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POLYTECHNIQUE MONTRÉAL

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Fate of cyanobacteria after pre-treatment using H_2O_2 and $CuSO_4$: a mesocosm experiment

PEGAH VATANI

Département des génies civil, géologique et des mines

Mémoire présenté en vue de l'obtention du diplôme de *Maîtrise ès sciences appliquées*

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Ce mémoire intitulé:

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présenté par **Pegah VATANI**

en vue de l'obtention du diplôme de *Maîtrise ès sciences appliquées*

a été dûment accepté par le jury d'examen constitué de :

Benoit BARBEAU, président
Sarah DORNER, membre et directrice de recherche
Michèle PREVOST, membre et codirectrice de recherche
Émilie BÉDARD, member

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RESUME

Au cours d'efflorescences de cyanobactéries (Bloom), en particulier dans les sources d'eau potable, les processus conventionnels de traitement de l'eau sont mis à l'épreuve par l'accumulation de cyanobactéries et la libération potentielle de cyanotoxines. Le pré-traitement a été proposée comme solution pour une suppression efficace des cyanobactéries et de leurs métabolites (y compris les cyanotoxines et les composés de goût ou d'odeur). Pour sélectionner le traitement le plus approprié, il est nécessaire de comprendre le comportement des cyanobactéries et la dynamique de leur composition dans l'ecosystéme après l'ajout du traitement. Peu de travaux sont disponibles pour comparer l'impact de pré-traitement, couramment utilisés, sur la taxonomie et la réponse fonctionnelle de la communauté des bactéries/cyanobactéries. Afin de mieux explorer ce volet, ce travail de maîtrise a été réalisé en utilisant du peroxyde d'hydrogène (H_2O_2) et du sulfate de cuivre ($CuSO_4$) comme pré-traitements en déployant des mésocosmes (étude à grande échelle) dans deux lacs canadiens, la baie Missisquoi et le Petit-lac-Saint-François (PLSF), durant l'été et l'automne 2019 (3 événements du 12 août au 23 octobre). L'objectif principal était d'évaluer l'impact de H_2O_2 et de $CuSO_4$ sur le contrôle de la communauté cyanobactérienne. Les objectifs spécifiques de cette étude étaient de déterminer (1) l'influence des traitements ajoutés sur la viabilité des cyanobactéries et l'intégrité cellulaire en utilisant la cytométrie en flux (FCM); (2) le changement de la concentration finale de composés organiques dissous (COD); (3) l'impact des traitements sur la production/libération de cyanotoxines (microcystines) et sur la teneur en gène de la microcystine synthétase (*mcyD*); (4) l'efficacité de H_2O_2 et $CuSO_4$ sur la modification de la composition de la communauté cyanobactérienne à l'aide du comptage taxonomique et cellulaire par la microscopie et du séquençage métagénomique; (5) l'influence des traitements sur la dynamique de la réponse taxonomique et fonctionnelle de la communauté bactérienne après l'ajout de H_2O_2 et $CuSO_4$, dans les 48 heures suivant l'oxydation/traitement.

Le projet comprenait trois séries d'expériences distinctes : 1) le traitement sur le terrain dans la baie Missisquoi en utilisant deux doses de H_2O_2 (10 mg/L et 20 mg/L), 2) le traitement sur le terrain dans PLSF en utilisant H_2O_2 (10 mg/L et 20 mg/L) et $CuSO_4$ (2 mg/L et 5 mg/L) et 3) au laboratoire avec H_2O_2 (10 mg/L et 20 mg/L) et $CuSO_4$ (2 mg/L et 5 mg/L) de l'eau brute collectée du PLSF.

Les résultats de la FCM suggèrent que le $CuSO_4$ a un impact négatif sur l'intégrité des cellules des cyanobactéries. En effet, moins de 5% des cyanobactéries étaient intactes 48 heures après l'ajout du traitement pour les deux doses (2 et 5 mg/L). Bien que le H_2O_2 a réduit l'intégrité cellulaire, les dommages n'ont pas conduit à la lyse cellulaire. On suggère que le pourcentage d'intégrité cellulaire suite à l'oxydation au H_2O_2 variait en fonction de la densité cellulaire initiale et de la dose ajoutée et de l'exposition à la lumière UV. Ces pourcentages variaient entre 3,7% et 72%. Par ailleurs, les résultats de COD ont démontré que dans la plupart des cas, l'ajout d'un traitement entraîne une augmentation de la concentration de COD dans les mésocosmes 48 heures l'oxydation. En ce qui concerne les cyanotoxines, l'oxydation par le H_2O_2 a montré un résultat prometteur comparativement au $CuSO_4$. En effet, dans la plupart des expériences utilisant le H_2O_2 comme pré-oxydant, la concentration finale de microcystines (MCs) était plus faible, probablement en raison d'une rupture cellulaire plus faible, d'une éventuelle oxydation des MCs et/ou d'une altération possible des gènes codant pour la synthèse de MCs. La quantification du gène codant pour la synthèse de MCs (*mcyD*) dans le PLSF a révélé que l'oxydation par H_2O_2 produit des cellules cyanobactériennes avec moins de concentration en copies *mcyD* par litre d'eau, produisant ainsi, potentiellement, des blooms moins toxiques. A l'inverse, lorsque $CuSO_4$ a été ajouté, une augmentation des MCs dissous a été observée, probablement, à la suite d'une rupture de la membrane cellulaire plus importante, suite à une augmentation des copies du gène *mcyD* et l'incapacité du $CuSO_4$ à oxyder les MCs. Nos résultats de ddPCR démontrent que les cellules contenant *mcyD* pourraient être un prédicteur des concentrations de MCs lors du traitement avec H_2O_2 ou $CuSO_4$.

