cyanobactéries sont aussi reconnues pour être avantagées par les apports excessifs en azote (N) et en phosphore (P) (Barica et al., 1980; Pick et al., 1987; Dolman et al., 2012) qui proviennent souvent du ruissèlement des engrais agricoles ou des résidences aux abords des lacs. Toutefois, les cyanobactéries seraient aussi capables de se développer avec de faibles concentrations en P et N, car elles auraient une plus grande affinité pour ces nutriments, c'est-à-dire qu'elles auraient la capacité de les assimiler lorsque les conditions sont limitantes (Sommer, 1985; Chorus and Bartram, 1999). De plus, les cyanobactéries ont un ratio optimal d'azote sur phosphore (N:P) plus faible que les algues eucaryotes (10-16 molécules de N : 1 molécule de P, comparativement à 16-23 N:P pour les eucaryotes), donc elles nécessitent moins d'azote (Schreurs, 1992).

Plusieurs genres de cyanobactéries (e.g. *Anabaena*, *Microcystis*) possèdent des vacuoles gazeuses qui agissent à la manière d'un système de bouées. Ces vacuoles leur permettent de se déplacer verticalement dans la colonne d'eau afin de se positionner adéquatement en fonction des conditions environnementales telles que la luminosité ou la disponibilité en nutriments (Reynolds et al., 1987).

Les cyanobactéries ont une grande tolérance aux fortes intensités lumineuses ce qui constitue un avantage important pour la photosynthèse. Cette caractéristique serait attribuable à leurs pigments de caroténoïdes (Paerl et al., 1983) qui les protègeraient des rayons UV. De plus, les cyanobactéries ont une activité photosynthétique très efficace, car en plus de posséder des pigments de chlorophylle-a (chl-a) communes à toutes les algues, elles ont des pigments additionnels de phycocyanine, phycoérithrine, ou d'allophycocyanine. Ces pigments particuliers leur permettent de capter la lumière à différentes longueurs d'onde, et ainsi d'augmenter leur capacité photosynthétique dans des conditions lumineuses variées.

Un autre avantage compétitif des cyanobactéries est leur grande affinité pour le CO_2. Grâce à un mécanisme de concentration du dioxyde de carbone (CO_2; *Carbon concentrating mechanism; CCM*), elles peuvent assimiler le CO_2 même lorsqu'il est en très faible concentration. Lors d'efflorescences en surface ou dans les lacs eutrophes ayant une forte productivité, on observe souvent une augmentation du pH de l'eau, ce qui entraine un changement dans le système des carbonates. Cela cause un changement dans la principale source de carbone dissous disponible, qui passe du CO_2 au CO_3^-. Contrairement à d'autres algues (p. ex. chrysophytes) les cyanobactéries peuvent utiliser le bicarbonate (CO_3^-) comme source de carbone (Kaplan and Reinhold, 1999) et sont plus tolérantes aux pH élevés.

L'azote est nécessaire aux organismes vivants afin de fabriquer les composantes essentielles au fonctionnement de la cellule. Cet élément sert à la construction des nucléotides et des acides aminés qui composent l'ADN et les protéines. Les cyanobactéries

ont la particularité de posséder différentes voies d'assimilation de l'azote, ce qui leur permet de puiser cet élément à partir de divers composés azotés inorganiques (ammonium, nitrate, nitrite) et organiques (urée, acides aminés) dissous (Figure 1.1.; Flores and Herrero 2005). Certaines cyanobactéries peuvent même assimiler l'azote atmosphérique (N_2**)**, ce qui leur confère un avantage majeur dans les milieux pauvres en azote dissous puisqu'elles sont les seules parmi les algues à pouvoir effectuer ce phénomène appelé diazotrophie. L'énergie requise pour l'assimilation de différentes formes d'azote augmente proportionnellement avec leur degré d'oxydation (Garcia-Fernandez et al. 2004), c'est-à-dire l'état électronique d'une forme par rapport à son état élémentaire neutre. Les cyanobactéries assimilent préférentiellement les formes les plus réduites (ayant perdu des électrons) telles que l'ammonium ou l'urée (Blomqvist et al., 1994; Flores and Herrero, 2005) car elles peuvent entrer directement dans la cellule et être utilisées sans nécessiter de réduction. L'énergie ainsi économisée peut être relocalisée pour répondre à d'autres besoins physiologiques (García-Fernandez et al., 2004) ou pour la production de protéines ou de peptides (courtes chaines d'acides nucléiques) par exemple, les microcystines.

1.3. Les microcystines : composition et conformation

Plusieurs genres de cyanobactéries aquatiques produisent des composés toxiques sous la forme de peptides de différentes compositions et conformations. Les cyanotoxines se divisent en cinq grandes familles ayant différents effets sur les organismes, soit elles affectent le foie, le système nerveux, le système digestif, ou la peau (Mankiewicz et al. 2003). La famille de cyanotoxines la plus recensée mondialement est celle des

microcystines (Carmichael, 1994; Chorus and Bartram, 1999; Tillmanns et al., 2007). Les MCs sont produites par une grande diversité de cyanobactéries, notamment par des souches appartenant aux genres *Microcystis, Anabaena, Oscillatoria* et *Planktothrix* (Cronberg and Annadotter, 2006). Ce sont des petits peptides formés de sept acides aminés regroupés de manière cyclique, qui affectent les fonctions du foie chez les mammifères. Toutes les microcystines possèdent l'acide aminé particulier ADDA (acide 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoïque) qui est essentiel à la toxicité de la molécule. Plus de soixante-dix variantes de MCs ont été identifiées et caractérisées à ce jour (Chorus and Bartram, 1999; Welker and von Dohren, 2006), dont la plus fréquente est la MC-LR (Figure 1.2). Les deux lettres identifiant la variante de microcystine font référence aux acides aminés variables occupant les positions deux et quatre de la molécule, selon le code d'identification classique des acides aminés (L : leucine, R : arginine). En plus d'avoir une combinaison unique d'acides aminés aux positions deux et quatre, chaque peptide a une conformation tridimensionnelle variable. La composition et la structure des microcystines sont des caractéristiques importantes, car elles confèrent une toxicité propre à chacune. MC-LR et MC-LA (2-leucine; et 4-alanine) sont les variantes les plus toxiques selon des bio-essais effectués sur des souris (Chorus and Bartram, 1999) et sont jusqu'à dix fois plus toxiques que leurs congénères (MC-RR). Les facteurs contrôlant la synthèse des variantes de MCs restent inconnus. Van de Waal (2009) a observé qu'une augmentation de l'azote inorganique dissout (AID) favoriserait les variantes de MC comportant des acides aminés plus riches en azote comme la MC-RR composée de deux molécules d'arginine aux positions variables.

1.4. Synthèse des microcystines

Les souches toxiques et non toxiques sont souvent apparentées et ne peuvent être distinguées que par l'utilisation d'outils moléculaires. Les outils développés en biologie moléculaire permettent l'identification des cyanobactéries et l'étude de leurs gènes fonctionnels, c'est-à-dire les gènes qui codent pour des enzymes assurant une fonction spécifique (p. ex. les enzymes nitrogénase et microcystine synthétase). Le groupement de gènes *mcyA-J* code pour l'enzyme microcystine synthétase qui produit les toxines. Cette série de gènes est volumineuse et sa réplication suivie de la production des toxines nécessite une importante quantité d'énergie (Kaebernick and Neilan, 2001; Neilan et al., 2008). Le séquençage complet du groupement de gènes *mcy* a été complété pour diverses souches toxiques, ce qui a permis la création d'amorces d'oligonucléotides (courts segments d'acides nucléiques) pour la détection de la série de gènes qui le composent (Ouellette and Wilhelm, 2003). Les cyanotoxines, dont les MCs, sont classées parmi les métabolites secondaires, car elles ne sont pas connues pour être utilisées par les cyanobactéries lors de fonctions métaboliques primaires telles la division cellulaire ou la photosynthèse (Carmichael, 1992). Une étude sur des cultures avec différents traitements de nitrate (NO_3^-) a toutefois démontré que la production de MCs par une souche de *Microcystis aeruginosa* était corrélée positivement au taux de croissance cellulaire (Orr and Jones, 1998), ce qui va à l'encontre de la théorie selon laquelle les MCs seraient des métabolites secondaires.

1.5. Fonction écologique des microcystines

Bien que les microcystines soient largement étudiées depuis plusieurs décennies, leur fonction exacte est encore mal comprise (Carmichael, 1994; Kaebernick and Neilan, 2001) et plusieurs hypothèses ont été avancées quant à leur réelle utilité. Plusieurs chercheurs (Lampert, 1987; Paerl, 1988) ont émis l'hypothèse qu'elles serviraient à contrer le broutage par le zooplancton. D'autres pensent que les MCs pourraient avoir des effets allélopathiques (effet biochimique d'un organisme sur un autre) qui inhiberaient la croissance des compétiteurs (Babica et al., 2006). Selon une étude récente, toutes les cyanobactéries auraient déjà possédé le groupement de gènes *mcy* pour la synthèse des microcystines, mais plusieurs l'auraient perdu au fil de l'évolution (Rantala et al., 2004). Cela suggère que la synthèse des toxines ne constituerait pas nécessairement un avantage en soi pour les cyanobactéries au niveau de leur croissance ou de leur adaptabilité. Aucune hypothèse ne peut être écartée quant à l'utilité de ces métabolites. Il est même permis de croire que la toxicité des cyanotoxines soit purement fortuite, ainsi les souches non toxiques produiraient des peptides de structure similaire qui n'auraient aucun potentiel toxique (Orr and Jones, 1998).

1.6. Facteurs contrôlant la synthèse des microcystines

Une multitude de facteurs influençant la synthèse des microcystines ont été identifiés, cependant les résultats rapportés sont souvent contradictoires. En cultures, une température élevée (Sivonen, 1990), de fortes intensités lumineuses (Tonk et al., 2005) et une concentration élevée de phosphore (Kaebernick and Neilan, 2001) ont favorisé

l'augmentation de la concentration des microcystines. Des études sur le terrain ont démontré l'importance du phosphore total (Dolman et al., 2012) et de l'azote total (Giani et al., 2005; Dolman et al., 2012) comme facteurs influençant la concentration de microcystines dans les lacs. D'autres études ont observé une relation positive entre l'urée et la concentration des microcystines dans les milieux naturels (Finlay et al., 2010; Donald et al., 2011), mais les raisons pour lesquelles ces facteurs sont importants restent à éclaircir. Le facteur de transcription (protéine initiant ou régulant la transcription des gènes) NtcA est central dans la régulation de l'assimilation de l'azote chez les cyanobactéries en promouvant l'expression de gènes alternatifs qui permettent l'assimilation d'autres sources d'azote en absence d'ammonium (Herrero et al., 2001). Très récemment, il a été suggéré que ce même transcripteur pourrait être impliqué dans la régulation de l'activation de l'enzyme microcystine synthétase (Ginn et al., 2009) et pourrait jouer un rôle dans le contrôle de la production de ces toxines.

1.7. La fixation d'azote

La fixation d'azote par les communautés diazotrophes peut contribuer à plus de 80 % du budget d'azote disponible pour les communautés phytoplanctoniques dans les systèmes aquatiques (Howarth et al. 1988a). Dans les lacs, l'activité diazotrophe serait particulièrement importante dans les lacs eutrophes riches en P et limités en N (Howarth et al., 1988b). Les cyanobactéries contribueraient à la majeure partie de cet ajout d'azote, bien que plusieurs autres groupes de bactéries non photosynthétiques (p. ex. les protéobactéries) puissent aussi effectuer cette fonction.

La fixation du diazote (N_2) requiert le bris du lien triple entre les deux molécules d'azote et la réduction complète du N_2 en ammonium. Étant donné le cout énergétique élevé requis (Postgate, 1982), ce mécanisme est activé uniquement lorsque les concentrations d'azote dissous sont faibles. La réaction complète de fixation du diazote catalysée par l'enzyme nitrogénase est décrite par l'équation 1.1. (Kirchman, 2008):

$$N_2 + 8\,[H] + 8\,e^- + 16\,ATP \rightarrow 2\,NH_3 + H_2 + 16\,ADP + 16\,P_i \qquad \text{(Eq. 1.1.)}$$

Où ATP; adénosine triphosphate, ADP; adénosine diphosphate, P_i; phosphate inorganique.

La fixation d'azote s'effectue obligatoirement en absence d'oxygène, car l'enzyme nitrogénase est inactivé par son contact. Chez les cyanobactéries, la fixation d'N_2 peut se faire dans des cellules spécialisées, les hétérocystes, ou directement à l'intérieur des cellules végétatives, selon les espèces. L'enzyme nitrogénase faisant partie d'un plus grand système enzymatique catalyse la réduction du N_2 en NH_3 (Kirchman, 2008). Les gènes appartenant à l'opéron (groupement de gènes) *nifHDK* sont hautement conservés et sont utiles pour évaluer la diversité des espèces bactériennes diazotrophes en milieu naturel (MacGregor et al. 2001). Plus particulièrement, le gène *nifH* est couramment utilisé comme marqueur pour l'étude de la fixation d'azote (Vintila et El-Shehawy 2007).

1.8. Facteurs contrôlant la fixation d'azote

La présence d'ammonium (NH_4^+) et de nitrate (NO_3^-) réprime généralement la fixation d'azote, mais à des degrés variables en fonction des espèces. (Vintila et El-Shehawy, 2007). Smith (1983) a observé que l'apparition des efflorescences de cyanobactéries dans les eaux de surface serait favorisée lorsque les ratios d'azote total sur

phosphore total (TN : TP) tombent sous la barre de à 29 : 1. Un faible ratio TN : TP favoriserait d'autant plus les cyanobactéries diazotrophes (MacGregor et al. 2001), toutefois les études à ce sujet sont contradictoires (Reynolds, 1998; Downing et al., 2001). La lumière est un facteur limitant la fixation de N_2 puisque, tel que mentionné plus haut, ce processus demande de grandes quantités d'énergie qui sont acquises par photosynthèse chez les cyanobactéries. De plus, la cellule a besoin de carbone (fixé par photosynthèse) pour transformer l'azote nouvellement fixé en biomasse. Une trop grande concentration d'ammonium à l'intérieur des hétérocystes peut s'avérer toxique pour la cellule (Flores and Herrero, 2005).

1.9. Objectifs et hypothèses

L'objectif global de cette étude est d'évaluer le rôle écologique et les facteurs qui régulent certains traits fonctionnels des cyanobactéries en période d'efflorescence en milieu lacustre. Nous focaliserons notre attention sur deux fonctions écologiques des cyanobactéries, soit la fixation d'azote et la synthèse de microcystines. Pour ce faire, nous avons effectué un suivi temporel des communautés phytoplanctoniques de trois lacs dominés par les cyanobactéries.

Le premier objectif (Chapitre 2) est de déterminer quels facteurs environnementaux exercent une influence sur la production des microcystines dans ces lacs. Nous concentrerons notre attention sur le rôle de l'azote puisque cet élément a déjà été identifié comme un facteur important favorisant la présence de cyanobactéries toxiques. Nous tenterons aussi d'établir des liens entre l'environnement, la structure des communautés

cyanobactériennes et la concentration et la composition des microcystines. Nous avons évalué la biomasse et identifié les cyanobactéries, quantifié les microcystines, et mesuré une multitude de facteurs environnementaux chimiques (phosphore, azote) et physiques (température, lumière, pH). Une approche corrélationnelle a été utilisée afin de déterminer les meilleures variables explicatives pour la synthèse des microcystines.

La suite de l'étude porte sur la fixation d'azote dans les mêmes communautés de cyanobactéries toxiques (Chapitre 3). Le deuxième objectif est, dans un premier temps, d'évaluer la diversité des cyanobactéries produisant les microcystines et la diversité des bactéries/cyanobactéries diazotrophes. Dans un deuxième temps, nous déterminerons si ces deux fonctions ont lieu simultanément, soit chez un individu ou à l'échelle de la communauté. Nous avons appliqué des méthodes moléculaires (PCR, séquençage nouvelle génération) dans le but d'examiner les gènes fonctionnels régulant la fixation d'azote (*nifH)* et la synthèse des microcystines (*mcyE*).

Nos hypothèses sont les suivantes : la concentration d'azote, soit en totalité ou sous ses différentes formes, dans les lacs non limités en phosphore contribuerait à structurer les communautés de cyanobactéries et influencerait indirectement les variantes de MCs synthétisées par le biais de la communauté. Les cyanobactéries diazotrophes contribueraient à l'apport d'azote dissous dans les milieux plus pauvres, ce qui contribuerait à supporter la communauté, dont les souches toxiques qui nécessitent de l'azote pour la synthèse des microcystines. Étant donné leur cout énergétique élevé, les deux fonctions ne sont probablement pas exprimées simultanément dans une cellule, mais pourraient coexister à l'échelle de la communauté. La fixation d'azote dans les milieux

lacustres a été peu étudiée et à notre connaissance, aucune étude n'a établi de liens entre l'activité diazotrophe des cyanobactéries et la synthèse des microcystines. Cette étude contribuera à une meilleure compréhension globale de la dynamique fonctionnelle des communautés de cyanobactéries toxiques en milieu lacustre.

1.10. Figures

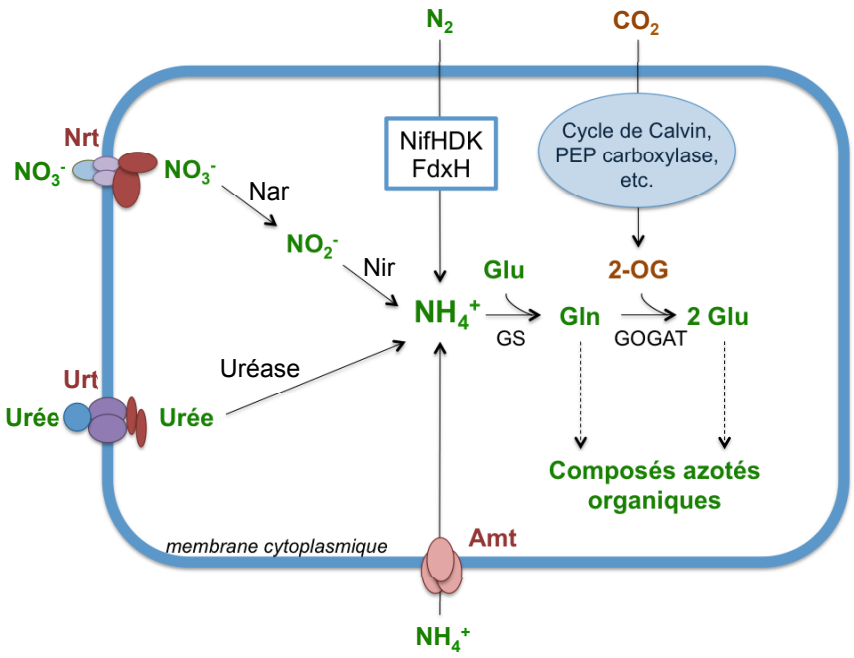

Figure 1.1. Principales voies d'assimilation de l'azote à l'intérieur d'une cellule de cyanobactérie. L'ammonium (NH_4^+) peut diffuser directement à travers la membrane cytoplasmique par les perméases. Le nitrate (NO_3^-) entre par un transporteur de type ABC pour ensuite être réduit en nitrite (NO_2^-) et en NH_4^+par les enzymes nitrate réductase (Nar) et nitrite réductase (Nir), respectivement. L'urée entre dans la cellule via le même type de transporteur, puis la molécule est fractionnée par l'uréase pour fournir deux molécules d'ammonium à la cellule. La fixation du diazote (N_2) est possible grâce à l'enzyme nitrogénase codée par les gènes de l'opéron *nifHDK*. (Figure modifiée de Flores and Herrero, 2005).