Les résultats de comptage taxonomique et cellulaire microscopique ont indiqué que tous les traitements ajoutés réduisaient le nombre total de cellules de cyanobactéries. Cependant, aucun changement important n'a été observé lorsque la dose de H_2O_2 est passée de 10 mg/L à 20 mg/L.

Enfin, les résultats du séquençage métagénomique, disponibles uniquement pour l'oxydation sur site dans le PLSF, suggèrent que chaque traitement a un mode d'action et un impact différent sur la communauté bactérienne/cyanobactérienne. *Cyanobacteria* était le phylum qui dominait les phyla détectés dans tous les mésocosmes contrôles (avant l'ajout de traitement au H_2O_2 ou au $CuSO_4$). Avec les deux traitements, l'abondance relative des cyanobactéries a diminué après 48 heures de pré-oxydation. Après l'ajout de H_2O_2, *Proteobacteria* semble être le phylum le plus

abondant alors que dans le cas de $CuSO_4$, les *Bacteroidetes* prédominent. Lorsque H_2O_2 a été ajouté aux mésocosmes, et en se basant sur l'indice de Shannon, une meilleure distribution de la communité microbienne a été observée après 48 heures, ce qui n'a pas été observé lorsque $CuSO_4$ a été ajouté. En comparant les résultats de la métagénomique et le nombre de cellules taxonomiques dans les mésocosmes traités avec $CuSO_4$-2 mg/L et $CuSO_4$-5 mg/L, il a été conclu que malgré un nombre de cellules de cyanobactéries inférieur après l'ajout de $CuSO_4$-5 mg/L pendant 48h, ce traitement a enregistré plus d'abondance relative des cyanobactéries. Il semble que la dose de $CuSO_4$ a créé une condition défavorable pour d'autres taxons bactériens, partageant avec les cyanobactéries le même écosystème, à savoir les *Actinobacter*. Dans ce sens et d'après le profil fonctionnel microbien présenté par les PCA fonctionnels, la condition $CuSO_4$-5 mg/L s'avère la condition la plus stricte (plus ''stringent'') par rapport à $CuSO_4$-2 mg/L et H_2O_2-20 mg/L, favorisant à son tour l'abondance des cyanobactéries par rapport aux autres taxons.

En conclusion, la comparaison des deux traitements $CuSO_4$ et H_2O_2 montre que le H_2O_2 pourrait être l'oxydant de choix pour contrôler les efflorescences des cyanobactéries dans les lacs, en raison de sa sécurité environnementale (ne s'accumule pas dans le milieu), de sa plus faible libération de MCs et de sa plus grande sélectivité envers les cyanobactéries.

ABSTRACT

During cyanobacterial bloom, especially in drinking water sources, conventional water treatment processes are challenged by cyanobacterial accumulation and potential cyanotoxins release. Pre-treatment has been proposed as a solution for efficient suppression of cyanobacteria and their metabolites (including taste and odor compounds or cyanotoxins). To select the most suitable pre-treatment, it is required to comprehend the dynamics of cyanobacteria and cyanobacteria behaviour following treatment addition. Little work is available comparing the commonly used pre-oxidants'/algicides' impact on the bacterial/cyanobacterial community and their functional response. To investigate this issue, this master's work was performed using hydrogen peroxide (H_2O_2) and copper sulfate ($CuSO_4$) as pre-treatments by deploying mesocosms (large-scale study) in lakes such as Missisquoi Bay and Petit-lac-Saint-François (PLSF) during the summer and fall 2019 (for 3 events starting the 12th of August until October 23rd). The main objective was to evaluate the impact of H_2O_2 and $CuSO_4$ on mitigating the cyanobacterial community. The specific objectives of this study were to determine: (1) the influence of added treatments on cyanobacteria viability and cell integrity using flowcytometry (FCM), (2) the change in the final concentration of dissolved organic compounds (DOC), (3) the impact of treatments on cyanotoxins (microcystins) production/release and the copy number of cyanotoxin gene synthesis (*mcyD* gene), (4) the efficacy of H_2O_2 and $CuSO_4$ on altering the cyanobacterial community composition using microscopic cell count and shotgun metagenomic sequencing, and the suppression of total cyanobacteria using microscopic cell count, and (5) the influence of treatments on the dynamics of the taxonomic and functional response of bacterial community to the added chemicals within 48 hours of the experiment.

The project included three separate sets of experiments: 1) field treatment in Missisquoi Bay using two doses of H_2O_2 (10 mg/L and 20 mg/L), 2) field treatment in PLSF using H_2O_2 (10 mg/L and 20 mg/L) and $CuSO_4$ (2 mg/L and 5 mg/L) and 3) lab oxidation using PLSF raw water with H_2O_2 (10 mg/L and 20 mg/L) and $CuSO_4$ (2 mg/L and 5 mg/L).

FCM results suggested that $CuSO_4$ induced severe damage to cyanobacterial cell integrity as fewer than 5% of the cyanobacteria were intact 48 hours after the treatment addition for both doses (2 and 5 mg/L). Though H_2O_2 impacted the integrity, the damage was less severe. The resulting cell

integrity percentage varied depending on initial cell density, added dose and exposure to UV light, with 3.7% and 72% being the lowest and highest integrity percentages, respectively. Results demonstrated that in most cases, treatment addition resulted in an increase in DOC concentration in mesocosms within 48 hours. With regards to cyanotoxins, H_2O_2 showed a promising result as compared to $CuSO_4$. When H_2O_2 was added, in most cases, the final concentration of microcystins (MCs) was lower due to milder cell rupture, the impact of H_2O_2 on decreasing the number of *mcyD* genes and possible MCs oxidation. Conversely, when $CuSO_4$ was added, a sudden increase in the dissolved MCs was observed as a result of severe cell rupture, with an increase in the number of *mcyD* genes and the inability of $CuSO_4$ to oxidize MCs.

Microscopic cell count results indicated that all the added treatments reduced the total cell counts. However, no significant change was seen when the dose of H_2O_2 increased from 10 mg/L to 20 mg/L. Finally, metagenomic shotgun sequencing results available only for onsite oxidation in PLSF suggested that each treatment has different mode of action and impact on bacterial/cyanobacterial community. The abundant phylum in all mesocosms were cyanobacteria before treatment addition. After H_2O_2 addition, with cyanobacteria decrease, *Proteobacteria* appeared to be the most abundant phylum while in the case of $CuSO_4$, *Bacteroidetes* predominated. When H_2O_2 was added, a better community distribution was seen after 48 hours, which was not seen when $CuSO_4$ was added. By comparing the shotgun metagenomics results and taxonomic cell counts in the mesocosm with $CuSO_4$-5 mg/L, it was concluded that treatment may create an unfavorable condition for some taxa, *Actinobacter*, which seemed to be more favorable for cyanobacteria persistence among the microbial community. Finally, when both added treatments are compared, H_2O_2 showed more promising performance as compared to $CuSO_4$ due to being environmentally safe, with lower metabolites release, and was more selective towards cyanobacteria.

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N	Nitrogen
P	Phosphorus
PLSF	Petit-Lac-Saint-François
H_2O_2	Hydrogen peroxide
$CuSO_4$	Copper sulfate
DOC	Dissolved organic compounds
MDDELCC	Ministère du Développement durable, de l'Environnement et de la Lutte contre les changements climatiques.
UV	Ultraviolet
PCR	Polymerase chain reaction
DNA	Deoxyribonucleic acid
WHO	World Health Organization
MCs	Microcystins
Fe^{2+}	Iron ion
MAC	Maximum acceptable concentration
OH•	Hydroxyl radical
ROS	Reactive oxygen species
Cu^{2+}	Copper ion
ATRAPP	Algal Blooms, Treatment, Risk Assessment, Prediction and Prevention Through Genomics
T0	Initial experimental time (before treatment addition)
T48	Final experimental time (48 hours after treatment addition)
$Na_2S_2O_3 \cdot 5H_2O$	Sodium thiosulfate

TOC	Total organic carbon
PETG	Polyethylene terephthalate glycol modified
SPE−UHPLC–HRMS	Ultra-high-performance liquid chromatography high resolution mass spectrometry (SPE−UHPLC–HRMS).
FCM	Flow cytometry
SG	SYBR Green I
PI	Propidium iodide
PCA	Principal Component Analysis
ddPCR	Droplet digital PCR

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CHAPTER 1 INTRODUCTION

The occurrence of harmful cyanobacterial blooms has increased as a result of climate change and increased nutrient inputs to water bodies (Crafton et al., 2021). The occurrence of cyanobacteria results in the degradation of water quality by producing taste and odour compounds and cyanotoxins (Crafton et al., 2021; Fan, 2013; Fan et al., 2014b; Jalili et al., 2021). Exposure to cyanotoxins can occur through recreational activities or drinking poorly treated water (McQuaid et al., 2011), which can give rise to severe health problems especially cyanotoxins can harm the liver or the neurological system (Matthijs et al., 2012a). Therefore, monitoring bloom occurrence, composition, frequency and intensity, as well as controlling their emergence in freshwater and coastal marine ecosystems, is crucial.

Several environmental factors can have a direct impact on cyanobacterial cell growth. For instance, warm temperature, appropriate light intensity, and the eutrophication (nutrient accumulation) of lakes accelerate cyanobacterial bloom appearance (Spoof et al., 2020). Therefore, bloom events occur due to cyanobacteria proliferation and favorable environmental conditions, leading to cyanobacteria accumulation in drinking water supply reservoirs (Health Canada, 2016; Naceradska et al., 2017). Cyanobacterial and their toxins can be challenging to remove during treatment processes, especially through coagulation and settling. Thus, with bloom occurrence, conventional water treatment processes may fail to maintain a desirable quality, especially in the case with the existence of dissolved cyanotoxins (Fan et al., 2014a; Fan et al., 2014b; Naceradska et al., 2017).

Limiting the availability of nutrients such as nitrogen (N) and phosphorus (P) in water sources is required to alleviate the problem of water quality degradation caused by cyanobacterial blooms (Zhou et al., 2013a). As a long-term solution, this strategy has been shown to be costly in most cases (Zhou et al., 2013a). Therefore, one alternative short-term solutions can be the addition of oxidants to the water source as a pre-treatment method (Moradinejad et al., 2020; Naceradska et al., 2017). Pre-oxidation can improve the efficiency of conventional treatment processes such as coagulation and the flocculation process to remove cyanobacteria (Coral et al., 2013; Fan et al., 2013b). The efficacy of oxidants is dependent on different factors that will be elaborated in the following sections of this project. It should be taken into consideration that intracellular metabolites release as a consequence of membrane damage is one of the outcomes of chemical addition (Zhou et al., 2013a).

This study was focused on evaluating the impact of oxidation on cyanobacteria using mesocosm study as it is useful *in situ* technique with benefits beyond studying cultured laboratory strains which should be grown in optimal conditions (Wood et al., 2012). Naturally grown bacteria usually exist in the form of multicellular communities that may resist environmental stress, especially during nutrient deficiency and temperature alteration (Palková, 2004). As a consequence, this work was carried on using natural cyanobacterial blooms exposed to their ecological conditions, such as the natural sunlight and temperature.