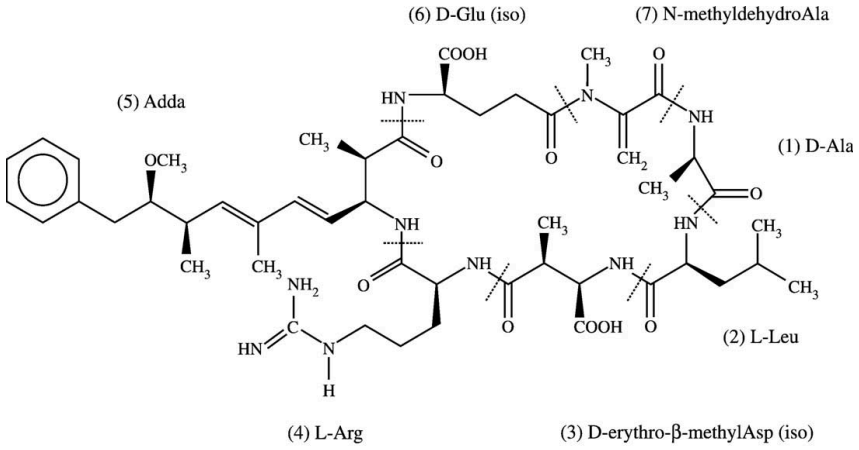

Figure 1.2. Structure générale de la microcystine-LR (MC-LR). Les acides aminés aux positions deux et quatre sont variables. (Figure tirée de Wiegand and Pflugmacher, 2005)

Chapitre 2: Variation in microcystin concentration and composition in relation to cyanobacterial community structure

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2.1. Abstract

In this study of three eutrophic lakes prone to cyanobacterial blooms, we examined the effects of nitrogen and other environmental factors in influencing cyanobacterial community composition, microcystin (MC) concentrations and composition. The identification of specific MC congeners was of particular interest as they vary widely in toxicity. Different forms of nitrogen appeared to influence cyanobacterial community composition leading to corresponding effects on MC concentrations and composition. Total microcystin concentrations across the lakes were largely explained by a combination of dissolved organic nitrogen (DON), water temperature and ammonium but *Microcystis* spp. biomass was overall the best predictor of MC concentrations. *Microcystis wesenbergii* and *Planktothrix agardhii* were also individually related to MC concentrations. Abiotic factors did not appear to affect MC congener composition directly but there were significant associations between specific MC congeners and particular species. Based on redundancy analyses the biomass of *M. wesenbergii* was associated with MC-LA whereas *M. aeruginosa* to MC-RR and *Aphanizomenon flos-aquae* was related to MC-YR. Interestingly, *M. wesenbergii* and *A. flos-aquae* are not typically associated with cyanotoxins production. We provide new understanding of how the main environmental factors identified (total nitrogen, water temperature, ammonium and DON) influence the structure of cyanobacterial community, which in turn results in differences in the dominant MC congener.

2.2. Introduction

The eutrophication of temperate lakes is associated with an increase in algal biomass, as well as a shift in community structure (Watson et al., 1997; Smith, 2006). Cyanobacteria often dominate nutrient enriched systems, sometimes causing unsightly and odorous scums at lake surfaces. Dense blooms increase turbidity and cause depletion of oxygen in the water column affecting the entire food web (Paerl and Huisman, 2009). In addition, several genera of cyanobacteria are capable of producing toxins harmful to humans and wildlife (Carmichael, 1994; Falconer, 1999). Thus, control of cyanobacterial biomass has become a major concern in the management of freshwater bodies.

The most ubiquitous cyanotoxin in freshwater systems is the family of hepatotoxic compounds known as microcystins (MC) (Carmichael, 1994; Chorus and Bartram,1999). They are small cyclic heptapeptides, which can be lethal to mammals if ingested (Carmichael, 1994; Falconer, 1999). This family of toxins comprises over 89 congeners (Welker and von Dohren, 2006), differing mainly in their composition of amino acids at position two and four of the peptide as well as in the conformation and methylation of the molecule. This contributes to a wide variation in toxicity among congeners (Sivonen and Jones, 1999; Welker and von Dohren, 2006). MCs are typically reported as total MC-LR equivalent concentrations (Chorus and Bartram, 1999), and few studies have examined the distribution of MC congeners in lakes, even though the toxicity of a bloom can vary significantly depending on the congener composition (Cerasino and Salmaso, 2012). Based on LD_{50} toxicological studies on mice, MC-LR and -LA are equally toxic, but are up to 12 times more toxic than other common variants such as -RR (Chorus and Bartram, 1999).

MC-YR is almost as toxic as -LR and -LA, and the demethylate form of MC-LR (MC-7dmLR) is 5 times less toxic than the non-methylate variant. Because of the wide range of toxicity between the different forms, the dominance of one variant over another in a bloom event will influence the overall toxicity. A wide range of cyanobacterial taxa can synthetize MC, including *Microcystis* (Chroococcales), *Anabaena* (Nostocales), and *Planktothrix* (Oscillatoriales) (Welker and Von Dohren, 2006). Some genera (e.g. *Aphanizomenon, Spirulina*) do not appear to have MC-producing strains, but molecular evolutionary evidence suggests that they likely possessed the *mcy* gene cluster for MC synthesis in the past (Rantala et al., 2004). Some cyanobacterial species include both toxic and non-toxic strains that are impossible to differentiate by microscopy, increasing the difficulty in monitoring toxic cyanobacterial blooms based on taxonomic evidence alone. Furthermore, an individual strain can produce several MC congeners (Chorus and Bartram, 1999) thereby hindering the capacity to predict the toxic potential of a bloom.

A large number of studies have examined the factors potentially triggering and controlling for total MC production (Orr and Jones, 1998; Kaebernick and Neilan, 2001; Tonk et al., 2005), Several empirical studies have shown that nitrogen may be particularly important via its effect on cyanobacterial biomass and composition (Rolland et al., 2005; Dolman et al., 2012). Comparative studies of individual lake regions found that one of the strongest predictors of MC concentrations was total nitrogen (TN) and this response appeared essentially linear (Giani et al., 2005; Rolland et al., 2005) or showed a peaked relationship at intermediate levels of TN (Graham et al., 2004). In a recent study on the comparative effects of urea, NH_4^+ and NO_3^+ in a hypereutrophic lake, Donald et al. (2011) found that under high P, the addition of both NO_3^- and urea increased MC concentration by

10 to 13 fold in mesocosm experiments. In contrast, an analysis of lakes across several regions concluded that lakes with significant MCs tended to be systems with very low nitrogen to phosphorus ratios (Orihel et al., 2012). In one of the few studies on MC congener composition, Van de Waal et al. (2009) concluded that nitrogen-replete waters appeared to lead to the production of more N-rich MC congeners. In general, the way N and its different forms affect cyanobacterial communities and their ability to produce different congeners of microcystin remains an open question. The objectives of this study were to test the hypothesis that different N forms, alone or in combination with other environmental variables influence the cyanobacterial community structure, the concentration of MC, as well as MC congener composition by following the dynamics of these variables in three eutrophic lakes.

2.3. Methods

2.3.1. Study sites

The study lakes were all located in the Eastern Townships in south-western Quebec, Canada. Lakes were selected based on their history of cyanobacterial blooms (Giani et al. 2005; Rolland et al. 2005) and their trophic status according to the OECD (Organization for Economic Cooperation and Development 1982) criteria as meso-eutrophic to hypereutrophic. Chosen lakes are relatively small and shallow, but differ in their catchment characteristics (Table 2.1.). Lake Bromont is located in a hilly and largely forested watershed. It is a meso-eutrophic lake and the deepest of the studied lakes (max. depth 7.5 m); it is thus the only one that thermally stratifies in the summer months. Lake Waterloo is eutrophic and polymictic, with a predominantly forested watershed as well, but with the highest population density (157 ind.$\cdot$km^{-2}). Lake Petit St François is hypereutrophic,

polymictic and has an agricultural watershed. It is the shallowest of the study lakes and has the shortest water residence time (Table 2.1.).

2.3.2. Sample collection

We sampled each lake on a monthly or fortnightly basis from May to October 2010 leading to a time series with eight data points per lake. Light penetration was measured using a photometer Li-Cor (Li-189) and light extinction coefficients (k) were calculated using the Lambert-Beer law (Wetzel, 1983) at each sampling. Water temperature profiles were obtained using a multiprobe sonde (YSI 556-MPS). Integrated water samples of the mixed (epilimnetic) layer were collected near midday at the center of each lake using a Tygon tube and samples were transferred to 4 L acid-washed dark plastic bottles pre-conditioned with lake water. When Lake Bromont was stratified, we observed a metalimnetic chlorophyll a (chl-a) peak using a Fluoroprobe (BBE-Moldaenke) and an additional sample was taken at that depth using a horizontal 2 L Van Dorn bottle. Whole water samples for phytoplankton identification and enumeration were preserved with Lugol's iodine in 125 mL flint glass bottles in the field. Samples for MC were filtered promptly in the field using a peristaltic pump (Masterflex L/S 7519-06, Cole-Parmer) at 80-100 $mL \cdot s^{-1}$ onto pre-ashed and pre-weighed 47 mm Whatman GF/C filters. After filtration of 200 to 1000 mL of lake water (or until filter clogged), filters were individually folded into 2 mL Cryovials and immediately flash-frozen in liquid nitrogen. Samples were kept at -20 °C in the dark until MC analysis.

Water samples for TN and TP analyses were stored in 100 mL acid-washed glass tubes without filtration. For total dissolved nitrogen (TDN) and soluble reactive phosphorus (SRP), 50 mL of water was syringe-filtered through 0.45 μm Acrodisc filters and stored in

the same manner as for TN and TP. Water for ammonium (NH_4^+), nitrate (NO_3^-) and nitrite (NO_2^-) analyses was also filtered through 0.45 μm Acrodisc filters and stored in 125 mL acid-washed Nalgene® bottles. All samples for nutrient analyses were either analyzed promptly upon return to the lab or stored frozen until processing.

2.3.3. Microcystin analyses

Prior to extraction, filters were oven-dried overnight at 60˚C, and weighed. MC extraction was performed using a pressurized liquid extraction method with the ASE 200 Dionex Corporation instrument (Bannockburn, IL, USA) at 60˚C and 14 MPa (Aranda-Rodriguez et al., 2005). Briefly, dried filters were unfolded and inserted in stainless steal cylindrical cells (11 mL) packed with prewashed Hydromatrix. Extracts recovered in 75% methanol (MeOH) were collected in amber vials previously rinsed with HPLC-grade solvent. Samples were then evaporated under a gentle ultra-high purity N flow using a Zymark Turbovap II Concentration Workstation and re-suspended in 50% MeOH in ultra-pure water. Recovered samples were filtered through pre-conditioned 0.22 μm Acrodisc filters (Palls Company) for purification prior to HPLC analysis. Further dilutions (50% MeOH in water) were done if necessary for the ELISA test.

Total MC concentration was measured with an ELISA kit for microcystin (Quantiplate^{TM}, Envirologix, ME, USA). This is a direct competitive ELISA using a polyclonal antibody bind to a microtiter plate. MC-LR is used as a standard; therefore all measurements are expressed in MC-LR equivalent. The test was run in duplicates and samples with coefficient of variation (CV) values over 15% were reanalyzed, according to the manufacturer's recommendation.

Since ELISA does not distinguish between congeners, we analyzed the same extracts using a reverse-phase high-pressure liquid chromatography (RP-HPLC) with UV-detection equipped with a photodiode array (PDA) for the quantification of five MC congeners (Aranda-Rodriguez et al., 2005). We used a HP series 1100 HPLC-PDA operated at 40°C, with a Zorbax Eclipse XDB-C18 column. The flow rate was of 0.5 mL per minute with a solvent gradient composition from 90% water and 10% acetonitrile, to 100% acetonitrile in 43 minutes. A volume of 0.05% trifluoroacetic acid, a pairing agent commonly used in reverse-phase HPLC peptide separation, was added to both acetonitrile and water. UV spectra between 200 nm and 300 nm were collected and concentrations were calculated from the absorbency at 239 nm by comparison against purified extracts of certified standards of MC-RR, -YR, -LR, -7dmLR and -LA, and nodularin, all obtained from Cedarlane and the National Research Council, Halifax, Canada. Nodularin was used as an internal standard and spiked prior to extraction to verify recovery, which averaged 74.5%.

2.3.4. Phytoplankton analyses

Total biomass of phytoplankton was calculated from counts of cells greater than 2 μm using a Zeiss AXIO A1 inverted microscope at X200, and X400 magnification. Aliquots (7 mL) of preserved phytoplankton sample were allowed to settle overnight in a 26 mm diameter chamber according to the Utermöhl method (Lund et al., 1958) before microscopic identification, cell measurements and enumeration**.** Phytoplankton biomass was estimated by converting cell volume to biomass assuming a specific density of 1 $g \cdot cm^{-3}$, which is by convention used for all phytoplankton taxa. The computer counting software Algamica (version 4.0) was used (Gosselain and Hamilton, 2000) to aid in counting and estimations of cell biovolume using assigned geometric shapes dimensions. Total volumetric biomass

($\mu g \cdot L^{-1}$) for cells >2 μm, cyanobacterial biomass and that of specific taxa were obtained for each sample.

2.3.5. Nutrient analyses

Nutrient concentrations (NO_3^-, NO_2^-, NH_4^+, and TP) were measured spectrophotometrically according to standard techniques (American Public Health Association, 2005). NH_4^+ was measured using the phenol-hypochlorite method relying on estimations of an indophenol blue compound after reaction with phenol and hypochlorite (Wetzel and Likens, 2000). Total dissolved N (TDN), NO_3^- and NO_2^- were all measured in the same manner, although TDN was first treated with a persulfate digestion to convert to NO_3^-, and all NO_3^- was then reduced to NO_2^- using a cadmium coil prior to analysis. NO_2^- concentration was measured with the Griess reaction. Briefly, sulfanilamide and N-naphthyl-ethylenediamine were successively added to a sample to form a stable azo compound that can be compared with a calibration curve treated in the same manner (Wetzel and Likens, 2000).

Dissolved organic nitrogen (DON) concentration was obtained by subtracting ($NO_3^- + NO_2^- + NH_4^+$) from TDN with standard deviation (STD) for [DON] incorporating measurement error (S^2) propagation according to $STD_{DON} = (S^2_{TDN} + S^2_{NH4} + S^2_{NO3+NO2})^{1/2}$ (Bronk et al., 2000). TN concentration was estimated as the sum of TDN and suspended particulate nitrogen (SPN) with STD_{TDN} calculated similarly to STD_{DON}**.** The coefficient of variability between sample replicates was less than 10% in all cases. Total phosphorus (TP) and SRP measurements were done with the molybdenum blue method using an Astoria analyzer (method # A050).

2.3.6. Statistical analyses

Data were examined to determine whether they met normality assumptions using a Shapiro-Wilks test and were transformed (log_{10} or square root) when necessary. All statistical analyses were performed using *R* (v. 2.14, the R Foundation for Statistical Computing). Simple and multiple linear regressions were used to determine which environmental and biotic factors could best predict the observed differences in MC concentrations among samples. For multivariate comparisons of the MC congener composition with environmental variables and community structure, we used principal components analysis (PCA) and redundancy analysis (RDA). Forward selection of environmental variables was used for all regression (including RDA) models. Species data to be used in the redundancy analyses were transformed to produce a matrix of Hellinger distances among sites (Legendre and Gallagher, 2001). To test for the significance of the RDAs, permutation tests on the global models were performed with a minimum of 1000 permutations, using the function *anova.cca* of the *Vegan* package in *R*.

2.4. Results

2.4.1. Nutrient concentrations

Nutrient dynamics over the sampling season are summarized in Fig. 2.1A-E for all lakes. TP was high in all lakes over the entire sampling period. In the meso-eutrophic Lake Bromont, TP varied seasonally from 0.55 to a maximum of 2.32 μM observed in autumn (Fig. 2.1A) with a mean value of 1.1 μM. Waterloo had an average concentration of 1.84 μM, ranging from 1.04 to 5.17 μM. Petit St François (PSF) was much more eutrophic than the other lakes with a maximum TP of 11.13 μM sustained from the beginning of August. Soluble reactive phosphorus (SRP) was relatively stable in all lakes with the

exception of a peak in August in PSF, probably due to internal loading in this polymictic system (Fig. 2.1B).

TN was also high in all lakes, with average concentrations ranging from 24.69 μM (Bromont epilimnion) to 239.46 μM (PSF) (Fig. 2.1C). DON concentrations were highest in PSF with a mean of 33.37 μM, followed by Waterloo (mean = 21.57 μM) and Bromont (mean = 14.91 and 13.28 μM for epi- and metalimnion, respectively). Total DON concentration was relatively stable over the summer sampling period in all lakes (Fig. 2.1D), accounting for an average of 40%, 47%, and 28% in Bromont, Waterloo and PSF, respectively. Nitrite and nitrate (NO_2^-+NO_3^-) decreased toward the end of spring in Bromont and Waterloo and increased again at the end of summer. In the metalimnion of Bromont, NO_2^-+NO_3^- stayed low until the lake destratified. PSF was depleted in (NO_2^- +NO_3^-), even in the early spring and remained low to virtually undetectable, throughout the summer until concentrations increased in October (Fig. 2.1E). A wide range of NH_4^+ concentrations were observed across all three lakes (1.59 to 9.51 μM). Higher concentrations and an obvious peak occurred in the metalimnion of Lake Bromont, located just above the anoxic hypolimnion (Fig. 2.1F).