This research was performed in Missisquoi Bay and Petit-Lac-Saint-François (PLSF) during summer and fall 2019 by installing mesocosms in the source water. This study aims to identify the impact of oxidation/treatment by $CuSO_4$ and H_2O_2 on the cyanobacterial composition within the microbial community by taxonomic cell counts and metagenomic shotgun sequencing. In this research the extent of DOC (dissolved organic compounds) and metabolites release after the induced stress was also evaluated.

CHAPTER 2 LITERATURE REVIEW

2.1 Cyanobacteria

Cyanobacteria, known as blue-green algae, are classified as oxygenic photosynthetic organisms (Hamilton et al., 2014), naturally appearing in marine, brackish, and freshwaters (Kimambo et al., 2019; Zamyadi, 2011). Cyanobacteria are considered problematic due to their ability to form a toxin-producing bloom if favorable growth conditions prevail (Kimambo et al., 2019). According to the « Ministère du Développement durable, de l'Environnement et de la Lutte contre les changements climatiques (MDDELCC) », a concentration above 20 000 cyanobacterial cells/ml, are considered to be a bloom (Groupe scientifique sur l'eau, 2017). Blooms form as a result of various environmental factors including high nutrient loads and optimal growth temperatures (Matthijs et al., 2012a). Figure 2.1 shows cyanobacterial bloom formed in Missisquoi Bay during summer 2019. Additionally, dry period duration and rainfall intensity influence bloom formation in freshwater (Kimambo et al., 2019).

When high density blooms form, other phytoplankton communities might be suppressed due to not receiving enough light intensity (Matthijs et al., 2012a). Cyanobacteria also create turbidity and other water quality problems (Matthijs et al., 2012a; Xu et al., 2019).

Due to cyanobacteria's high tolerance against stresses, such as ultraviolet (UV) radiation in natural light and warm temperatures, around 60% of the freshwater bodies suffer from toxin-producing cyanobacteria blooms (Hamilton et al., 2014; Kimambo et al., 2019). Consequently, Canadian government guidelines recommended that utilities monitor their drinking water sources visually, particularly around the intake points and shorelines during the peak season, which usually starts in May and extends to October. Visually, cyanobacteria bloom color may vary from green to blue to red. In calm waters, scum can be formed on the surface of the water (Health Canada, 2016).

Figure 2.1 Cyanobacterial bloom at Missisquoi Bay, August 2019

2.2 Biodiversity of cyanobacteria

2.2.1 Cyanobacteria morphological and physiological-based classification

Over time, mutations and metabolic changes enhanced the survival of cyanobacteria when encountering environmental change. Cells can demonstrate morphological variation within the population (Sharma et al., 2013). The classification of cyanobacteria was revised, emphasizing cell division and polyphasic information (Perkerson III et al., 2011). This classification is summarized in Table 2.1. Under this classification, Cyanobacteria are composed of five subsections: *Subsection I (Order Chroococcales)*: this subsection is composed of individual cells (unicellular) coccoids that reproduce by binary fission. The representative genera of this subsection are *Aphanocapsa, Aphanothece, Gloeocapsa, Merismopedia, Microcystis, Synechococcus, Synechocystis* (Vincent, 2009).

Subsection II (Order Pleurocapsales): is composed of unicellular cyanobacteria that are reproduced by multiple fission producing small, dispersed cells called baeocytes. The representative genera of this subsection are *Chroococcidiopsi* and *Pleurocapsa* (Vincent, 2009).

Subsection III (Order Oscillatoriales): is comprised of only vegetative and filamentous cyanobacteria. They undergo binary fission in one plane (false branching), leading to the formation of hormogonia. The representative genera of this subsection are *Lyngbya, Leptolyngbya, Microcoleus, Oscillatoria, Phormidiu* and *Planktothrix* (Vincent, 2009).

Subsections IV (Order Nostocales) and V (Order Stigonematales): are composed of filamentous cyanobacteria that differentiate in more than one plane into morphologically and ultrastructurally distinct heterocysts, akinetes and the formation of hormogonia. Akinetes can survive different stressful conditions such as cold and desiccation (Nicholson et al., 2000). Representative genera within *Subsections IV (Order Nostocales)* are *Dolichospermum* (also named *Anabaena)*, *Aphanizomenon, Calothrix, Cylindrospermopsis, Nostoc, Scytonema* and *Tolypothrix* (Vincent, 2009).

Subsection V is considered as among the most complex and highly developed prokaryotes. Representative genera in *Subsection V (Order Stigonematales)* are *Mastigocladus (Fischerella)* and *Stigonema* (Vincent, 2009).

This traditional classification is not supported by recent studies because molecular phylogeny has become a powerful tool in elucidating evolutionary pattern, and taxonomy classification of all organisms, including cyanobacteria.

Table 2.1 Morphological classification of cyanobacteria (Sharma et al., 2013)

Classification Subsection	***Morphological aspect***
Subsection I *(order Chroococcales)*	Unicellular cyanobacteria; deploying binary fission division
Subsection II *(order Pleurocapsales)*	Unicellular cyanobacteria; deploying multiple division
Subsection III *(order Oscillatoriales)*	Filamentous cyanobacteria; binary fission
Subsection IV *(order Nostocales)*	Filamentous cyanobacteria
Subsection V *(order Stigonematales)*	Filamentous cyanobacteria; most complex and highly developed prokaryotes

2.2.2 Classification based on molecular techniques

Recently microscopic counting method was combined with molecular methods that have been developed for research monitoring cyanobacterial blooms and their associated cyanotoxins.

2.2.2.1 PCR amplification and sequencing of genetic markers

Polymerase-chain reaction (PCR) analysis is a frequently used molecular technique in environmental microbiology. It is a relatively simple technology that amplifies a target DNA (deoxyribonucleic acid) sequence, which is previously selected by specific primers. The high specificity and sensitivity of some primers make the PCR the technology of choice for bacterial monitoring. PCR-based approaches have been used in several cyanobacterial bloom detection projects, toxicity assessment and many other cyanobacterial research topics (Fortin et al., 2010a). Several genetic markers have been used in various studies to resolve taxonomic issues, determine the cyanobacterial composition or even identify isolated strains thanks to species or strain-specific primers for identification. Table 2.2 illustrates the genetic markers that were used in previous studies to study cyanobacteria taxonomy, phylogeny, phylogeography, evolution or biogeography (Moreira et al., 2013; Ragon et al., 2014). Some studies used more than one genetic marker rather than a single one.

Table 2.2 List of the genetic markers used and their respective application in phylogeny and biogeography studies of cyanobacteria (Moreira et al., 2013)

Genetic marker	Taxonomy	Phylogenetic	Phylogeography	Evolution	Biogeography
PC-IGS	√	√	√		√
ftsZ		√			
glnA		√			
gltX		√			
gyrB		√			
pgi		√			
recA		√			
tpi		√			
16S-23S ITS1-L		√	√		
16S-23S ITS1-S		√			
rpoB	√				
rpoC1	√	√	√	√	
nifH	√	√	√	√	
nifD	√				
16 rRNA	√	√	√	√	√
16S-23S ITS	√	√	√		√
rbcLX	√				
hetR	√				
psbA		√			
rbcL		√			
rbcS		√			

For taxonomic studies, 16S rRNA (ribonucleic acid) gene marker has been widely used for several taxonomic studies, which means detection of cyanobacterial genera/species presence within the whole microbial community (Giovannoni et al., 1990). It has been shown that 16S rRNA gene sequences supported the traditional classification where baeocytes (subsection II) and heterocysts (subsections IV and V) are each phylogenetically coherent (Giovannoni et al., 1988; Turner et al., 1999). However, subsections I and III appear intermixed in the phylogenetic tree. This result suggests that heterocystous cyanobacteria may have diverged later in the cyanobacteria lineage or have multiphylege, in other words, filamentous morphology may have polyphyletic origins within cyanobacteria. However, unlike 16S rRNA gene marker, using *nifH* structural gene, which encodes for nitrogenase (catalysis of biological nitrogen fixation), as a genetic marker, subsections I, IV and V seem to be coherent (Zehr et al., 1998). These controversial results reflect an evolutionary phenomenon within the cyanobacterial population and thus, molecular phylogenetic and paleontological studies should be performed in tandem.

For microcystin-producing algae, primers are designed to amplify six gene fragments of the microcystin synthetase *mcy* cluster *mcyA-G* (Hisbergues et al., 2003; Vaitomaa et al., 2003) (Figure

2.2). By using these primers, *Anabaena*, *Planktothrix* and *Microcystis* genera are identified to be microcystin-producing genera. Detecting the presence of toxin producing gene in a bloom sample does not mean necessary toxin production. It only gives an indication of the potential for toxin production in this bloom.

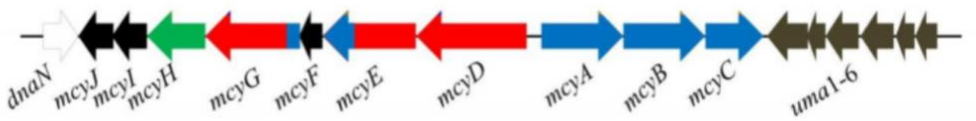

Figure 2.2 The Microcystin gene Operon. Tailoring enzymes are presented in black, polyketide synthase are presented in red, non-ribosomal peptide synthases are presented in blue, non microcystin synthetase are in dark green and ABC transporter genes are in Green (Rastogi et al., 2015)

2.2.2.2 Metagenomic sequencing approaches

methods are necessary to identify more than 99% of the remaining non-identified bacteria in nature, including some taxa within the cyanobacterial community. Indeed, high-throughput sequencing technologies, such as metagenomics are extensively used in recent years (Sanschagrin and Yergeau, 2014). Metagenomic sequencing allows the studying of various sets of genes and genetic information existing in a sample (Sanschagrin and Yergeau, 2014). Unlike genomics, the study of a single organism genomic DNA (Gilbert and Dupont, 2010), the metagenomic study is not limited to just one genome analysis. In fact, it includes all microorganisms existed in a community, even those that may not grow among lab cultured strains. (Hugenholtz and Tyson, 2008).

Living organism genes are formed by DNA. DNA, which is made up of adenine (A), cytosine (C), guanine (G), and thymine (T) bases, is used as a storage for cell information (Medicine, 2020). To identify the organisms that are present in a specific environment, it is necessary to determine the genes associated with them. The established taxonomy method may suffer from errors that can be corrected by genomics in order to obtain the cyanobacteria classification. For instance, misidentification of cyanobacteria or overlooking some species may occur due to morphological similarity when using microscopic cell count methods (Moradinejad et al., 2020). This correction

is attainable thanks to the recent DNA sequencing methods, which enable access to a broader biological dataset by engaging computer science (Alvarenga et al., 2017).