All lakes had relatively low mean TN: TP molar ratios (between 8.7 and 15.2) except for Bromont's metalimnion where the ratio was 28. Overall, mean DIN: SRP ratios were slightly higher at 26.8 (Waterloo), 14.1 (PSF), 13.7 (Bromont), 18.3 (Bromont metalimnion). pH varied between lakes, being on average highest in hypereutrophic PSF at 9.1 (range: 7.5-9.5) intermediate in Waterloo at 8.0 (range: 7.1-9.0) and lowest in both the epilimnion (7.3) and metalimnion (7.2) of Bromont. Accurate pH measurements were only

done on the last five sampling dates, therefore pH was not included in subsequent statistical analyses.

2.4.2. Cyanobacterial community composition

Total phytoplankton biomass averaged at 3,372.4 and 8,386.8 $\mu g \cdot L^{-1}$ in Bromont (epi- and metalimnion, respectively). In Waterloo, the average for the sampling season was 4,190.3 $\mu g \cdot L^{-1}$ and in PSF, the total phytoplankton biomass averaged at 23,079.9 $\mu g \cdot L^{-1}$. Cyanobacteria were the dominant taxonomic division on most sampling dates contributing up to approximately 90% of mean total biomass in all lakes, with the exception of the epilimnion of Bromont where they contributed 67% of total algal biomass. PSF had the highest overall cyanobacterial biomass throughout the sampling period (Fig. 2.2A). The structure of the cyanobacterial communities varied through time within Bromont and among lakes (Table 2.2.). Waterloo and PSF had similar composition, sharing several of the same taxa, but with different relative abundances. Several cyanobacterial species were found uniquely in Bromont (e.g. *Anabaena smithii, Planktothrix agardhii,* and *Spirulina* spp.) or only present at very low biomass levels in lakes PSF and Waterloo (e.g. *Anabaena planktonica*). Conversely, *Microcystis wesenbergii* was dominant in hyper-eutrophic PSF and found only at very low biomass in August in Waterloo. High biomass of *Planktothrix* spp. was detected in lakes Bromont and Waterloo. *Anabaena* spp. as well as *Aphanizomenon* spp. were both present in all lakes, but more abundant in PSF and Bromont than in Waterloo.

2.4.3. Total Microcystin concentration

MCs were detected on every sampling occasion in all locations by ELISA (Fig. 2.2B). Total concentrations ranged from 0.06 $\mu g \cdot L^{-1}$ in meso-eutrophic lake Bromont

to 1.68 $\mu g \cdot L^{-1}$ in eutrophic lake Waterloo. In PSF, the maximum concentration of the toxin was measured in mid-July, while in Waterloo, high values occurred in late July, early August, and mid-September. Lakes Waterloo and PSF displayed similar seasonal patterns and overall concentrations of MC despite the clear differences in nutrient concentrations (Fig. 2.1A-F) and cyanobacterial species composition and biomass between the two lakes (Fig. 2.2A). This resulted in a much higher biomass-specific MC content in Waterloo, reaching a maximum of 624.67 $\mu g \cdot g^{-1}$ dry weight compared to 428.27 $\mu g \cdot g^{-1}$ in PSF (Fig. 2.2C). In Bromont, MC concentrations were quite low at all times in both the epilimnion as well as in the metalimnion (max. 79.40 and 74.92 $\mu g \cdot g^{-1}$, respectively).

We used a simple linear regression approach to determine if MC concentration could be predicted from environmental variables and cyanobacterial species biomass. We observed that TP, TN, NO_3^-+NO_2^-, NH_4^+, DON, the light extinction coefficient (*k*), and water temperature (t°) were all positively and significantly related to total MC concentration (Table 2.3.). One of the strongest relationships observed was between *k* and MC concentration ($R^2_{adj.}$=0.52, p<0.0001, n=24). Of all of the nutrients measured, DON emerged as the best predictor explaining the most variability (40%; p<0.001, n=24) in MC concentration. In term of species effects, the biomass of *Microcystis wesenbergii*, *Microcystis* spp. and *Planktothrix agardhii* were also all related to MC concentration, with *Microcystis* spp. showing the strongest relationship ($R^2_{adj.}$=0.63; p<0.00001, n=24).

We then used a multiple linear regression approach with forward selection in order to develop a more robust model to explain the variation in MC concentrations. Highly correlated (r>0.60) explanatory variables were excluded. Because TP and *k* were most often highly correlated with other variables, they were both excluded from further analysis.

Furthermore, when TP was included in forward selection it was not retained as an explanatory variable. The final list of variables included in the analyses was: TN, TN: TP ratio, SRP, $NO_3^-+NO_2^-$, NH_4^+, DON, and water temperature. The final regression model ($R^2_{adj.}$=0.61; p<0.001, n=24) indicated a positive influence of DON, NH_4^+, and water temperature on total MC concentration:

Log (MC-LR equivalent ($\mu g \cdot L^{-1}$)) = -21.12 + 2.89t° + 1.80logDON + 0.50logNH_4^+

(Eq. 2.1.)

Variation partitioning demonstrated that DON, water temperature, and NH_4^+ explained 33%, 16%, and 11% of the variation in MC concentration respectively.

No significant multiple regression model emerged when we used biomass of different cyanobacteria species alone as explanatory variables (Table 2.2.). However when a regression was performed using both the environmental and species variables, the following model predicted 76% ($R^2_{adj.}$=0.76; p<0.0001, n=24) of the variation of the observed MC concentration with:

Log (MC-LRequivalent ($\mu g \cdot L^{-1}$)) = -1.11 + 1.04*Microcystis* spp. + 0.41log(TN+1) + 0.43t° + 0.45*Aphanizomenon flos-aquae*. (Eq. 2.2.)

This final regression constituted the best model to predict total MC in our lakes. Variation partitioning demonstrated that *Microcystis* spp. explained the most variation (31%) followed by TN (12%). *A. flos-aquae* and water temperature both entered the model significantly but each explained a relatively small amount of the total variation (5 and 3% respectively).

2.4.4. Microcystin congener composition

The dominant MC congeners varied both among and within lakes (Fig. 2.3A-C) over time. MC-LA was only found in PSF while MC-7dmLR and -YR were only detected in Bromont. MC-RR was present in Bromont but dominant in Waterloo, while MC-LR was present in all lakes. MCs in meso-eutrophic Lake Bromont were comprised mainly of MC-YR although early in the season the dominant variant was -LR, switching to -YR in August and for the rest of the sampling season. The total sum of MC concentration as a function of the different congeners measured using HPLC was at times lower than the concentration determined using ELISA.

One of our main goals was to determine how the congeners varied as a function of environmental conditions and community composition. The PCA showed distinct patterns in the distribution of environmental descriptors, cyanobacteria species and MC composition among lakes with sites well separated in the ordination space (Fig. 2.4.). The first axis was mainly driven by DON and explained 27% of total variation. Axis 2 was mainly related to community structure where *Microcystis aeruginosa* and *Anabaena spiroïdes* dominance in Lake Waterloo, contributed another 15% of total variation. Lake PSF was associated with the presence of *Microcystis wesenbergii* and was, not surprisingly given its higher relative trophic state, associated with indicators of nutrient enrichment (TN, TP, DON). Several other cyanobacterial taxa such as *Planktothrix agardhii*, *Aphanizomenon flos-aquae,* and *Anabaena smithii* were important in Lake Bromont. The most important environmental descriptor in Bromont was water temperature, which was negatively related to this lake. Each lake was characterized by a dominant microcystin congener: MC-LA, -RR and -YR emerged in PSF, Waterloo and Bromont respectively. This pattern suggests an association

of the MC congener composition with the community structure and environmental conditions of different systems.

We did a series of redundancy analyses (RDA) with forward selection in order to determine the factors potentially affecting MC congener composition in the lakes. We first tried to determine the effect of environment on congener composition, but no environmental variables emerged as significant (p>0.05; results not shown). However, species composition appeared to be associated with congener composition (Fig. 2.5A). Together, the biomass of *Microcystis wesenbergii* and *Aphanizomenon flos-aquae* explained 31.9% of the variation in congener composition across all lakes (p=0.001, n=24) According to the results of this RDA, the biomass of *M. wesenbergii* was associated with MC-LA in PSF whereas *A. flos-aquae* was significantly related to MC-YR in Bromont and *M. aeruginosa* was related to MC-RR in Waterloo. No other cyanobacterial taxon appeared to significantly influence the congener distribution across the study lakes.

Given that environmental factors did not appear to affect MC congener composition directly, but that species did, we examined the relationship between environmental variables and community structure using a third RDA. Our results indicate that TN and DON were both significantly and positively related to the biomass of non-heterocystous cyanobacterial species ($R^2_{adj.}$=0.28). TN was closely associated with *M. wesenbergii* biomass (dominant species in PSF) but negatively to *Anabaena planktonica* in Bromont, and DON was related more to *Microcystis* spp. (dominant species in Waterloo) (Fig. 2.5B). These were the only two environmental variables to enter the RDA model, and the only taxa to show significant relationships.

Nitrogen is shown as an important environmental factor along with water temperature in regulating MC concentrations both directly and indirectly. However, congener composition was influenced directly by species composition and indirectly by N, through its influence on community structure.

2.5. Discussion

Several studies have attempted to understand how environmental factors affect toxic cyanobacteria dominance and total microcystin in freshwaters (Giani et al., 2005; Donald et al., 2011), but few have described the phytoplankton community dynamics in relation to the MC congener concentration and composition. In this study, uni- and multivariate analyses suggested that nitrogen and various forms of N influenced the relative biomass of different cyanobacterial species and relationships with MC concentration and composition. There was no direct effect of environmental variables on MC congener composition but only one mediated through species composition.

2.5.1. Cyanobacteria biomass and composition in response to environmental factors

Cyanobacteria dominated the phytoplankton communities in all three eutrophic study lakes and, as anticipated (Downing et al., 2001; O'Neil et al., 2012), the highest biomass levels were encountered in the most eutrophic lake (PSF), where nitrogen and phosphorus concentrations were about eight times higher than in the other two lakes. In addition, water temperature and pH were also highest in this hypereutrophic lake. The cyanobacterial species composition varied among lakes (Table 2.2.). The most common cyanobacterial taxa were the non-nitrogen-fixing *Microcystis wesenbergii*, *Microcystis* spp., and *Planktothrix agardhii*, except in the epilimnion of Lake Bromont where heterocystous *Aphanizomenon*

flos-aquae represented a significant fraction of the total biomass. N-fixing cyanobacteria were present in all lakes given the average low measured DIN in all systems (with the exception of the metalimnion of Bromont) and represented 15-25% of the total biomass in our more eutrophic systems, Waterloo and PSF.

Our hypothesis that N influenced the cyanobacterial composition between lakes was largely supported. The multivariate models showed that relative biomass of *Microcystis* spp. and *Microcystis wesenbergii* was related to DON, whereas only the latter species appeared to respond to TN. Although it is known that phytoplankton rely mainly on DIN, DON constitutes the largest pool of fixed nitrogen in most aquatic systems (Berman and Bronk, 2003) and could be an important source of N to these communities. Previous studies have shown that DON enhances cyanobacterial development (Glibert et al., 2004) and more precisely, the growth of non-heterocystous taxa (Finlay et al., 2010). The DON pool is usually composed of refractory N-containing compounds as well as more labile molecules such as urea and dissolved free amino acids (DFAA) (Bronk et al., 2007) both of which can be assimilated by cyanobacteria at relatively low cost (Flores and Herrero, 2005).

Ammonium (NH_4^+) is believed to play an important role in shaping the cyanobacterial community of some lakes (Blomqvist et al., 1994; McCarthy et al., 2009). It did not emerge as a significant variable in the multivariate model estimating cyanobacterial composition in this study. One possible explanation is that the concentrations of this N form were low and relatively invariable in these lakes, with the exception of the metalimnion in Bromont. NH_4^+ could also have been an important source of DIN to the cyanobacteria through rapid remineralization (Dugdale and Goering, 1967; McCarthy et al., 2007) in the shallow and polymictic lakes Waterloo and PSF. The PCA model however did suggested

that NH_4^+ was related to both heterocystous cyanobacteria (*Aphanizomenon flos-aquae*, *Anabaena* spp.) and non-N-fixing filamentous taxa (*Planktothrix agardhii* and *Spirulina* spp.) in Lake Bromont. In contrast, dominant taxa from lakes Waterloo and PSF were not associated (*M. wesenbergii*), or negatively associated (*M. aeruginosa* and *Microcystis* spp.) to NH_4^+, suggesting that different taxa respond in different ways to the concentration of this N-species in the environment (Table 2.2.).

2.5.2. MC concentration predicted from environmental variables and species composition

Similar to other studies (Giani et al., 2005), we found that N influences MC concentration; TN concentration alone explained a significant fraction of total MC (Eq. 2.2.). However, we also observed that specific N-species (DON and NH_4^+) emerged as important environmental explanatory variables (Eq. 2.1.). Interestingly, the most elevated total MC concentrations were observed in Lake Waterloo where DON represented the highest fraction of the total N pool among systems. We typically observed higher MC concentration in relatively more NH_4^+-depleted and DON-replete waters (Waterloo and PSF), regardless of the total cyanobacteria biomass. This suggests that synthesis of microcystins could be indirectly related to the availability of different N forms through cyanobacterial species composition (Glibert et al., 2005; Ginn et al., 2010; Beversdorf et al. 2013).

The biomass of certain species of cyanobacteria was also good predictors of MC concentration with *Microcystis* spp. explaining almost two thirds of the variance in this study (Table 2.3.). Again, other authors have linked biomass composition to MC concentration (Giani et al., 2005; Rolland et al., 2005). All the genera identified, with the

exception of *Spirulina* spp., *Aphanizomenon flos-aquae*, *Microcystis wesenbergii* and a few other rare species (data not shown) are known to be potential MC producers (Chorus and Bartram, 1999). *Microcystis aeruginosa*, known to be a high MC-producer and one of the most widespread hepatotoxic species in freshwater bodies (Long et al., 2001), was only identified in Lake Waterloo. Although *M. aeruginosa* was not related to overall total MC concentration across lakes its dominance in that particular system was associated with comparably high MC concentrations with relatively lower overall biomass. This resulted in the highest biomass specific MC content in this study (635 $\mu g \cdot g^{-1}$ in Waterloo). This biomass specific MC concentration is similar to what observed in eutrophic-hypereutrophic Canadian (Kotak and Lam, 1995) and Japanese lakes (Watanabe et al., 1992), and on the higher end of what has been reported in a large survey of German lakes (Fastner et al., 1999) for whole water samples, supporting the notion of high toxicity associated with this species. By comparison, the biomass specific MC content was lower in PSF, as this was largely diluted by the very high biomass of *Microcystis wesenbergii,* which is not known to produce toxins. *Planktothrix agardhii*, another well-known MC producer was also significantly correlated with MCs in Lake Bromont but had relatively low biomass-specific MC in comparison to what is reported in the literature (see Chorus and Bartram 1999).

The best overall model to predict MC concentration, however, combined both environmental variables and species relative biomass. *Microcystis* spp. and TN emerged as the most important variables with *A. flos-aquae* and water temperature also explaining part of the variation again supporting the potential role of both N and species composition in influencing bloom toxicity. Temperature consistently emerged as a significant variable. This

finding is not surprising as most cyanobacteria have higher growth rates in warm waters (Robarts and Zohary, 1987; Paerl and Huisman, 2008).

2.5.3. Microcystin congener composition

One of the main goals and original aspects of this study was to relate MC congener composition to environmental variables and species composition. No environmental variables emerged as predictive variables to describe MC congener type and concentration, but species composition and their biomass did. What was more of a surprise, however, was that, other than *M. aeruginosa,* the species significantly associated with MC congeners were two taxa not normally associated with MC production: *Aphanizomenon flos-aquae* and *Microcystis wesenbergii.* The possibility that these species could also include toxic strains cannot be excluded (Yasuno et al. 1998, Rantala et al., 2004). A study using both molecular and chemical approaches, demonstrated that *M. wesenbergii* was not a MC-producing species in Chinese waters (Xu et al., 2008) although it was often the dominant species in hepatotoxic blooms. This observation has been supported by other studies in both European (Via-Ordorika et al., 2004) and Japanese lakes (Otsuka et al., 1999). Our results do not indicate that these species are producing microcystins. However, the RDA models suggest that the main explanation for the differences in MC congener composition observed in this study was cyanobacterial species composition where *Aphanizomenon flos-aquae* was related to MC-YR in Bromont*, Microcystis wesenbergii* with MC-LA in lake PSF and MC-RR with *M. aeruginosa* in lake Waterloo. Thus, the presence and biomass of toxic and non-toxic cyanobacteria taxa affects the overall toxicity, and the community structure also affects the specific congeners produced.

Despite a non-association of congener composition with environmental variables, N forms influenced the cyanobacterial community composition and therefore indirectly influenced the congener type synthesized (Fig. 2.5B). There is a growing amount of evidence, at multiple scales of inquiry, supporting the potential influence of N and N speciation in contributing to the presence and toxicity of cyanobacteria. In a large-scale latitudinal study, both temperature and N were the main explanatory variables for cyanobacterial dominance in shallow lakes (Kosten et al., 2012). Our study agrees with this pattern and supports both field and laboratory experiments that have related MC concentration to nitrogen (Lee et al., 2000; Dolman et al., 2012; Beversdorf et al., 2013) and water temperature (Davis et al., 2009; Paerl and Huisman, 2009; Tillmanns and Pick, 2011). This study also supports others (Giani et al., 2005; Rolland et al., 2005) where the presence/absence and the biomass of toxigenic taxa constituted the main explanation for the observed changes in bloom toxicity.

2.6. Acknowledgements

We would like to thank Stéphanie Massé, Supriya Tandan, Dan Nguyen (Université de Montréal), and Katherine Velghe (McGill University) for field assistance and Arthur Zastepa (University of Ottawa) for help with the microcystin analyses. We extend a special thank to Morgan Botrel (Université de Montréal) for her coordination of sampling, nutrients analyses and for statistical advice and to Linda Ley (University of Ottawa) for phytoplankton identification and counts. Funding was provided by a *Fonds de Recherche Québécois sur la Nature et les Technologies* FQRNT-Team grant to RM, BB and FP and the *Groupe de Recherche Interuniversitaire en Limnologie et Écologie Aquatique* (GRIL).

Table 2.1. Morphological and physical characteristics for the three study lakes.