The metagenomic approach includes metagenomics based on 16S rRNA gene sequencing and metagenomic shotgun sequencing (or whole genome sequencing). The first one is based on extracting the DNA of the whole community within a sample, followed by 16S rRNA gene amplification (by PCR) and sequencing (Muyzer et al., 1993). In this approach, variable region of 16S rRNA gene of the highly conserved regions is amplified, sequenced and analysed by bioinformatics tools (Sanschagrin and Yergeau, 2014). In general, 16S rRNA gene of 1500 base-pair comprises 9 hypervariable regions (V1-V9) that enable the determination of differential sequence discriminating the community members. The regions V2, V3 and V4 are the most used to detect the diversity between sequences. Indeed, these regions have the maximum heterogeneity between taxa (Chakravorty et al., 2007). Once these regions are sequenced, thousands of 16S rRNA gene fragments are analysed simultaneously. One of the disadvantages of this approach is its inability to give a precise indication of the taxa at the genus and species level (Ranjan et al., 2016).

Shotgun metagenomic sequencing relies on a global analysis of the microbial population within a sample, providing deep analysis on phylogenetic diversity, metabolic pathways and functional capacities (Barker and Pagel, 2005). This approach enables studying complex microbial communities, their phylogenetic composition (taxonomic profile), as well as their metabolic and functional pathways (functional profile). Functional profile allows understanding the microbial community respond to the induced oxidation stress (Gomez-Smith et al., 2016). Interestingly, it can identify new genes that cannot be amplified by simple PCR. Additionally, shotgun metagenomic sequencing allows accurate classification at the genus and species level of taxa (Ranjan et al., 2016). Statistical analysis of metagenomic shotgun sequencing gives better information on the taxonomic and community composition, diversity and similarity of functional profiles, analysis of variation, and differential abundance. The disadvantage of this approach is the high cost compared to metagenomics based on one target gene sequencing (i.e., 16S rRNA gene sequencing) and the software's training for statistical and bioinformatics analysis.

2.3 Cyanotoxins

According to the World Health Organization (WHO), the consequences of ingesting microbially contaminated drinking water could be fatal in some cases (WHO Chronicle, 2008). In fact, the appearance of cyanobacteria in the water reservoir can be used as a sign of water contamination (Health Canada, 2016). When cyanotoxins are released into a drinking water source, the deterioration of the water quality may extend to even after the bloom cycle (Hamilton et al., 2014). Also, cyanotoxins are categorized within the high risk toxic biological elements; therefore, it is imperative to eliminate the proliferation of cyanobacteria cells to prevent toxins release (Dixit et al., 2017). More than 20 different genera of cyanobacteria have been identified as toxin-producing species worldwide (Health Canada, 2016). It should be taken into consideration that in case of direct contact with a bloom, skin irritation and asthma are predicted (Harris and Graham, 2017).

Cyanobacteria may exist in the form of different genera such as *Microcystis*, *Anabaena*, *Oscillatoria*, *Nostoc*, *Anabaenopsis*, and *Planktothrix*. Some genera within cyanobacteria such as *Microcystis*, *Dolichospermum* (also named *Anabaena)*, *Oscillatoria*, *Nostoc*, *Anabaenopsis*, and *Planktothrix* are responsible for microcystins (MCs) toxins production, which is the most studied and common toxin in freshwater ecosystems (Rodríguez et al., 2007).

MCs (12 microcystins (Microcystin-RR, YR, HtyR, LR, HilR, LA, LY, LW, LF, [Asp3Dhb7]MC-RR and [Asp3]MC-LR) are heptapeptides in a cyclic form composed of amino acids that are considered relatively stable as they are able to tolerate high temperatures or even boiling (Fan et al., 2014b). There are more than 90 different types of MCs, varying in their structure and toxicity (Dixit et al., 2017). MCs are also likely to have elevated concentrations during bloom appearance (Health Canada, 2016).

MCs are not only detrimental to human health by damaging the DNA, but they can impair the liver and neurological system (Bartram and Chorus, 1999; Spoof et al., 2020). Therefore, the WHO organization has established a provisional guideline value for MC-LR (one of the MCs) at 1.0µg/L (WHO Chronicle, 2008). Also, the government of Canada has published guidelines indicating the maximum acceptable concentration (MAC) of total microcystin to be 1.5µg/L, which is in accordance with the guidelines set in Quebec (Health Canada, 2016). In spite of these adverse

effects of MCs, humans are generally exposed to nonfatal doses of MCs by utilizing drinking water (Spoof et al., 2020).

Other problematic forms of cyanotoxins are known as anatoxins, classified in neurotoxins that disrupt the nervous system. *Dolichospermum (Anabaena)*, *Microcystis*, *Aphanizomenon*, and *Oscillatoria* are mostly responsible for anatoxins production (Health Canada, 2016). According to the WHO, there was only enough data to set a provisional guideline value for anatoxins due to its frequent occurrence during bloom appearance (WHO Chronicle, 2008).

Table 2.3 Cyanotoxins, the responsible cyanobacteria genera, and the main target of the mentioned toxin

Toxin	***Responsible genera***	***Main target***	***References***
Microcystins	*Microcystis*, *Anabaena*, *Oscillatoria, Nostoc*, *Anabaenopsis*, and *Planktothrix*	Liver	(Kaebernick and Neilan, 2001; Rodríguez et al., 2007)
Anatoxin-a	*Anabaena*, *Microcystis*, *Aphanizomenon*, and *Oscillatoria*	Nervous system	(Health Canada, 2016; Kaebernick and Neilan, 2001)
Saxitoxins	*Aphanizomenon, Anabaena, Planktothrix, Cylindrospermosis, Lyngbya*	Nervous system	(Kaebernick and Neilan, 2001)
Cylindrospermopsin	*Aphanizomenon*, *Cylindrospermosis*, *Umezakia*	Liver and Gastrointestinal problem	(Kaebernick and Neilan, 2001; Sharma et al., 2013)
Aplysiatoxins	*Oscillatoria*, *Lyngbya*, *Schizothrix*	Skin irritation	(Kaebernick and Neilan, 2001; Sharma et al., 2013)

More research is needed to gather sufficient information to establish new regulations for other types of cyanotoxins (Health Canada, 2016). Table 2.3 provides a summary of cyanotoxins and responsible genera for toxin production as well as their targeted organs in humans.

It is imperative to take into account that the toxicity of a bloom depends on the toxin-producing strains population and toxin gene expression as well as environmental conditions (Health Canada, 2016). It was observed that both high light intensity and enough nutrient concentration would result in higher MCs level which can be attributed to gene expression and synthetic activity inside cells. Additionally, nutrients such as phosphorus (P) and Nitrogen (N) are considered to be essential elements for the toxin production process (Dai et al., 2016).

Table 2.4 indicates the cyanobacterial cell count limit and cyanotoxin thresholds for human health according to WHO and Health Canada.

Table 2.4 Safe drinking water guidelines based on the WHO and Health Canada (Health Canada, 2016; WHO Chronicle, 2008)

Type of water system	***Indicators***	***Thresholds***	***References***
Drinking water	Cyanobacteria cell count	20 000 cells/ml	(Groupe scientifique sur l'eau, 2017; Health Canada, 2016)
	Cyanotoxins (MC-LR)	WHO: 1.0µg\L Health Canada and Quebec regulations: 1.5µg\L	(Health Canada, 2016; WHO Chronicle, 2008)

2.4 Cyanobacteria Oxidation

Cyanobacteria can be controlled in water resources using various methods, including nutrient reduction. Nevertheless, this method has proven to be time and energy consuming, especially in nutrient rich lakes. As a consequence, oxidant addition can be considered as an effective short-term strategy for cyanobacteria and cyanotoxins reduction (Pflaumer, 2016).

Cyanotoxins are not usually evenly dispersed in water (Spoof et al., 2020). In fact, as long as cyanobacteria cells are intact, toxins are most likely to stay mainly within cyanobacterial cells. However, toxins may be released under chemical stress, aging, environmental stress, or membrane damage (Fan et al., 2013b). It has been recommended that intact cyanobacteria cells should be removed to prevent water quality deterioration; however, whole cells removal may not always be possible because oxidants are required to fulfill other treatment purposes (Fan et al., 2013a). As a result, oxidants can be used to impair cyanobacteria cells and remove their toxins prior to entering into water treatment plants (Fan et al., 2014a). Chlorine, ozone, hydrogen peroxide, potassium permanganate, copper sulfate, and many other oxidants/algicides can be applied as pre-oxidants or as an element for source treatment (Zhou et al., 2014). The cyanobacteria cells appear to lyse when exposed to oxidants at a certain dose, which could result in intracellular toxins leakage into the environment, cause an increase in dissolved metabolites concentrations (Fan et al., 2013a). Depending on the added oxidant dose, when the toxin oxidation and degradation rate is greater than the toxin release rate, no accumulation of dissolved extracellular toxin takes place (Fan, 2013). This fact highlights the significance of studying the dose or exposure of added oxidant in order to identify the trade-off required to achieve the most efficient pre-oxidation conditions (Fan et al., 2013b). Therefore, it is crucial to investigate the impact of the added oxidant on cell integrity (Fan et al., 2014b). Consequently, the proper dose of oxidant and oxidation efficiency can vary for different sites depending on the quality of the water matrix (e.g. pH, dissolved organic carbon content (DOC)), species composition, light, meteorological conditions and mode of action of each oxidant (Health Canada, 2016; Moradinejad et al., 2020). In addition to the concentration of the oxidant or algicide, determining the appropriate contact time plays an important role as well (Fan et al., 2014a). Fan et al. (2013) studied the impact of oxidants on cyanobacterial cells and they concluded that with a rise in oxidant concentration, the number of lysed cells will increase as well. It may be practically more beneficial to apply the oxidants in the bloom's early stages to avoid intracellular toxin release (Fan et al., 2013b).

Finally, it should be noted that when cyanobacteria cells undergo oxidative stress, they tend to produce toxins, especially microcystin, as a defence mechanism. Therefore, oxidation could induce *mcy* genes inside the cells to produce microcystins (Pflaumer, 2016)

2.4.1 Hydrogen peroxide as an oxidant

In this study, the pre-oxidation process was carried out using hydrogen peroxide (H_2O_2), which is considered environmentally friendly due to its dissociation to water and oxygen. What makes H_2O_2 a promising oxidant is its remarkable toxicity and selectivity towards cyanobacteria compared to green algae (Mikula et al., 2012). Moreover, previous work has demonstrated that H_2O_2 with a dose of 20 mg/L will not be considered as toxic to fish (Zhou et al., 2013a). Also, based on previous studies, it has been shown that H_2O_2 is able to prevent cyanobacteria photosynthesis by disrupting electron transfer (Fan et al., 2013b). Additionally, hydroxyl radicals are formed by the decomposition of H_2O_2 in the water, which is able to impair DNA, protein, and existing lipids in the cells (Fan et al., 2013b; Latifi et al., 2009; Moradinejad et al., 2020). It was observed that H_2O_2 has the ability to also prevent microcystin release in water to some extent as a result of impairing transcription of the responsible genes (Mikula et al., 2012).

It is possible that the naturally occurring iron ion (Fe^{2+}) can react with H_2O_2 through Fenton reaction (Equation1.) and produce hydroxyl radical (OH•), which can directly target the DNA of bacteria and living organisms (Latifi et al., 2009).

$$Fe^{2+} + H_2O_2 \rightarrow OH^- + FeO^{2+} + H^+ \rightarrow Fe^{3+} + OH^- + OH\cdot$$

Equation 1

Additionally, another benefit of using this oxidant is the fact that the mentioned produced reactive oxygen species (ROS), mostly damage the cyanobacteria biomass rather than the existing phytoplankton community in the water sources (Pflaumer, 2016).