Lake	Bromont	Waterloo	Petit St-François
Mean depth (m)	4.0	2.7	1.1
Maximum depth (m)	7.5	4.8	1.8
Water temperature range (˚C)	14.8-23.1 (epi) 10.6-14.6 (meta)	15.9 - 24.7	15.1 - 27.8
Watershed area (km^2)	24.8	28.7	19.5
Lake surface area (km^2)	0.46	1.47	0.87
Lake volume (km^2)	$1.87 \cdot 10^6$	$4.03 \cdot 10^6$	$9.74 \cdot 10^5$
Water residence time (days)	39	116	22
Population density ($ind \cdot km^{-2}$)	35	157	54
Forested area (%)	72.6	65.9	22.6
Agricultural area (%)	6.7	10.1	47.9

epi: epilimnion

meta: metalimnion

Table 2.2. Average biomass (Biomass $\mu g \cdot L^{-1}$) and mean percent of total cyanobacteria biomass (% cyano) of the dominant cyanobacterial taxa observed in the three study lakes across all sampling dates

Mean species biomass	Bromont epilimnion		Bromont metalimnion		Waterloo		Petit St François	
$\mu g \cdot L^{-1}$	Biomass	% cyano	Biomass	% cyano	Biomass	% cyano	Biomass	% cyano
*Anabaena crassa**	1.38	<0.1	ND	-	218.31	6.1	298.15	1.5
*Anabaena planktonica**	206.93	9.1	253.32	3.3	69.88	1.9	ND	-
*Anabaena smithii**	222.31	9.8	550.46	7.1	ND	-	ND	-
*Anabaena spiroïdes**	ND	-	ND	-	660.29	18.3	ND	-
*Aphanizomenon flos-aquae** †	491.07	65.6	1,081.03	14.0	25.96	0.7	2,497.76	12.2
Microcystis aeruginosa	ND	-	ND	-	900.42	25.0	ND	-
Microcystis spp.	6.25	0.3	27.05	0.4	1,706.14	47.4	5,403.46	26.3
Microcystis wesenbergii†	ND	-	ND	-	20.76	0.6	12,363.86	60.1
Planktothrix agardhii	342.61	15.1	5,700.24	73.7	ND	-	ND	-
Spirulina spp.†	2.16	0.1	120.12	1.6	ND	-	ND	-

notes: ND, not detected; Rare species with low biomass that were observed <3 times are not listed here and were not used in the statistical analyses. Species marked with * are potentially nitrogen fixing, and those with † are considered non MC-producing.

Table 2.3. Relationships between log MC ($\mu g \cdot L^{-1}$) and limnological variables and cyanobacterial species biomass for the overall study period (n=24). Regression coefficient, R^2; p-values, *p*; not statistically significant, NS.

Variable	Regression model function	R^2	*p*
TP	= -2.23+0.44 $\log_{10}$(TP)	0.33	0.002
SRP	NS	-	-
TN	= -3.33+0.43 $\log_{10}$(TN)	0.19	0.02
NO_3^-+NO_2^-	= -0.14-0.17 ln(NO_3^-+NO_2^-+1)	0.17	0.03
NH_4^+	NS	-	-
DON	= -4.98+0.80 $\log_{10}$(DON)	0.40	<0.001
Light extinction coefficient (*k*)	= -0.83+0.63(k)	0.35	0.001
Water temperature (t˚)	= -2.03=0.08(t˚)	0.28	0.004
Anabaena crassa	= -1.41+0.23 ln(*A. crassa*+1)	0.13	0.05
Anabaena planktonica	NS	-	-
Anabaena spiroïdes	NS	-	-
Aphanizomenon flos-aquae	NS	-	-
Microcystis aeruginosa	NS	-	-
Microcystis wesenbergii	= -1.70+0.20 ln(*M. wesenbergii*+1)	0.31	<0.001
Microcystis spp.	= -2.63+0.32 ln(*M. spp.* +1)	0.63	0.0001
Planktothrix agardhii	= -0.64-0.19 ln(*P. agardhii*+1)	0.17	0.02

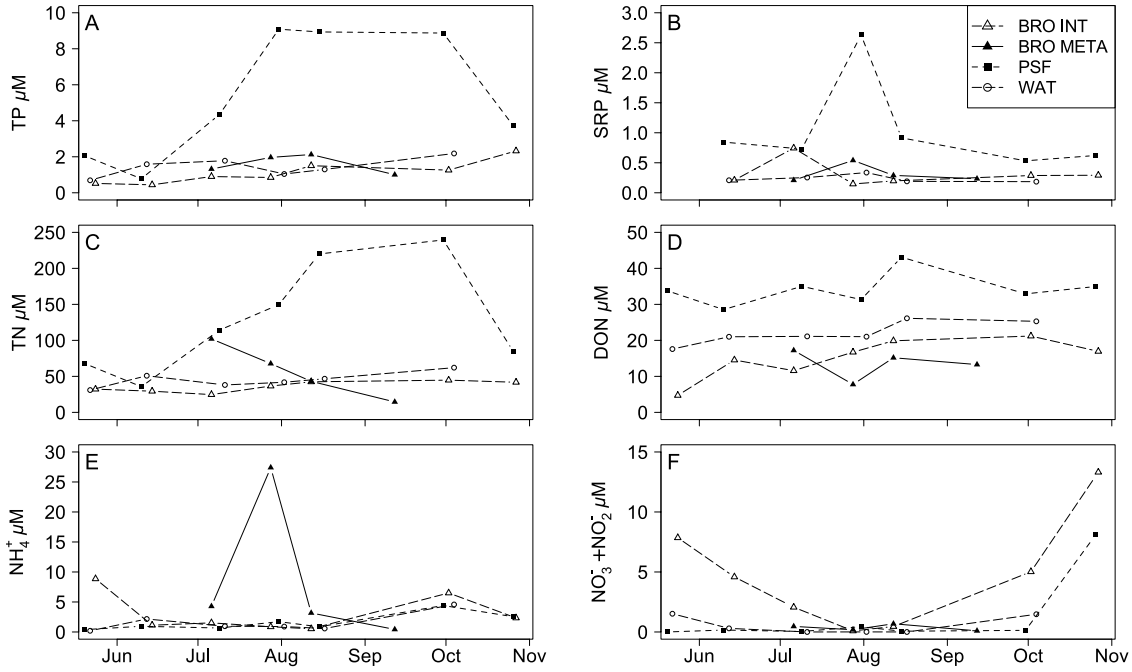

Figure 2.1. Concentration of nutrients A) TP, B) SRP, C) TN, D) DON, E) NH_4^+, and F) NO_3^-+NO_2^- in μM from April to October for the three study lakes. BRO INT are integrated samples from the epilimnion of lake Bromont; BRO META is from metalimnion of lake Bromont; PSF is the integrated sample from lake Petit St François; WAT, lake Waterloo.

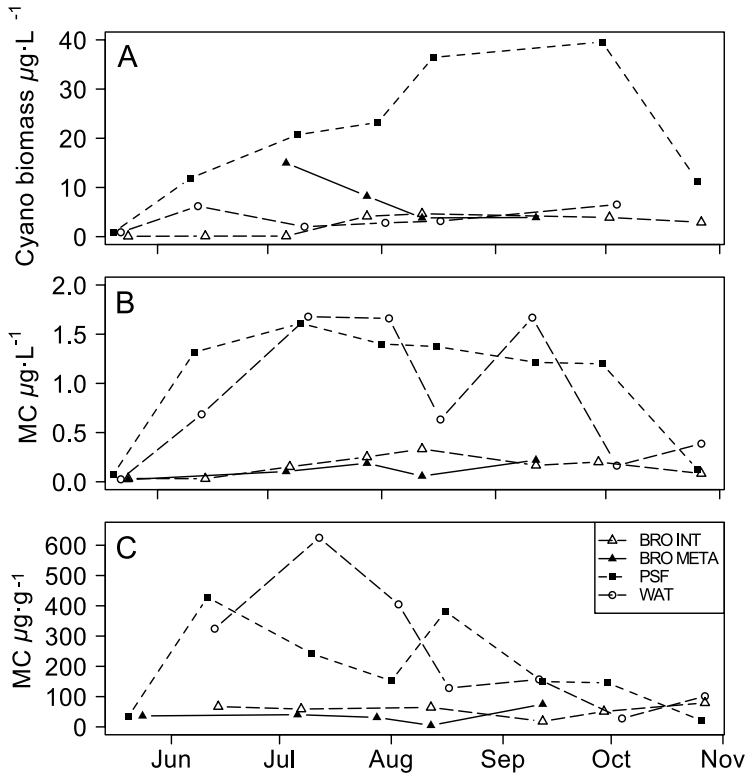

Figure 2.2. A) Cyanobacterial biomass ($\mu g \cdot L^{-1}$); and evolution of total microcystin concentration expressed as B) $\mu g \cdot L^{-1}$ and C) $\mu g \cdot g^{-1}$ dry weight from May to October in the three study lakes.

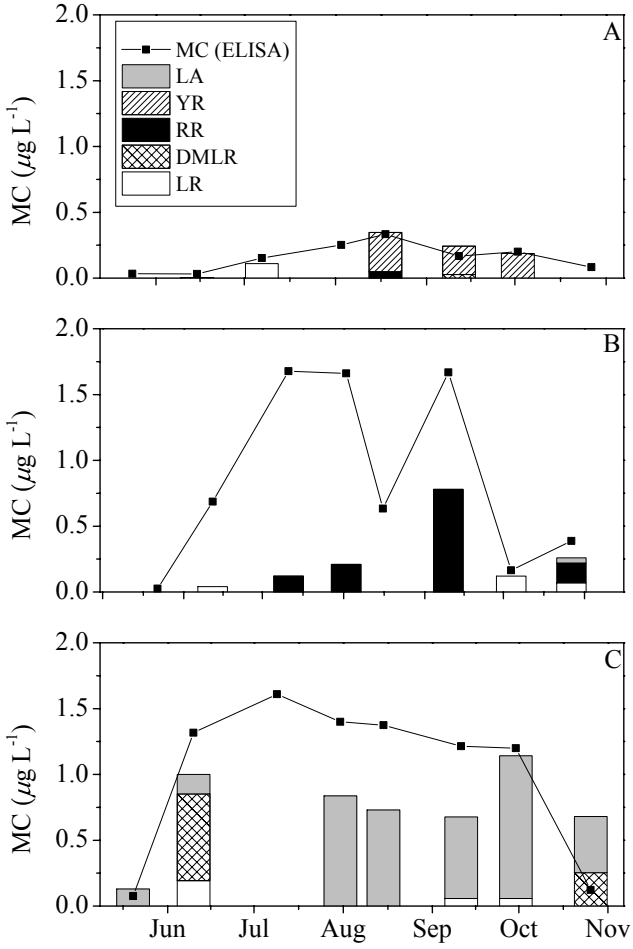

Figure 2.3. Temporal variation of total MC concentration measured by ELISA (full squares) and MC variants measured by HPLC-UV (stacked bars), from May to October in lakes A) Bromont, B) Waterloo, and C) Petit St François.

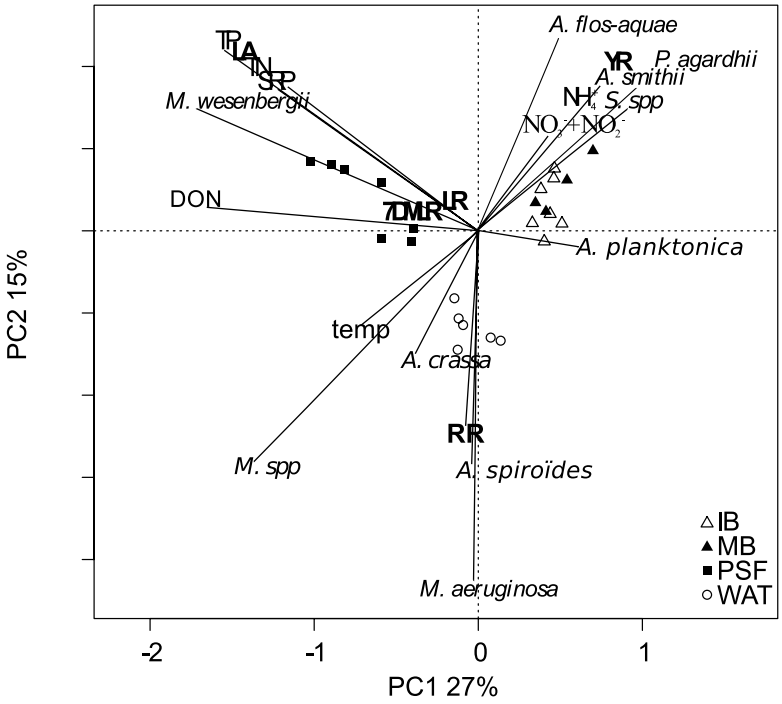

Figure 2.4. Principal component analysis (PCA) (scaling 1) of environmental variables, cyanobacteria species and microcystin congeners. The length of a vector is proportional to the importance of this descriptor to the sites. (IB: Bromont integrated epilimnion, MB: Bromont metalimnion, PSF: Petit St François, WAT: Waterloo). Cyanobacterial species are in italic, MC variants are in bold.

Legend

Code	*Species*
A. crassa	*Anabaena crassa*
A. planktonica	*Anabaena planktonica*
A. smithii	*Anabaena smithii*
A. spiroïdes	*Anabaena spiroïdes*
A. flos-aquae	*Aphanizomenon flos-aquae*
M. aeruginosa	*Microcystis aeruginosa*
M. wesenbergii	*Microcystis wesenbergii*
M. spp.	*Microcystis* spp.
P. agardhii	*Planktothrix agardhii*
S. spp.	*Spirulina* spp.

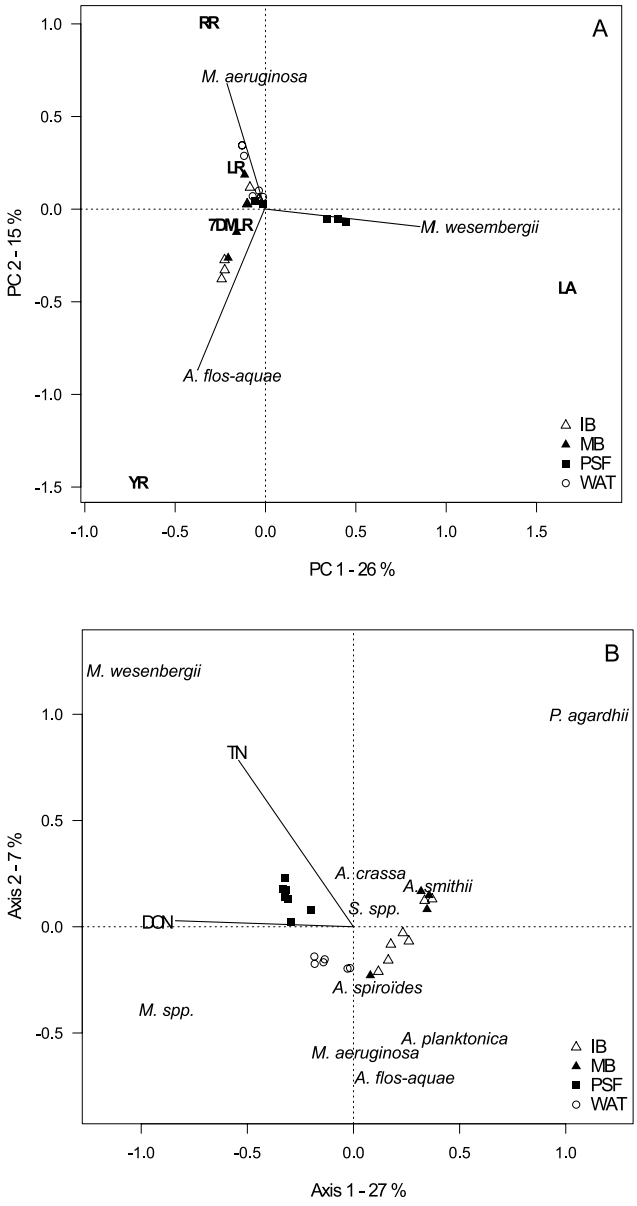

Figure 2.5. RDA performed with forward selection by permutation (n-perm=999) on A) Species relative biomass as explanatory variables for MC congener composition ($R^2_{adj.}$ =0.39, p=0.001). *Microcystis aeruginosa, Microcystis wesenbergii and Aphanizomenon flos-aquae* combined explain 39% of the variation in MC congener concentration; and B) environmental factors as explanatory variables for cyanobacterial species biomass. ($R^2_{adj.}$=0.28, p=0.001) where combined TN and DON explain 28% of the variation in species composition.

Chapitre 3 : Diversity and activity of diazotrophs in lakes with episodes of hepatotoxic cyanobacterial blooms

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Article en préparation pour publication dans *Journal of Applied and Environmental Microbiology*

Abstract:

In this study of three lakes dominated by cyanobacteria, we examined the diversity of the diazotrophic and toxic populations of cyanobacteria by targeting two functional genes, *nifH* (nitrogen-fixation), and *mcyE* (microcystin synthesis). The presence and expression of *nifH* in 28 integrated water samples collected from May to October 2010 were examined using polymerase chain reaction (PCR) and reverse transcription PCR (RT-PCR). Phytoplankton was identified, and counted along with the heterocysts of cyanobacteria using microscopy. DNA samples were selected from each lake for high-throughput sequencing to assess the diversity of nitrogen-fixing and microcystin-producing species. Microcystins were detected at all times, and *mcyE* was detected in a majority of samples throughout the sampling season. High-throughput sequencing revealed high similarity (97-99%) between our *mcyE* sequence reads and toxic strains of cultured *Microcystis* sp. and *Planktothrix* sp. *NifH* was also successfully amplified from all DNA samples. Interestingly, no *nifH* transcripts were detected by RT-PCR in any of the study lakes, indicating that the potentially N_2-fixing organisms were not active at the time of sampling, even when inorganic nitrogen concentrations were low and high heterocyst densities suggested N_2-fixation activity. The diversity of diazotrophs varied in time and among lakes. Surprisingly, most *nifH* sequence reads corresponded to proteobacterial diazotrophs, whereas only few cyanobacterial sequences were detected with the exception of two dates in the season in the two least eutrophic lakes where cyanobacterial *nifH* sequences were dominant (90%). Although N_2-fixation and

microcystin synthesis might co-occur at the community level, they apparently did not in this study.

3.1. Introduction

Eutrophication of freshwater bodies worldwide has accelerated in the past decades mainly due to anthropogenic activities (Schindler, 2001; Paerl and Huisman, 2009). Over-enrichment of lakes and reservoirs with nitrogen (N) and phosphorus (P) tend to favour cyanobacteria dominance over eukaryotic phytoplankton (Schindler, 1974; Watson et al., 1997; Kosten et al., 2012). Aquatic cyanobacteria are photoautotrophic prokaryotes known for two important characteristics. First, several common taxa (e.g. *Microcystis, Anabaena, Planktothrix, Lyngbya. Oscillatoria*; Chorus and Bartram, 1999; Cronberg and Annadotter, 2006) can produce toxigenic compounds known as cyanotoxins that are harmful to mammals (Carmichael, 1994; Chorus and Bartram, 1999). Second, a restricted number of cyanobacteria have the capacity to fix atmospheric N_2 under N-limiting conditions. This function can also be performed by other autotrophic and heterotrophic prokaryotes including taxa from the *Archaea*, spirochetes, *alpha- beta-* and *gamma-proteobacteria* (Short and Zehr, 2005). Some strains of cyanobacteria can potentially perform both functions (e.g. *Anabaena*); others only carry out one of these functions or none. It is known that toxic and non-toxic strains can coexist in freshwater populations (Kurmayer et al., 2002; Via-Ordorika et al., 2004). Cyanobacteria as a group have been extremely well studied, however, the distribution of populations from a functional trait perspective has only recently been addressed (Dokulil, M., Teubner, K., 2000; Dolman et al., 2012).

The microcystins (MCs), a family of hepatotoxic cyclic peptides, are the most common group of cyanotoxin produced in freshwaters (Chorus and Bartram, 1999). Among the MC-producing taxa, *Microcystis*, *Planktothrix* and *Anabaena* are most frequently observed in lakes worldwide (Chorus and Bartram, 1999). Toxic and non-toxic strains are impossible to differentiate visually, thus molecular tools can be used to assess the presence of toxic strains in natural waters. The *mcyE* gene, part of the *mcyA-J* gene cluster responsible for the transcription of the microcystin synthetase enzyme, may be used to assess the presence of MC-producing strains in natural cyanobacterial communities (Rantala et al., 2004). The *mcy* gene cluster is thought to be constitutively expressed, (but see Wood et al., 2011). The factors controlling MC synthesis are numerous, although recent studies have demonstrated in natural environments that nitrogen source may play a key role in controlling MC concentration in freshwater lakes (Donald et al., 2011; Dolman et al., 2012; Chapter 2).

Another characteristic of cyanobacteria is the capacity for N_2-fixation in some species, made possible by the nitrogenase enzyme complex encoded by the *nifHDK* operon. The *nifH* gene encodes the di-nitrogenase reductase sub-unit of the enzyme complex and is often used as a marker for N_2-fixation (Vintila and El-Shehawy, 2007). In contrast to *mcy*, it is not constitutively expressed, in other words its expression is under environmental control and function of cell needs. Under low combined-nitrogen conditions, Nostocales filamentous cyanobacteria produce heterocysts (Fogg, 1949), specialized cells for N_2-fixation where the nitrogenase enzyme is protected from oxygen. Other cyanobacteria (e.g. *Lyngbya*) and some bacteria can fix N_2 directly in their

vegetative cells without differentiating heterocysts but always under anaerobic conditions (Kirchman, 2008). The NtcA protein is a general transcription factor involved in nitrogen assimilation in cyanobacteria that is required for heterocysts differentiation (Flores and Herrero, 2005). In a recent study, NtcA has also been shown to act as a transcription factor for the microcystin synthetase genes (Ginn et al., 2010).

Along with phosphorus (P), nitrogen (N) availability in lakes contributes to structure the phytoplankton communities (Watson et al., 1997; Smith, 2006). Diazotrophic bacteria potentially increase the level of dissolved inorganic nitrogen (DIN) available for phytoplankton (Horne and Fogg, 1970), thus affecting community composition and ecosystem functioning. However, the contribution of N_2-fixation to the total nitrogen (TN) pool in freshwaters has scarcely been studied (Horne and Fogg, 1970; Howarth, 1988a). The influence of nitrogen on MC synthesis in toxic strains of cyanobacteria has been suggested at the molecular level (Ginn et al., 2010). The influence of N on both the concentration of MC and the cyanobacterial community composition has also been validated in field studies conducted in Canadian (Rolland et al., 2005; Donald et al., 2011; Chapter 2) and European lakes (Dolman et al., 2012; Kosten et al., 2012). Cyanobacteria preferentially assimilate inorganic nitrogen under reduced forms because they require less energy to transform into intracellular ammonium (NH_4^+; Flores and Herrero, 2005). Planktonic N_2-fixation by cyanobacteria is thought to be important in the N budget of eutrophic lakes (Howarth et al., 1988a) but yet little is known about the diversity of lacustrine diazotrophs and their importance to freshwater phytoplankton communities.

The main objective of this study was to characterize cyanobacterial populations in three eutrophic lakes dominated by cyanobacteria, and determine the occurrence of nitrogen fixation and microcystin synthesis in these communities. We hypothesized that diazotrophic cyanobacteria were responsible for newly fixed nitrogen in these lakes mostly depleted in DIN, contributing to sustain the growth of non-diazotrophic taxa, including that of toxic strains. To our knowledge, The relationships between N-fixation and microcystin synthesis in cyanobacterial communities has never been explored in shallow lakes. This study provides new understanding of the functional dynamics of freshwaters cyanobacterial communities in toxic bloom events.

3.2. Methods

3.2.1. Sites description and sample collection

Three eutrophic lakes located in the Eastern Townships, south-eastern Quebec (Canada) were sampled monthly or fortnightly from May to October 2010. Lake Petit François (PSF) is hypereutrophic, lake Waterloo is eutrophic and Bromont is considered meso-eutrophic according to the OECD (Organization for Economic Cooperation and Development, 1982) criteria. Sampling sites are described in details in Monchamp et al., 2012; Chapter 2).

All samples were collected at the deepest point toward the center of lake. Whole water samples for phytoplankton identification, enumeration, and heterocyst counts were preserved with Lugol's iodine in 125 mL flint glass bottles in the field. Samples were kept in the dark at 4˚C until analyses. For microcystin analysis, between 100 mL and 1000 mL of water was filtered gently using a peristaltic pump (Masterflex L/S 7519-06,

Cole-Parmer) at 80-100 $mL \cdot s^{-1}$ onto pre-ashed and pre-weighed 47 mm Whatman GF/C filters. Samples were immediately flash-frozen in the field and kept at -20 ˚C until analysis. Water samples were also taken to measure concentration of total phosphorus (TP), soluble reactive phosphorus (SRP), total nitrogen (TN), and several N-species (ammonium [NH_4^+], nitrite [NO_3^-], and nitrate [NO_3^-]). Sampling methods for nutrient are detailed in Monchamp et al. 2012, Chapter 2.

Water for molecular analyses was filtered in the field as soon as possible to prevent degradation of gene material. Samples were treated with care to avoid DNA or RNA contamination. Work surfaces and instruments were cleaned with RNAse/DNAse-free solution and rinsed with RNAse/DNAse-free water (Sigma Aldrich). Between 250 ml and 1000 ml of water was filtered gently using a peristaltic pump (Masterflex L/S 7519-06, Cole-Parmer) at 80-100 $ml \cdot s^{-1}$ through pre-ashed 47 mm Whatman GF/C Glass Fiber 1.2 μm pore size filters. Filters were then individually folded into 2 ml Cryovials and immediately flash-frozen in liquid nitrogen. Samples for RNA analysis were additionally treated with 1 ml Trizol reagent (Sigma Aldrich) prior to flash-freezing for preservation. Back to the laboratory, samples were stored at -80 ˚C until analysis.

3.2.2. Phytoplankton, nutrients and microcystin analyses

Total biomass and diversity of microalgae was estimated from counts of cells greater than 2 μm using a Zeiss AXIO A1 inverted microscope at X200 and X400 magnification (method detailed in Monchamp et al. 2012, Chapter 2). Heterocysts counts were performed simultaneously.

Concentrations of total phosphorus (TP), soluble reactive phosphorus (SRP), total nitrogen (TN), nitrate and nitrite (NO_3^-+NO_2^-), dissolved organic nitrogen (DON) and ammonium (NH_4^+) were measured at each sampling time. Methods for nutrient analyses are detailed in Monchamp et al. 2012, Chapter 2. Total MC concentrations were measured by ELISA (enzyme-linked immunosorbent assay) as described in Monchamp et al., Chapter 2.

3.2.3. DNA extraction

DNA was extracted from the biomass of 100-1000 mL lake water collected on GF/C glass fibre filters (Whatman). Filters were dispersed in 250 μL of TEN (50mM Tris-HCl pH 8.0, 10 mM EDTA pH 8.0, and 150 mM NaCl) containing 250-mg glass beads (0.25–0.50 mm). Cells were lysed using a FastPrep homogenizer (MP Biomedicals, Solon, OH, USA) for two 45 second intervals at setting 4.0 and then put on ice. The homogenate was centrifuged for 15 min at 13,000×g. One microliter RNAse (10 mg/mL) was added to the supernatant, which was then extracted once with phenol–chloroform–isoamyl alcohol (25:24:1) and once with chloroform–isoamyl alcohol (24:1). DNA was precipitated by adding ammonium acetate (2 M final concentration) and two volumes of ethanol and incubated for 15 min at −20°C. After centrifugation at 13,000×g for 15 min, the DNA pellet was washed with 70% ethanol and dissolved in 50 μL water. DNA quantification and purity assessment of DNA extracts were performed using a NanoDrop ND-1000 instrument (NanoDrop Technologies inc., Wilmington, DE). Further quantification of double-stranded DNA (dsDNA) was

performed using Quant-iT^{TM} PicoGreen® dsDNA reagent. Between 20 and 100 ng·μL^{-1} dsDNA was recovered from each sample.

3.2.4. RNA extraction

For RNA extraction of samples preserved in Trizol reagent, we used the RNEasy extraction kit from Qiagen (Valencia, CA, USA) with a modified protocol from the manufacturer. Briefly, filters were cut in pieces and separated into two sterile tubes containing 200 mg glass beads 0.4–0.5 mm and Trizol reagent was added to reach a final volume of 1 mL in all tubes. Samples were homogenized with a bead beater (FastPrep-24, MP Biomedicals) for two 20 seconds intervals and let to stand for 5 minutes on ice. Two hundred μL of chloroform were added and samples were centrifuged at 12 000×g for 15 min at 4˚C. The remaining steps were performed according to the Qiagen RNA-mini kit protocol. After extraction, we performed a final step of DNase Treatment (Ambion, Austin, TX, USA) on the RNA extracts. After extraction, RNA was visualised on a 1% agarose gel stained with ethidium bromyde.

3.2.5. PCR and RT-PCR amplification

NifH- Nested PCR amplification was performed on the *nifH* gene to assess diazotrophic diversity among prokaryotic organisms. The first round of PCR was done using the pair of primers nifH4; (5'-TTYTAYGGNAARGGNGG-3') and nifH3; (5'-ATRTTRTTNGCNGCRTA-3') that were designed based on conserved sequences outside of nifH1 and nifH2 (Zani et al. 2000). Each 25 μL PCR reaction contained 2.5 μL Taq Buffer (10X), 0.5 μL BSA (20 μg·μL^{-1}), 0.5 μL dNTPs (10 mM), 1.0 μL of each *nifH* primer (10 mM), and 0.25 μL Taq Polymerase (5U). The PCR was carried out

with 35 cycle of denaturation at 94˚C for 0.5 min., 47˚C (NifH3 and NifH4) or 57˚C (NifH1 and NifH2) for 0.5 min., and 72˚C for 1 min. The other pair of degenerate oligonucleotide PCR primers NifH1; (5'-TGYGAYCCNAARGCNGA-3'), and nifH2; (5'-ADNGCCATCATYTCNCC-3') (Zehr and McReynolds 1989) was used for the second round of the nested PCR to amplify an approximately 359-bp region of the *nifH* gene inside the first segment. The second round of the nested PCR was performed with 1 μL of the first round product in the same mixture. Positive control for both *nifH* DNA and RNA nested PCR consisted of *Anabaena flos-aquae* strain CPCC 67 maintained in a mix of BG11 and BG11o (without NO_3^-) medium. Cultures were kept at room temperature under a 12:12 diel cycle.

For RT-PCR, we used the Qiagen OneStep RT-PCR kit (#210210) according to the manufacturer's manual. RT-PCR amplification of the *nifH* gene was performed using the same protocol and primers as for the nested PCR amplification of *nifH* described in this section, with addition of a reverse transcriptase step using the following protocol : 50˚C for 30 min (transcription (RT)); 50˚C for 10 seconds (waiting); 95˚C for 15 min (Taq polymerase activation/reverse-transcriptase inactivation) followed by 35 cycles of denaturation as described for the *nifH* nested-PCR amplification in the previous section. Positive and negative controls were added to check for DNA contamination, and for the transcription step to prevent false positive results.

McyE - PCR amplification of the *mcyE* gene was performed using the set of primers mcyE-F2 (5'-GAAATTTGTGTAGAAGGTTGC-3') and mcyE-R4 (5'-AATTCTAAAGCCCAAAGACG-3') (Rantala et al., 2004) with the following

program : 95˚C for 3 min., 35 cycle of denaturation at 94˚C for 0.5 min., 56˚C for 0.5 min., 72˚C for 0.5 min, and 72˚C for 10 min.

16S - For the amplification of the 16S rDNA gene sequences, primers 27f (5'-AGAGTTTGATYMTGGCTCAG-3') and 534r (5'-ATTACCGCGGCTGCTGG-3') (Auclair et al., 2010) were used to amplify a 510-bp segment with the following program: 95˚C for 5 min., 55˚C for 5 min., 30 cycles of denaturation at 72˚C for 0.75 min., 94˚C for 0.75 min., 55˚C for 0.75 min., and a final step at 72˚C for 10 min.

3.2.6. Pyrosequencing

The *16S* rDNA, *nifH* and *mcyE* genes were amplified, purified and sequenced using the 454 FLX Genome Sequencer system (Roche Diagnostics, Indianapolis, IN, USA) at Research and Testing Laboratories (LLT, TX, USA). We provided 10 *µL* DNA extracts from two samples from each lake to reveal the overall bacterial diversity and identify N_2-fixing and MC producing cyanobacteria. One sample from early season and one from late season were selected for each lake in order to examine community changes among and within different systems. Dates were selected based on observed microcystin concentrations in the field and quality of extracted DNA to ensure successful sequencing. Primers used to amplify the functional genes *nifH* and *mcyE* were the same as described in section 2.5. Primers 28F (5'-GAGTTTGATCNTGGCTCAG-3') and 519R (5'-GTNTTACNGCGGCKGCTG-3') were used to amplify the variable regions V1-3 of the 16S rDNA gene. After sequencing, all sequence reads were checked for anomalies and poor quality reads were removed. The remaining sequences were trimmed, aligned and checked for chimeras using Mothur software (Schloss, 2009).

Alignments were visually checked and additional alignment was done using Mafft when necessary to ensure accuracy of multiple sequence alignment (Katoh et al., 2005). Mothur was also used to construct the distance matrices and assign sequences to operational taxonomic units (OTUs) (Guo et al., 2012) using a cut-off of 97% similarity.

3.3. Results

3.3.1. Nutrients and microcystin concentration

Phosphorus and nitrogen were never limiting in the three lakes over the sampling season (Table 3.1.). Total phosphorus (TP) averages per lake ranged from 34 $\mu g \cdot L^{-1}$ in mesotrophic Lake Bromont to over 196 $\mu g \cdot L^{-1}$ in hypereutrophic Lake Petit St François. Lake Waterloo, which is considered eutrophic, had an average TP of 57 $\mu g \cdot L^{-1}$. Soluble reactive phosphorus (SRP) concentrations were always detectable and averaged at 9, 12 and 35 $\mu g \cdot L^{-1}$ following the trophic gradient of these lakes.

Total nitrogen (TN) average concentrations were high in all lakes (Table 3.1.) although the dissolved inorganic fraction (DIN) was overall low during the season, implying that these systems were at times limited by nitrogen. NO_3^- and NO_2^- together averaged at 81.93 $\mu g \cdot L^{-1}$ in the less eutrophic system, and were lower in lakes Waterloo and PSF (mean = 32.41 $\mu g \cdot L^{-1}$ and 16.44 $\mu g \cdot L^{-1}$ respectively). In all lakes, $NO_3^- + NO_2^-$ concentrations near zero were observed (Table 3.1.) during the growing season. As for NH_4^+, the average concentration was again relatively higher in Bromont (42.03 $\mu g \cdot L^{-1}$) than in the two more eutrophic lakes (23.03 and 22.33 $\mu g \cdot L^{-1}$ in Waterloo and PSF, respectively). At times, NH_4^+ concentrations were below 10 $\mu g \cdot L^{-1}$ (minimum 7.42, 2.80, and 5.46 $\mu g \cdot L^{-1}$ in Bromont, Waterloo and PSF respectively).

Microcystins were always detectable. On average, the hypereutrophic lake (PSF) had the highest MC concentration (Table 3.1.). Even though the average concentration was slightly higher in Lake PSF, MC concentration in Lake Waterloo reached a higher maximum concentration during the sampling season (Table 3.1.).

3.3.2. Phytoplankton and cyanobacteria biomass

The total biomass of phytoplankton estimated from cell counts was similar in lakes Bromont and Waterloo over the season (average 3372.39 $\mu g \cdot L^{-1}$ and 4190.24 $\mu g \cdot L^{-1}$ respectively; Fig. 3.1A-B). In lake Petit St François it was up to 5 times higher (average 23,079.85 $\mu g \cdot L^{-1}$; Fig. 3.1C) than in the other two lakes. Cyanobacteria were the dominant taxonomic group during most of the sampling season (Table 3.2.). In the epilimnion of Lake Bromont, the community was initially dominated by chrysophytes (data not shown) and cyanobacteria became dominant in late July (Fig. 3.1A). In Waterloo, *Microcystis aeruginosa* accounted for most biomass from May to September (Fig. 3.1B; Table 3.2). In Lake PSF, diatoms represented the largest biomass in May (data not shown), although from June until the end of autumn cyanobacteria became dominant (Fig. 3.1C) with *Microcystis wesenbergii* as the dominant species (Table 3.2.). From early June until the end of sampling, the biomass of phytoplankton in both lakes Waterloo and PSF comprised from 78% to 99% of cyanobacteria (Fig. 3.1B-C).

3.3.3. Heterocyst counts

With the exception of two sampling dates where no heterocysts were observed in any of the lakes (end of May and early July), all other samples from early June to end of October had heterocyst densities between 19 and 9,000 units$\cdot mL^{-1}$ (Fig. 3.1A-C).

Heterocysts in all lakes reached a maximum in late July or August when DIN concentrations were the lowest. In PSF, the seasonal average was much higher (1,935 units·mL^{-1}) than in eutrophic Lake Waterloo (average 93 units·mL^{-1}) and in Lake Bromont epi- and metalimnion (average 314 and 458 units·mL^{-1}, respectively). In the latter lake, *Anabaena planktonica* and *Aphanizomenon flos-aquae* were the main heterocystous species while in Waterloo, heterocysts were found in various *Anabaena* species (*A. spiroïdes*, *A. planktonica*, *A. crassa* and other species) and on occasion in *Aphanizomenon flos-aquae*. The latter was the main heterocystous taxa in Lake PSF throughout the sampling season, with the presence of a few heterocysts from *Anabaena* taxa from June to mid August.

3.3.4. Microscopic identification and counts

We specifically chose two dates during the sampling season, where microscopic and phylogenetic diversity could be compared. Dates were selected as a function of high differences in toxin concentration. Identification of cyanobacterial taxa by microscopy revealed a variation in the community composition in time and among lakes (Fig. 3.2A-F). In Lake Bromont, taxa from the family *Phormidiaceae* accounted for 86% of the cyanobacterial biomass in the early season while *Nostocaceae* equalled 14% of the biomass (Fig. 3.2A). However, a shift occurred in August when *Nostocaceae* became the dominant family (94%) over *Phormidiaceae* (6%). In eutrophic Lake Waterloo, the dominant taxa were mainly *Microcystaceae* for both dates (99%; Fig. 3.2C-D). Species composition in the most eutrophic lake (PSF) was similar to that of Lake Waterloo as *Microcystaceae* dominated from June (Fig. 3.2E). However, the composition changed in

August when *Microcystaceae* remained dominant, but *Nostocaceae* followed closely with 44% of the biomass (Fig. 3.2F).

3.3.5. 16S pyrosequencing

Although the number of sequence reads cannot directly be converted into the number of cells, it gives a relative abundance of the different taxa at the time of sampling. In Lake Bromont, *Phormidiaceae* accounted for 96% of the sequence reads in July (Fig. 3.3A) similarly to what observed using microscopy, whereas in September *Merismopediaceae* were dominant (40%) according to the molecular analyses (Fig. 3.3B). In Lake Waterloo, the 16S diversity based on the number of pyrosequencing reads was greater than the diversity observed microscopically in both dates (Fig. 3.3C-D**)**. Taxa grouped as "others" were either reads that could be identified but represented a small proportion of the whole or were unclassified because they did not show sufficient similarity to the sequences in the database to be formally classified. In Lake PSF, the proportions of taxa obtained with the molecular analyses were similar to those obtained via microscopy. In early season, half of sequences were classified as *Microcystaceae*, with 19% identified as *Phormidiaceae* and the rest was mostly unclassified sequences (Fig. 3.3E). In August, *Microcystaceae* accounted for 34%, and *Nostocaceae* were more important with 49% (Fig. 3.3F**)**.

3.3.6. Functional genes

Both functional genes studied were successfully amplified and sequenced in our lake water samples. However, the number of pyrosequencing reads obtained was irregular and relatively low in most samples for both *mcyE* (Annexe B, Table BI) and

nifH (Annexe B, Table BII). This can suggest a low number of genes copies, although it is impossible to assess here.

The *nifH* gene was amplified successfully from all DNA extracts (Table 3.2). However, no transcripts were detected by RT-PCR throughout the sampling season in any of the lakes, indicating that although the gene was present, it was not being expressed at detectable levels at the time of sampling (Table 3.2). OTUs were divided in classes following the BLAST search results; cyanobacteria, alpha-, beta-, delta- and gamma-proteobacteria, and unidentified N_2-fixing microorganisms (Fig.3. 4A-F). Structure of diazotrophic communities varied among lakes and temporally within lakes. In early summer, *nifH* sequences amplified from Lake Bromont's samples were almost all cyanobacterial (*Anabaena* sp., 97% of reads) whereas later in the season the diazotrophic community became mostly bacterial and dominated by gamma-proteobacteria (70%; Fig. 3.4A-B). As for Waterloo, the community composition of diazotrophs was 25% cyanobacteria (*Lyngbya* sp.; data not shown), 30% delta-proteobacteria, and 35% unidentified N_2-fixing in the early season (Fig. 3.4C). Interestingly, the latter lake became dominated by cyanobacterial sequences in the late season (92%; *Lyngbya* sp.; Fig. 3.4D). In hypereutrophic Lake PSF, no cyanobacterial *nifH* sequences were detected (Fig. 3.4E-F). Diazotrophs carrying the *nifH* gene were over 87% gamma-proteobacteria in early season whereas in late season this class accounted for 39% and over half of the reads were unidentified sequences that could have been either bacterial or cyanobacterial. This could be due to the limits of available

sequenced *nifH* genes in the database compared to the variety of strains in natural environments.

The *mcyE* gene was successfully amplified from the majority of DNA samples in the study lakes (Table 3.2). Sequences of *mcyE* reads were classified in OTUs that were subsequently searched (BLAST) in GenBank to find the highest similar cultured organism corresponding. In Lake Bromont, all of the sequence reads corresponded to either *Planktothrix* sp. or to *Microcystis* sp**.** (Fig. 3.5A). Early in the season, the majority of the reads were associated with *Planktothrix* sp. (84%), however subsequently the number of reads from *Microcystis* and *Planktothrix* spp. was very similar (45%, and 55% respectively). In the two most eutrophic lakes Waterloo and PSF, 99% of sequence reads were highly similar to *Microcystis* taxa (Fig. 3.5B-C).

3.4. Discussion

Nitrogen has previously been demonstrated to play a key role in structuring phytoplankton communities and regulating microcystin synthesis by cyanobacteria both in laboratory (Ginn et al., 2009) and field studies (Donald et al. 2011; Chapter 2). However, how toxicity may be related to N_2-fixation from a community perspective has yet to be assessed. The present work contributes to our understanding of how the different functional roles of the cyanobacteria are structured in phytoplankton communities and challenges some of the strongly held assumptions of what conditions support cyanobacterial N_2-fixation.

3.4.1. Evidence for potential diazotrophic activity

The three study lakes showed favourable conditions for the growth of cyanobacteria, especially nitrogen-fixing taxa. All lakes were rich in phosphorus, with high pH and mostly depleted in dissolved inorganic nitrogen (DIN; Table 3.1). Furthermore, moderate ratios of TN:TP were observed in all lakes (average 15, 11 and 9 in lakes Bromont, Waterloo and PSF, respectively). Low DIN concentration and TN:TP ratios (< 29:1) are thought to favour diazotrophic cyanobacteria (MacGregor et al., 2001) and trigger the formation of cyanobacterial blooms (Smith, 1983) that can offset N deficits to aquatic systems (Schindler, 1977). These findings seem to support the overall dominance in cyanobacterial biomass observed in these study lakes (Figs. 3.2 and 3.3). However, although N-fixing species were present in all systems and at each sampling date, with the exception of Bromont, the dominant cyanobacteria in terms of biomass were non-fixing taxa. Cyanobacterial communities differed among lakes and during the course of the growing season, where taxa capable of nitrogen fixation and microcystin synthesis co-existed at all times (Chapter 2).

The overall low concentrations of dissolved inorganic nitrogen (DIN) and the presence of heterocystous cyanobacteria (Chapter 2) would suggest the presence of active N_2-fixing organisms, and indeed the successful amplification of the *nifH* gene from all DNA extracts suggested that all assemblages had the potential for nitrogen fixation. A BLAST search in GenBank performed on *nifH* amplicons revealed that the N_2-fixing community composition of lakes Bromont and Waterloo was variable. Both cyanobacterial and bacterial *nifH* sequences were identified, with the exception of

Bromont in the late season when only bacterial sequences were observed (Fig. 3.4A-D). However, the low number of *nifH* sequence reads obtained by pyrosequencing in most of our samples (data not shown) and the need to proceed to a nested-PCR to visualize *nifH* amplicons in all samples both suggest an overall low number of gene copies in the environment.

Further support for the presence of potential nitrogen fixation comes from the heterocyst densities, which were relatively high throughout the sampling period in all three lakes (Fig. 3.1A-C). Heterocyst densities were highest in the hypereutrophic Lake Petit St François, in fact over seventeen fold higher compared to Waterloo whereas cyanobacterial biomass was only up to five fold higher. Surprisingly, even though heterocyst densities were highest in Lake PSF no *nifH* gene sequences were cyanobacterial (Fig. 3.4E-F).

Previous studies have found that N_2-fixation arose when heterocyst densities were greater than 2 units$\cdot mL^{-1}$, and positive relationships between N_2-fixation rates (measured with the acetylene reduction method) and heterocyst density have been reported (Levine and Lewis, 1987; Findlay and Hecky, 1994). However, in this study, no such relationship was observed. No *nifH* transcripts were detected by PCR amplification of RNA (RT-PCR), which indicated that the gene was not actively being transcribed at the time of sampling in any of the sites. Positive RNA controls were amplified successfully. Possible inhibition was also tested with 1 μl of control DNA control spiked in lake water samples. In a recent survey of the same lakes, no obvious change in isotopic signature of the particulate organic matter was observed (Botrel, 2011) nor were there any detectable

rates of N_2-fixation using isotopic tracer techniques (Maranger unpublished data). Both molecular and isotopic evidence suggest that N_2-fixation was not occurring in these systems, despite the low ambient DIN and the presence of heterocystous cyanobacteria. The negative RT-PCR results could also be due to the low number of *nifH* transcripts in the samples.

3.4.2. Diversity of diazotrophs and toxic cyanobacteria

Heterocystous N_2-fixing cyanobacteria (e.g. *Anabaena* spp., *Aphanizomenon flos-aquae*) were observed at all times in the study lakes but usually in relatively low biomass (Chapter 2). Cyanobacterial-corresponding sequences of *nifH* were only found in early season in Lake Bromont, and both times in Lake Waterloo. In the hypereutrophic Lake (PSF), there was no clear evidence that cyanobacteria were present in the diazotrophic community as no sequence reads had a close cyanobacterial relative in GenBank (Fig. 3.4E-F). This is surprising as the highest heterocysts densities were observed in the latter lake (Fig. 3.1C). Although heterocysts are specialized cells where N_2-fixation is performed, their mere presence does not conclusively support that this function is active. Nitrogen fixation is further regulated by several environmental factors such as macro and micro-nutrients concentration, oxygen, and turbulence (Howarth et al. 1988b).

Genera known to include toxigenic strains (e.g. *Microcystis* and *Planktothrix*) were present at high levels in all lakes (Chapter 2). In the two most eutrophic lakes (PSF and Waterloo), almost all *mcyE* sequence reads were highly similar to *Microcystis* sp. in GenBank (Fig. 3.5B-C). This was consistent with the microscopic enumerations. In the

meso-eutrophic Lake Bromont, *Planktothrix* sp. was the dominant taxa in terms of number of reads in the early season, however in late season the number of reads corresponding to *Microcystis* sp. was almost the same as *Planktothrix* sp. (Fig. 3.5A). However, the total number of reads obtained from Lake Bromont samples was relatively low, suggesting that the number of gene copies was probably also low in these samples (Annexe B, Table BI). Both *Microcystis* and *Planktothrix* are often the major microcystin producers in lakes (Cronberg and Annadotter, 2006) and important biomass-specific content of microcystins have been associated with the their biomass in lakes worldwide (Kotak and Lam, 1995; Fastner et al., 2001; and others).

3.4.3. Link between nitrogen fixation and microcystin synthesis in cyanobacteria

All *mcyE* sequences identified were closely related to the non-fixing taxa *Microcystis* and *Planktothrix.* Interestingly the majority of *nifH* sequences were related to non-cynaobacterial taxa dominated primarily by gammaproteobacteria. When cyanobacterial *nifH* sequences were present they were either related to *Anabaena* sp. or *Lyngbya* sp. Some strains of *Anabaena* have been shown to produce microcystins (Cronberg and Annadotter, 2006), however, we found no evidence for this in the present study as no *mcyE* sequence reads corresponded to this genus in any of the study lakes. As for *Lyngbya* sp., this species accounted for a negligible amount of biomass in our total counts in one of the lakes (Waterloo) and no strains are known to produce MCs (Cronberg and Annadotter, 2006) although other cyanotoxins may be produced (Lajeunesse et al., 2012).

3.5. Conclusion

Using molecular-based methods and high-throughput sequencing of the functional *nifH* gene coding for the nitrogenase enzyme transcription, this study showed that N_2-fixation was not a significant source of nitrogen in the three study lakes, despite high densities of heterocyst and low DIN during most of the sampling season. This is in contrast to other studies suggesting that heterocysts density can be used to infer N_2-fixation rates (Horne and Goldman, 1972; Findlay and Hecky, 1994; Gondwe et al. 2008). Pyrosequencing data showed that proteobacteria were the dominant diazotrophs in these communities. To our knowledge, this is one of the first studies to assess the diversity and activity of both diazotrophic cyanobacteria/bacteria and toxic cyanobacteria in freshwater. More studies on the assimilation of nitrogen in cyanobacteria are needed to further understand the community functioning in toxic cyanobacterial bloom events in freshwater.

3.6. Acknowledgements

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Table 3.1. Concentration ranges of nutrients, total microcystin ($\mu g \cdot L^{-1}$) and pH, over the sampling season in the three study lakes.

	Bromont epilimnion	Waterloo	Petit St François
TP ($\mu g \cdot L^{-1}$)	13.69 – 71.98	21.45 – 67.62	24.07 – 281.48
SRP ($\mu g \cdot L^{-1}$)	4.55 – 23.07	5.76 – 10.43	16.49 – 81.46
TN ($\mu g \cdot L^{-1}$)	345.66 – 626.50	433.58 – 869.40	499.80 – 3352.44
DON ($\mu g \cdot L^{-1}$)	66.08 – 296.8	246.26 – 365.82	399.68 – 603.54
$NO_3^- + NO_2^-$ ($\mu g \cdot L^{-1}$)	1.26 – 186.41	0.01 – 21.28	<0.01 – 6.16
NH_4^+ ($\mu g \cdot L^{-1}$)	7.42 – 124.04	2.80 – 64.12	5.46 – 61.74
Microcystin ($\mu g \cdot L^{-1}$)	0.03 – 0.33	0.63 – 1.68	0.08 – 1.61
pH	6.30 – 8.01	7.08 – 8.96	7.53 – 9.52

Table 3.2. Presence (+/-) of *mcyE* and *nifH* (PCR), expression (+/-) of *nifH* (RT-PCR), microscopic identification of the main toxigenic species (Main toxigenic sp.), and taxa accounting for the highest biomass (Highest biomass), are shown for each lake.

Site	Date	*mcyE* PCR	*nifH* PCR	*nifH* RT-PCR	Main toxigenic sp.	Highest biomass
Bromont	24 May	+	+	-	*Planktothrix agardhii*	*Planktothrix agardhii*
epilimnion	14 June	+	+	-	*Anabaena planktonica*	*Anabaena planktonica*
	7 July	+	+	-	*Planktothrix agardhii*	*Planktothrix agardhii*
	28 July	+	+	-	*Anabaena planktonica*	*Aphanizomenon flos-aquae*
	12 August	+	+	-	*Planktothrix agardhii*	*Aphanizomenon flos-aquae*
	12 Sept.	+	+	-	*Anabaena planktonica & Planktothrix agardhii*	*Aphanizomenon flos-aquae*
	4 Oct	+	+	-	*Planktothrix agardhii*	*Aphanizomenon flos-aquae*
	12 Sept	+	+	-	*Anabaena planktonica*	*Aphanizomenon flos-aquae*
Waterloo	22 May	+	+	-	*Microcystis aeruginosa*	*Microcystis aeruginosa*
	13 June	+	+	-	*Anabaena spiroïdes & Microcystis aeruginosa*	*Anabaena spiroïdes & Microcystis aeruginosa*
	12 July	+	+	-	*Microcystis spp.*	*Microcystis spp.*
	3 August	+	+	-	*Microcystis spp.*	*Microcystis spp.*
	17 August	+	+	-	*Microcystis spp.*	*Microcystis spp.*
	11 Sept	+	+	-	*Microcystis spp.*	*Microcystis spp.*
Petit St	20 May	-	+	-	*Microcystis spp.*	*Microcystis wesenbergii*
François	**11 June**	+	+	-	*Microcystis spp.*	*Microcystis spp.*
	9 July	-	+	-	*Microcystis spp.*	*Microcystis wesenbergii*
	1 August	+	+	-	*Microcystis spp.*	*Microcystis wesenbergii*
	16 August	+	+	-	*Microcystis spp.*	*Microcystis wesenbergii*
	19 Sept	+	+	-	*Microcystis spp.*	*Microcystis wesenbergii*
	30 Sept	-	+	-	*Microcystis spp.*	*Microcystis wesenbergii*

note: MB 6-07, DNA under detection limit; *na*: not applicable Dates in bold are those chosen for pyrosequencing based on observed differences in toxin concentration and on the quality of DNA.

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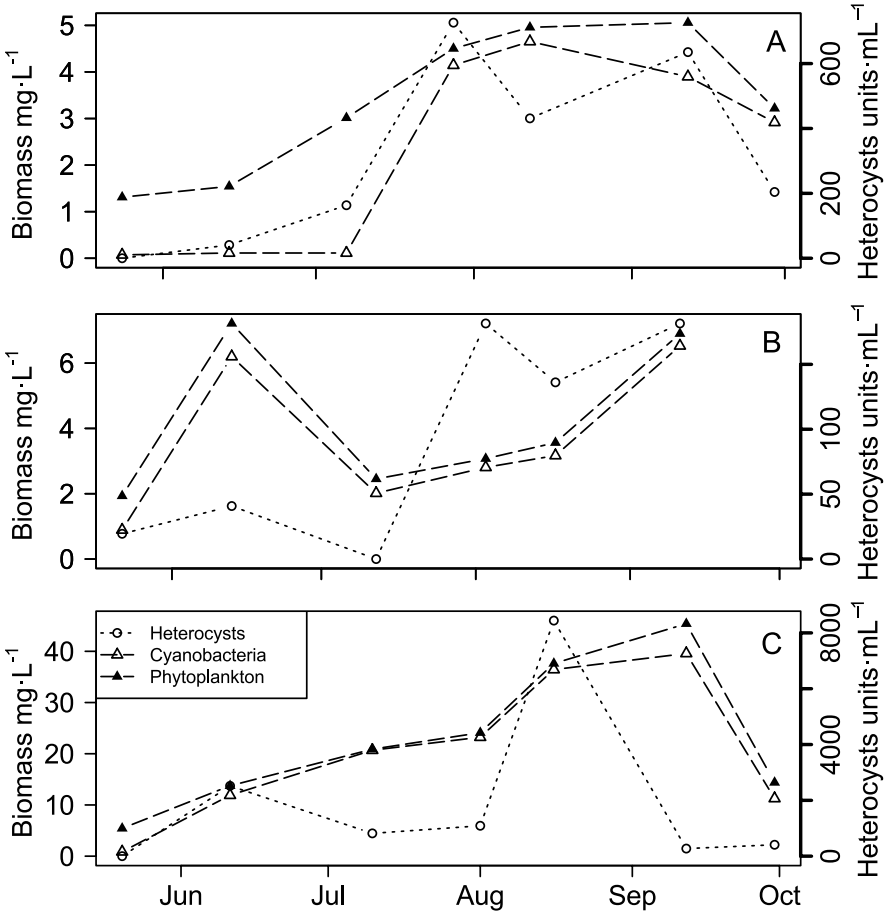

Figure 3.1. Heterocyst counts (units·mL^{-1}), phytoplankton total biomass (mg·L^{-1}), and cyanobacteria biomass (mg·L^{-1}) in integrated water samples over the sampling season of A) Lake Bromont, B) Lake Waterloo, and C) Lake Petit St François.

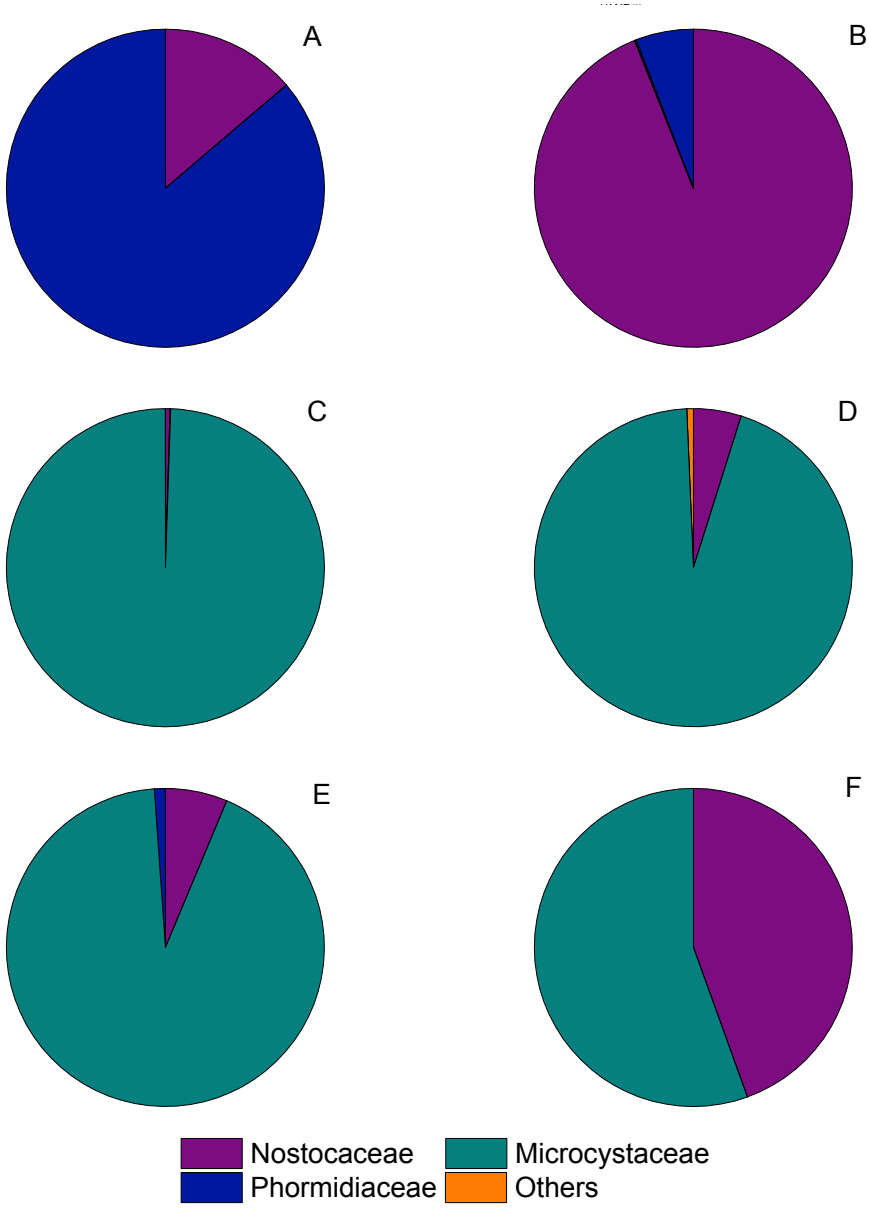

Figure 3.2. Variation in the cyanobacterial diversity at the family level using percentage of microscopic counts for Bromont A) early and B) late season; Waterloo C) early and D) late season; Petit St François E) early and F) late season are shown.

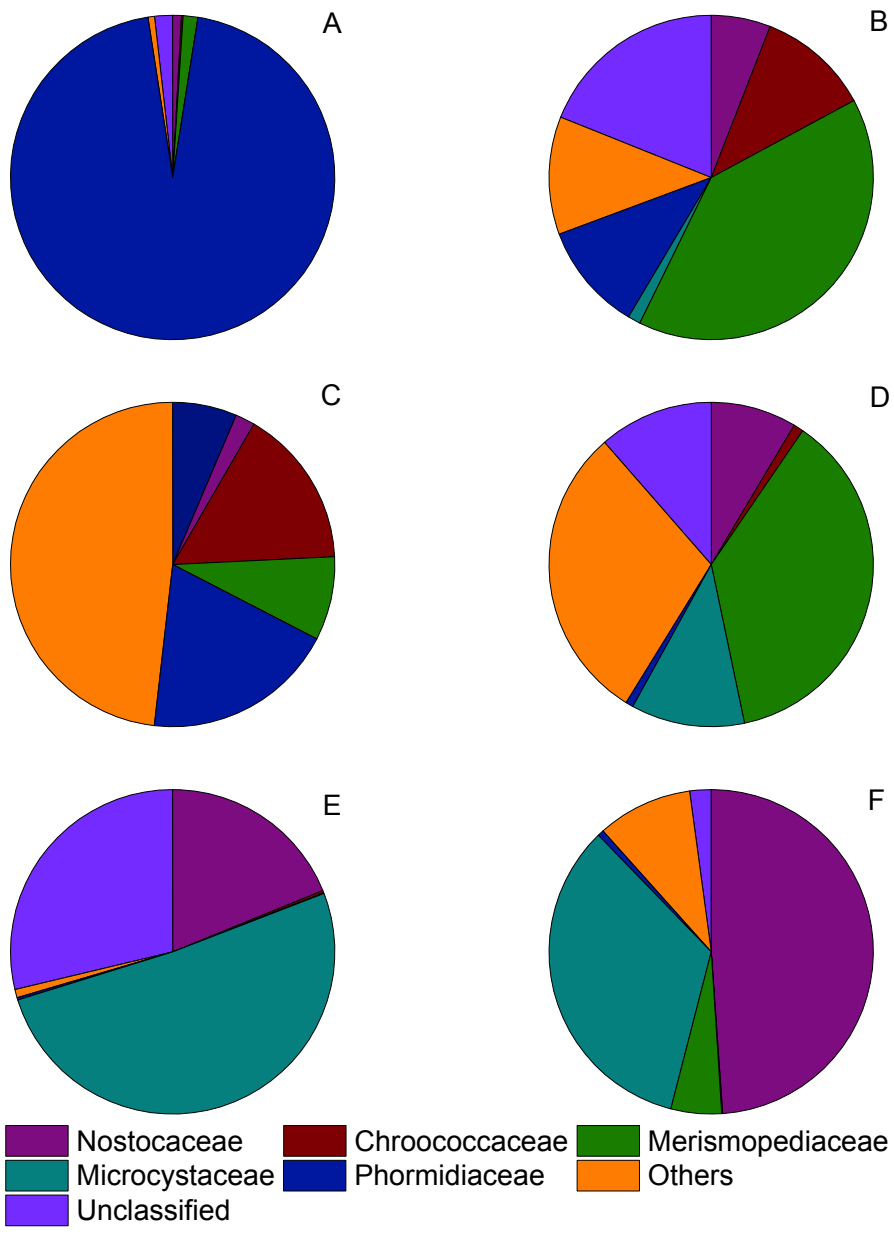

Figure 3.3. Variation in the cyanobacterial diversity at the family level using percentage of sequence reads from pyrosequencing of the 16SrDNA gene for Bromont A) early and B) late season; Waterloo C) early and D) late season; and Petit St François E) early and F) late season are shown

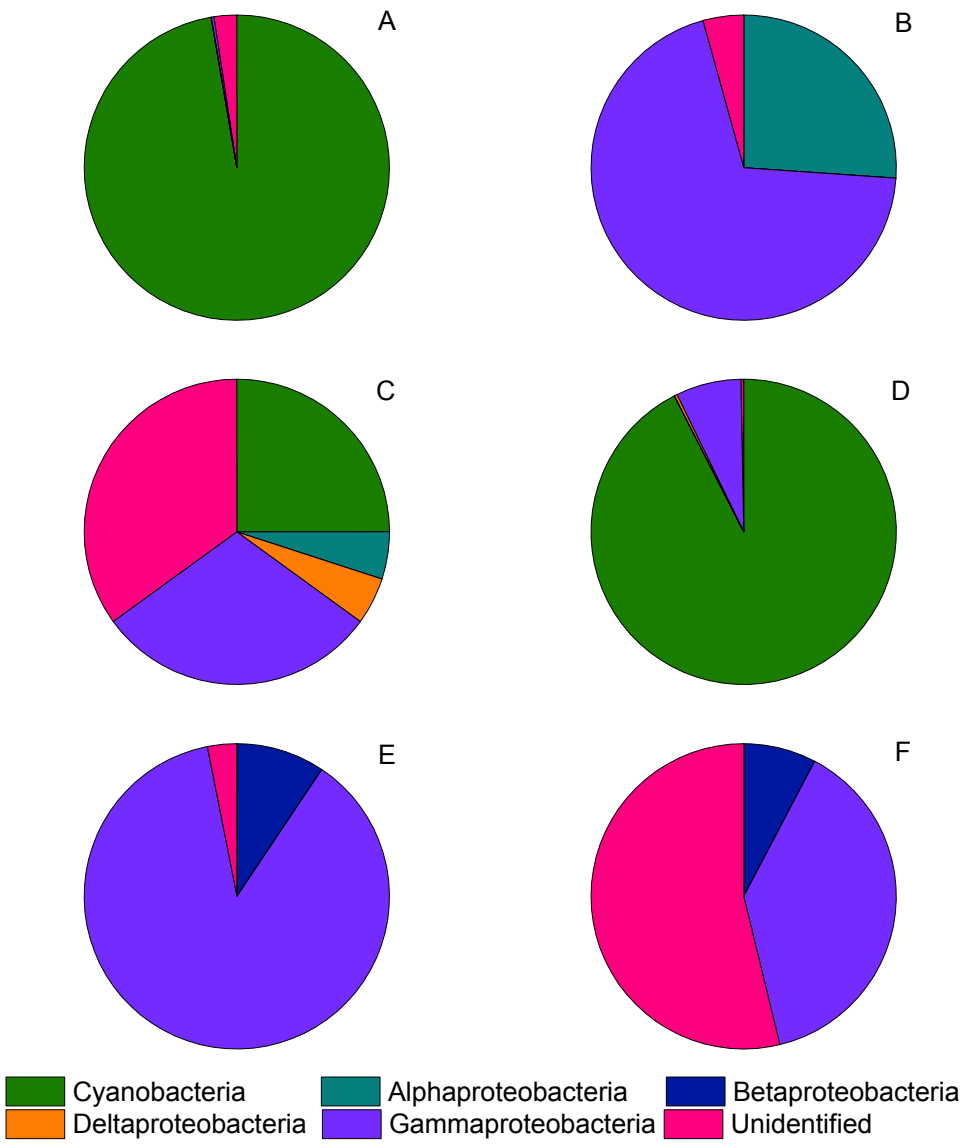

Figure 3.4. Relative diversity of the *nifH* gene at the class level based on the reads number classified in OTUs (cut-off 97%) in lakes A) Bromont, B) Waterloo, C) Petit St François, are shown for early season and late season. The OTUs were classified as bacterial, cyanobacterial or unidentified.

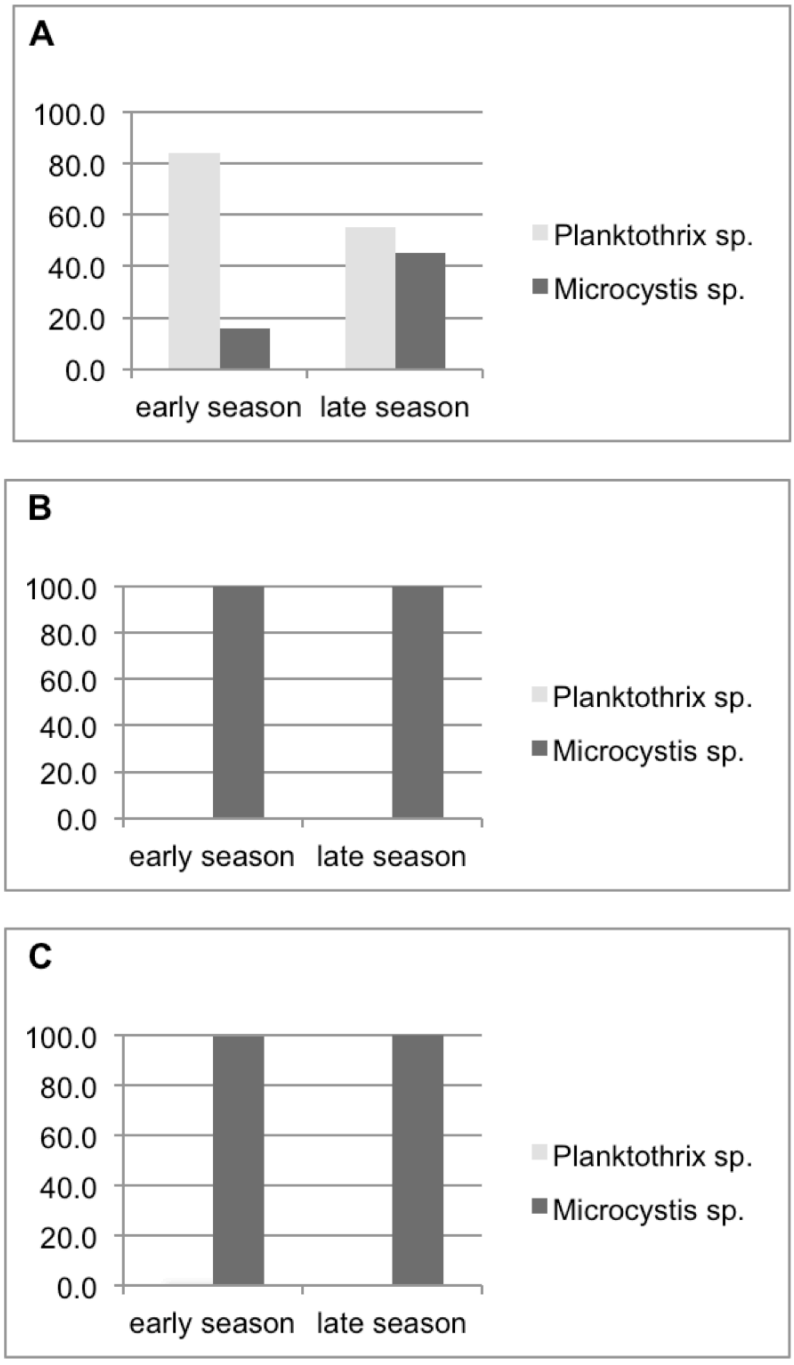

Figure 3.5. Relative diversity of the *mcyE* gene based on the reads number classified in OTUs (cut-off 97%) expressed in percent of reads (%reads) for lakes A) Bromont, B) Waterloo, C) Petit St François in early and late season. The highest similarity with a BLAST search in GenBank for each OTU is shown.

Chapitre 4 : Conclusion générale

4.1. Retour sur les objectifs

Les objectifs de cette étude étaient, dans un premier temps, d'identifier les communautés phytoplanctoniques de trois lacs eutrophes dominés par les cyanobactéries pendant toute une saison de croissance, dans le but de déterminer les facteurs chimiques et environnementaux contrôlant la composition de la communauté, ainsi que la concentration et la composition des microcystines. Dans un second temps, nous avions pour objectif de déterminer la diversité des bactéries diazotrophes et de celles productrices de microcystines dans les mêmes lacs. Finalement, cette étude avait comme objectif global de comprendre la structure des communautés de cyanobactéries au niveau fonctionnel et de déterminer l'existence de relations entre la fixation d'azote et la synthèse des microcystines à l'échelle de l'individu et de la communauté.

Dans le deuxième chapitre, nos résultats ont démontré que, parmi les variables environnementales, l'azote était le meilleur prédicteur de la concentration de microcystines dans les trois lacs étudiés. L'azote était aussi le facteur expliquant le mieux la composition des communautés cyanobactériennes. C'est finalement la composition taxonomique des cyanobactéries qui était le meilleur prédicteur des variantes de MC produites dans ces lacs. Cette étude est la première à révéler l'interrelation entre l'azote, la structure des communautés et la composition des microcystines dans les eaux douces.

Au chapitre 3, nous avons décrit en détail la diversité des communautés de cyanobactéries à l'intérieur des trois mêmes lacs. Grâce à des outils moléculaires, nous

avons catégorisé les taxons en deux groupes fonctionnels, soit les bactéries diazotrophes et les cyanobactéries synthétisant les microcystines. Cette étude révèle que les cyanobactéries diazotrophes n'étaient pas actives au moment de l'échantillonnage malgré des périodes de limitation en azote inorganique dissous (DIN) et la présence d'hétérocystes dans les trois lacs. Le fait que la majorité des séquences identifiées du gène *nifH* appartenaient à d'autres groupes taxonomiques que les cyanobactéries suggère que ce groupe ne pouvait contribuer que peu ou pas à la fixation d'azote dans les lacs étudiés. Toutefois, l'amplification positive des gènes fonctionnels *nifH* et *mcyE* à partir de l'ADN sur la presque totalité des échantillons indique qu'à l'intérieur d'une même communauté phytoplanctonique, les deux traits fonctionnels étaient généralement présents simultanément.

4.2. Directions futures

Les objectifs de cette étude ont globalement été atteints, toutefois plusieurs questions demeurent sans réponse. De nouvelles avenues pourraient être explorées dans des recherches futures afin d'approfondir les résultats détaillés dans ce mémoire. Par exemple, il serait intéressant de refaire une étude similaire avec un nombre plus élevé de lacs, ce qui permettrait d'effectuer des analyses statistiques puissantes qui pourraient révéler de nouvelles relations entre l'environnement, les communautés, et les microcystines. Dans cette étude, les concentrations d'azote organique dissous (AOD) semblaient jouer un rôle majeur autant dans le façonnement des communautés que pour dicter la concentration des microcystines dans les lacs à l'étude. En ce sens, il serait intéressant d'analyser non

seulement les concentrations des formes inorganiques d'azote, mais aussi la fraction assimilable de l'AOD comme l'urée.

La diversité des microorganismes diazotrophes et la contribution de la fixation d'azote au budget d'azote dans les lacs ont été peu étudiées, et on comprend encore mal les facteurs contrôlants, ainsi que l'importance réelle de cette fonction des cyanobactéries pour l'écologie des lacs. Plus d'études sont nécessaires pour investiguer la présence et l'expression du gène *nifH* afin d'obtenir un meilleur portrait global de l'importance de la fixation d'azote dans des écosystèmes lacustres.

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Annexe A : Suppléments pour le chapitre 2

Table AI. Total concentration of microcystins (MC) measured using ELISA and HPLC-UV ($\mu g \cdot L^{-1}$), concentration of 5 variants of microcystins (MC-LR ; MC-7DMLR ; MC-RR, MC-YR ; MC-LA ; $\mu g \cdot L^{-1}$) from May to October 2010 in lakes Bromont, Waterloo and Petit St. François (PSF).

Date	Code	MC (ELISA)	MC (HPLC)	MC-LR	MC-7DMLR	MC-RR	MC-YR	MC-LA
(aa-mm-jj)		$\mu g \cdot L^{-1}$	$\mu g \cdot L^{-1}$	$\mu g \cdot L^{-1}$	$\mu g \cdot L^{-1}$	$\mu g \cdot L^{-1}$	$\mu g \cdot L^{-1}$	$\mu g \cdot L^{-1}$
10-05-24	IB1	0.033	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
10-06-14	IB2	0.031	0.008	<0.001	0.008	<0.001	<0.001	<0.001
10-07-07	IB3	0.153	0.110	0.110	<0.001	<0.001	<0.001	<0.001
10-07-28	IB4	0.251	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
10-08-12	IB5	0.333	0.346	<0.001	<0.001	0.048	0.298	<0.001
10-09-12	IB6	0.167	0.242	<0.001	0.027	<0.001	0.215	<0.001
10-09-29	IB7	0.201	0.187	<0.001	<0.001	<0.001	0.187	<0.001
10-07-06	MB3	0.104	0.037	<0.001	0.020	0.017	<0.001	<0.001
10-07-30	MB4	0.189	0.100	0.038	0.017	<0.001	0.045	<0.001
10-08-12	MB5	0.058	0.017	<0.001	0.017	<0.001	<0.001	<0.001
10-09-12	MB6	0.220	0.140	0.034	<0.001	<0.001	0.101	<0.001
10-05-20	P1	0.076	0.130	<0.001	<0.001	<0.001	<0.001	0.130
10-06-11	P2	1.317	0.659	0.193	0.659	<0.001	<0.001	0.148
10-07-10	P3	1.610	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
10-08-01	P4	1.400	0.837	<0.001	<0.001	<0.001	<0.001	0.837
10-08-16	P5	1.374	0.730	<0.001	<0.001	<0.001	<0.001	0.730
10-09-12	P6	1.213	0.677	0.058	<0.001	<0.001	<0.001	0.620
10-09-30	P7	1.199	1.340	<0.001	0.253	<0.001	<0.001	1.083
10-05-22	W1	0.025	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
10-06-13	W2	0.686	0.080	0.020	<0.001	0.060	<0.001	<0.001
10-07-12	W3	1.678	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
10-08-03	W4	1.660	0.210	<0.001	<0.001	0.210	<0.001	<0.001
10-08-17	W5	0.633	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
10-09-11	W6	1.668	0.780	<0.001	<0.001	0.780	<0.001	<0.001

IB; Lake Bromont epilimnion, MB; Lake Bromont metalimnion, W; Lake Waterloo, P; Lake Petit St. François.

Table AII. Total biomass of cyanobacterial species ($\mu g \cdot L^{-1}$) identified by microscopy, and estimated abundance of heterocysts (units$\cdot L^{-1}$) from May to October 2010 in lakes Bromont, Waterloo and Petit St. François.

Date	Site	*A. cra*	*A. pla*	*A. smi*	*A.spi*	*Ap. flo*	*M. aer*	*M. sp.*	*M. wes*	*P. aga*	*S. sp.*	*Heterocysts*
(yy-mm-dd)		$\mu g \cdot L^{-1}$	$\mu g \cdot L^{-1}$	$\mu g \cdot L^{-1}$	$\mu g \cdot L^{-1}$	$\mu g \cdot L^{-1}$	$\mu g \cdot L^{-1}$	$\mu g \cdot L^{-1}$	$\mu g \cdot L^{-1}$	$\mu g \cdot L^{-1}$	$\mu g \cdot L^{-1}$	units$\cdot L^{-1}$
10-05-24	IB1	9.7	<0.1	<0.1	<0.1	5.9	<0.1	<0.1	<0.1	56.6	<0.1	<0.1
10-06-14	IB2	<0.1	97.2	<0.1	<0.1	14.5	<0.1	0.9	<0.1	<0.1	<0.1	40 841
10-07-07	IB3	<0.1	14.5	<0.1	<0.1	0.9	<0.1	<0.1	<0.1	97.2	<0.1	163 363
10-07-28	IB4	<0.1	1096.2	934.5	<0.1	1483.7	<0.1	<0.1	<0.1	630.1	<0.1	726 057
10-08-12	IB5	<0.1	<0.1	481.1	<0.1	3156.9	<0.1	2.2	<0.1	996.9	15.1	431 096
10-09-12	IB6	<0.1	240.7	<0.1	<0.1	3420.8	<0.1	5.5	<0.1	231.0	<0.1	635 300
10-09-29	IB7	<0.1	<0.1	140.6	<0.1	2354.9	<0.1	35.1	<0.1	386.4	<0.1	204 204
10-07-06	MB3	<0.1	70.1	<0.1	<0.1	35.4	<0.1	78.6	<0.1	14715.6	84.5	27 227
10-07-30	MB4	<0.1	525.1	2201.8	<0.1	<0.1	<0.1	<0.1	<0.1	5372.7	138.1	476 475
10-08-12	MB5	<0.1	154.9	<0.1	<0.1	700.9	<0.1	<0.1	<0.1	2712.7	244.8	238 237
10-09-12	MB6	<0.1	263.2	<0.1	<0.1	3587.8	<0.1	29.6	<0.1	<0.1	13.1	1 089 085
10-05-20	P1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	141.9	711.6	<0.1	<0.1	<0.1
10-06-11	P2	<0.1	<0.1	<0.1	<0.1	305.5	<0.1	8744.3	2848.1	<0.1	<0.1	2 518 510
10-07-10	P3	550.7	<0.1	<0.1	<0.1	463.4	<0.1	5484.9	14192.3	<0.1	<0.1	816 814
10-08-01	P4	15.1	<0.1	<0.1	<0.1	593.3	<0.1	8455.9	14166.7	<0.1	<0.1	1 089 086
10-08-16	P5	1521.3	<0.1	<0.1	<0.1	14684.5	<0.1	1905.3	18321.4	<0.1	<0.1	8 440 412
10-09-12	P6	<0.1	<0.1	<0.1	<0.1	856.0	<0.1	9412.1	29310.7	<0.1	<0.1	272 271
10-09-30	P7	<0.1	<0.1	<0.1	<0.1	581.6	<0.1	3679.9	6996.3	<0.1	<0.1	408 407

Table AII (continued)

Date	Site	*A. cra*	*A. pla*	*A. smi*	*A.spi*	*Ap. flo*	*M. aer*	*M. sp.*	*M. wes*	*P. aga*	*S. sp.*	*Heterocysts*
(yy-mm-dd)		$\mu g \cdot L^{-1}$	$\mu g \cdot L^{-1}$	$\mu g \cdot L^{-1}$	$\mu g \cdot L^{-1}$	$\mu g \cdot L^{-1}$	$\mu g \cdot L^{-1}$	$\mu g \cdot L^{-1}$	$\mu g \cdot L^{-1}$	$\mu g \cdot L^{-1}$	$\mu g \cdot L^{-1}$	units$\cdot L^{-1}$
10-05-22	W1	<0.1	<0.1	<0.1	325.1	<0.1	508.3	57.6	<0.1	<0.1	<0.1	19 447
10-06-13	W2	<0.1	42.9	<0.1	3594.5	<0.1	2172.7	389.2	<0.1	<0.1	<0.1	40 841
10-07-12	W3	<0.1	<0.1	<0.1	9.4	<0.1	55.0	1951.3	<0.1	<0.1	<0.1	<0.1
10-08-03	W4	<0.1	15.2	<0.1	32.6	84.5	970.5	1620.1	84.5	<0.1	<0.1	181 514
10-08-17	W5	372.3	179.7	<0.1	<0.1	71.3	765.0	1740.4	40.1	<0.1	<0.1	136 136
10-09-11	W6	937.5	181.5	<0.1	<0.1	<0.1	931.1	4478.3	<0.1	<0.1	<0.1	181 514

A. cra; Anabaena crassa, A pla.; Anabaena planktonica, A. smi; Anabaena smithii, A. spi; Anabaena spiroïdes, Ap. flo ; Aphanizomenon flos-aquae, M. aer ; Microcystis aeruginosa, M. sp. *Microcystis* sp., *M. wes; Microcystis wesenbergii, P. aga ; Planktothrix agardhii, S.* sp.*; Spirulina* sp.

Table AIII. Nutrient and chlorophyll-a concentrations ($\mu g \cdot L^{-1}$), temperature (Temp. ; ˚C), and light extinction coefficient (k) from May to October 2010 in lakes Bromont, Waterloo and Petit St. François.

Date	Site	TP	TN	DON	DIN	NH_4^+	NO_3^-+NO_2^-	CHL-A	k	Temp.
(yy-mm-dd)		$\mu g \cdot L^{-1}$	$\mu g \cdot L^{-1}$	$\mu g \cdot L^{-1}$	$\mu g \cdot L^{-1}$	$\mu g \cdot L^{-1}$	$\mu g \cdot L^{-1}$	$\mu g \cdot L^{-1}$		˚C
10-05-24	IB1	16.07	451.97	66.08	233.94	124.04	109.9	5.61	0.67	14.98
10-06-14	IB2	13.69	411.68	203.75	80.03	15.82	61.88	5.02	0.77	17.5
10-07-07	IB3	27.91	345.70	162.09	50.12	21.28	27.44	7.28	0.75	21.24
10-07-28	IB4	26.34	511.96	234.06	13.46	12.18	<0.01	14.17	1.10	23.09
10-08-12	IB5	46.57	594.17	278.34	13.70	7.42	3.36	9.49	1.37	20.77
10-09-12	IB6	32.98	575.37	211.19	364.17	33.77	10.19	11.06	1.37	19.02
10-09-29	IB7	39.00	626.46	296.79	161.29	91.00	69.02	5.79	1.56	14.83
10-07-06	MB3	40.85	1428.64	241.03	1187.6	59.92	6.16	11.93	0.75	10.63
10-07-30	MB4	60.87	947.85	108.65	66.55	383.6	1.96	40.3	1.1	14.61
10-08-12	MB5	65.80	603.47	212.32	386.11	44.38	9.52	20.3	1.37	12.64
10-09-12	MB6	31.05	559.05	187.72	53.82	5.96	1.34	3.89	1.37	14.21
10-05-20	P1	63.71	949.08	472.75	5.49	5.46	<0.01	8.12	1.20	16.37
10-06-11	P2	13.69	499.80	484.35	15.45	13.30	2.24	26.03	2.89	17.71
10-07-10	P3	134.80	1599.81	490.24	9.84	9.24	0.56	29.03	3.19	27.79
10-08-01	P4	281.48	2094.64	438.66	30.34	24.08	6.02	53.38	6.01	21.02
10-08-16	P5	276.76	3080.35	603.49	12.93	12.04	0.7	81.79	6.24	22.18
10-09-12	P6	344.88	3222.42	491.39	2731.03	15.46	8.36	26.46	7.34	17.17
10-09-30	P7	274.83	3352.41	460.88	63.70	61.74	1.82	61.61	5.98	15.09

Table AIII. (continued)

Date	Site	TP	TN	DON	DIN	NH_4^+	NO_3^-+NO_2^-	CHL-A	k	Temp.
(yy-mm-dd)		$\mu g \cdot L^{-1}$	$\mu g \cdot L^{-1}$	$\mu g \cdot L^{-1}$	$\mu g \cdot L^{-1}$	$\mu g \cdot L^{-1}$	$\mu g \cdot L^{-1}$	$\mu g \cdot L^{-1}$		˚C
10-05-22	W1	21.45	433.56	246.30	24.18	2.80	21.28	5.79	1.06	15.88
10-06-13	W2	49.12	712.41	293.93	34.52	30.24	4.20	11.23	1.84	17.37
10-07-12	W3	55.14	532.83	295.00	13.42	13.44	<0.01	8.52	1.58	24.69
10-08-03	W4	32.27	585.72	293.70	12.62	12.60	<0.01	11.46	1.11	22.00
10-08-17	W5	40.21	653.97	365.35	8.17	8.12	<0.01	14.77	1.75	22.20
10-09-11	W6	56.19	772.35	308.88	463.47	14.64	<0.01	22.47	2.45	18.39

TP , Total Phosphorus ; TN, Total Nitrogen, DON ;Dissolved Organic Nitrogen, DIN ; Dissolved Inorganic Nitrogen, NH_4^+ ; Ammonium, NO_3^- +NO_2^-, Nitrate + Nitrite, Chl-a, Chlorophyll-a ; k ; Light extinction coefficient; Temp., temperature.

Annexe B : Suppléments pour le chapitre 3

Table BI. Number of mcyE pyrosequencing reads and closest relative obtained by BLAST search in GenBank on samples from lakes Bromont, Waterloo and Petit St. François. Number of reads for each OTU, total number of reads, closest relative found in GenBank and maximum identity are shown.

Date (yy-mm-dd)	Site	Number of reads	Total reads	% of total reads	Closest relative in GenBank (Accession number and name)		Max. ID (%)
10-07-07	B-early	42		84.0	AY382554.1	*Planktothrix agardhii* (or *P. rubescens*)	99
		1		2.0	JQ290095.1	*Microcystis aeruginosa*	97
		7	50	14.0	JN936964.1	*Microcystis aeruginosa*	99
10-09-12	B-late	158		55.4	AY382554.1	*Planktothrix agardhii* (or *P. rubescens*)	99
		127	285	44.6	DQ514535.1	*Microcystis* sp.	99
10-06-11	P-early	738		99.6	JQ290095.1	*Microcystis aeruginosa*	99
		3	741	0.4	AY382554.1	*Planktothrix agardhii*	99
10-08-01	P-late	818		99.6	JQ290095.1	*Microcystis aeruginosa*	99
		3	821	0.4	AY382554.1	*Planktothrix agardhii*	99
10-07-12	W-early	166	166	100.0	JQ290095.1	*Microcystis aeruginosa*	99
10-08-03	W-late	1		0.1	JQ290095.1	*Microcystis aeruginosa*	97
		1		0.1	FJ393328.1	*Microcystis* sp.	97
		734	736	99.7	JQ290095.1	*Microcystis aeruginosa* (or *M. viridis*)	99

B; Lake Bromont, W; Lake Waterloo, P; Lake Petit St. François (PSF)

Table BII. Number of *nifH* pyrosequencing reads and closest relative obtained by BLAST search in GenBank on samples from lakes Bromont, Waterloo and Petit St. François. Number of reads for each OTU, total number of reads, closest relative found in GenBank and maximum identity are shown.

Date (yy-mm-dd)	Site	Number of reads	Total reads	% of total	Closest relative in GenBank (Accession number and name)		Max ID (%)
10-07-07	B-early	22		0.2	DQ294218.1	*Anabaena azotica* (nostoc)	81%
		15		0.1	AF378716.1	*Methylomonas* sp.	85%
		4		0.0	AF484672.1	*Methylomonas methanica*	81%
		21		0.1	DQ294218.1	*Anabaena azotica* (nostoc)	80%
		15		0.1	HM149325.1	*Ectothiorhodospira* sp.	83%
		49		0.3	AF216923.1	Unidentified	83%
		3216		18.6	DQ294218.1	*Anabaena azotica* (nostoc)	81%
		14		0.1	AY937260.1	*Methylobacter tundripaludum*	89%
		12		0.1	DQ294218.1	*Anabaena azotica* (nostoc)	79%
		10798		62.8	DQ294218.1	*Anabaena azotica* (nostoc)	81%
		2675		15.6	DQ294218.1	*Anabaena azotica* (nostoc)	80%
		359	17200	2.1	na	others	na
10-09-12	B-late	5		21.7	GQ289566.1	*Bradyrhizobium japonicum*	75%
		1		4.3	AM110700.1	*Azorhizobium* sp	87%
		9		39.1	AF378717.1	*Methylomonas methanica*	96%
		1		4.3	na	unidentified	na
		7	23	30.4	AF378715.1	*Methylomonas* sp.	89%

Table BII. (continued)

Date (yy-mm-dd)	Site	Number of reads	Total reads	% of total	Closest relative in GenBank (Accession number and name)		Max ID (%)
10-06-11	P-early	3		9.4	EU072030.1	*Vibrio porteresiae*	79%
		2		6.3	HQ398520.1	*Burkholderia* sp.	75%
		1		3.1	FJ668319.1	*Burkholderia cenocepacia*	79%
		1		3.1	na	not found	na
		25	32	78.1	AF378717.1	*Methylomonas methanica*	95%
10-08-01	P-late	5		38.5	na	not found	na
		2		15.4	CP001654.1	*Dickeya dadantii*	
		1		7.7	na	not found	na
		1		7.7	EU072030.1	*Vibrio porteresiae*	77%
		2		15.4	CP002738.1	*Methylomonas methanica*	88%
		1		7.7	na	Unidentified	na
		1	13	7.7	JN698218.1	*Herbaspirillum* sp.	93%
10-07-12	W-early	1		5.0	CP001197.1	*Desulfovibrio vulgaris*	79%
		4		20.0	na	not found	na
		3		15.0	na	not found	na
		1		5.0	AF378715.1	*Methylomonas* sp.	88%
		1		5.0	GQ289577.1	*Bradyrhizobium japonicum*	80%
		5		25.0	AF378717.1	*Methylomonas methanica*	96%
		5	20	25.0	AY768413.1	*Lyngbya majuscula*	95%

Table BII (continued)

Date (yy-mm-dd)	Site	Number of reads	Total reads	% of total	Closest relative in GenBank (Accession number and name)		Max ID (%)
10-08-03	W-late	1		0.3	AF378717.1	*Methylomonas methanica*	93%
		1		0.3	AF378715.1	*Methylomonas* sp.	91%
		1		0.3	AP010904.1	*Desulfovibrio magneticus*	100%
		1		0.3	AF378715.1	*Methylomonas* sp.	90%
		1		0.3	na	not found	na
		267		88.1	AY768413.1	*Lyngbya majuscula*	96%
		18		5.9	AF378717.1	*Methylomonas methanica*	95%
		13	303	4.3	AY768413.1	*Lyngbya majuscula*	95%

na; not applicable