In addition to the sole potential of H_2O_2 in cyanobacterial bloom control, it has been demonstrated that UV light enhances the decomposition of H_2O_2 and results in the creation of hydroxyl radicals with a stronger oxidizing ability (Fan et al., 2014a). Therefore, the ability of H_2O_2 to impair cyanobacteria and cyanotoxins will be accelerated with the cooperation of the UV radiation existing in the natural light, especially on the lake surface (Fan et al., 2013b). Table 2.5 summarizes the results of selected studies using H_2O_2 as an oxidant to control cyanobacteria.

Table 2.5 Description of studies that used H_2O_2 as an oxidant to remove cyanobacteria

Type of the study	*Location of the study*	*Studied condition*	*Conclusion*	*Reference*
Lab scale study using cultured cyanobacteria strains	-	• H_2O_2 with 3 different concentrations (10.2, 51 and 102 mg/L) was added to the samples with cyanobacteria strains. • The changes were monitored within a period of 8 days.	• The lowest percentage of the intact cells can be marked 48 hours after the oxidant addition (less than 20%) using the highest dose (102 mg/L). • The oxidant was more effective on cells viability rather than the cell density. • Not only this oxidant compromised the cell integrity, but also effectively degraded the MCs.	(Fan et al., 2013b)
Lab scale study using cultured cyanobacteria strains and 50% of raw water collected from wastewater treatment plants (WWTP)	WWTP collected from outlet of a plant in Adelaide, South Australia	• H_2O_2 with 3 different concentrations (10.2, 30.6 and 51 mg/L) was added to the samples with cyanobacteria strains.	• H_2O_2 effectively controlled cyanobacteria and cyanotoxins (MCs). • 80% of the cells in control sample remained intact after 48h. • After 2 days the highest dose reduced the intact cells to 4% and two other doses inactivated 94% of the cells.	(Fan et al., 2014a)
Lab scale study using cultured *M. aeruginosa* strains	-	• H_2O_2 with 5 different concentrations (0, 0.01, 0.025, 0.05 and 0.1 mg/L) was added to the samples containing *M. aeruginosa* strains.	• 93.4% of inactivation with the highest dose exposure after 72h	(Qian et al., 2010)

The application of H_2O_2 as a pre-oxidation control strategy suffers from a few drawbacks. Firstly, homogenous dispersion of the oxidant in large volume lake would be costly. Therefore, this approach may just be effective for small lakes (Pflaumer, 2016). Another major drawback regarding H_2O_2 as a control method is the fact that its dissociation is so rapid; therefore, the application of this oxidant should be done repeatedly (Health Canada, 2016). Finally, though H_2O_2 is selective toward cyanobacteria compared to eukaryotic phytoplankton organisms, the addition of this oxidant with high doses (usually above 3.0 mg/L) in some cases could be destructive to desirable living organisms including zooplankton such as *Daphnia* (Fan et al., 2014a).

2.4.2 Copper sulfate as a treatment

Copper sulfate ($CuSO_4$) is the second chemical used as an algicide in this study. Copper, being a metal, can have toxic characteristics at high doses towards cells as it can damage the membrane integrity (Nongrum and Syiem, 2012). Copper ion (Cu^{2+}), which is generated by $CuSO_4$, was studied as one of the mostly used chemicals due to its reasonable price, efficiency and accessibility. Additionally, the usual doses of $CuSO_4$ (lower than 5 mg/L) are considered to be safe for human (Qian et al., 2010). Copper sulfate is more toxic for the cyanobacterial community as compared to other living organisms in the aquatic biota (Matthijs et al., 2016).

Existing Cu^{2+} ion in copper compounds is capable of denaturing the enzymes, impact the cell membrane permeability as well as impairing photosynthetic activity, nitrogen fixation, and phosphorus uptake (Matthijs et al., 2016). Additionally, copper addition could result in a decrease in nucleoplasm electron-density as well (Crafton et al., 2021). However, it should be taken into consideration that the accumulation of copper in sediments can be toxic. Furthermore, copper toxicity may result in killing fish in natural lakes. The application of this chemical is usually performed by spraying the $CuSO_4$ solution into reservoirs (Fan et al., 2013b). Table 2.6 summarizes the results of selected studies using $CuSO_4$ as an oxidant chemical to control or remove cyanobacteria.

Table 2.6 Summary of studies that used $CuSO_4$ to remove cyanobacteria

Type of the study	***Location of the study***	***Studied condition***	***Conclusion***	***Reference***
Lab scale study using cultured cyanobacteria (*M. aeruginosa*) strains	-	• $CuSO_4$ with 4 different concentrations (0, 0.5, 1.0 and 1.5 mg/L) was added to the samples with cyanobacteria strains. • The changes were monitored within a period of 7 days.	• The highest oxidant dose inactivated 100% of cells after 3 days. • All $CuSO_4$ doses inhibit further growth of the cyanobacteria community and toxin production. • This algicide is applicable when the bloom is at its early stage and when long period of time is available for treatment to allow biodegradation take care of released metabolites.	(Fan et al., 2013b)
Lab scale study using cultured *M. aeruginosa* strains	-	• $CuSO_4$ with 4 different concentrations (0, 0.1, 0.5, 1 and 1.5 mg/L) was added to the samples containing *M. aeruginosa* strains. • The changes were monitored within a period of 6-96 hours.	• The highest oxidant dose inactivated almost 61% of cells after 96 hours.	(Qian et al., 2010)

CHAPTER 3 RESEARCH OBJECTIVES AND METHODOLOGY

3.1 General objective

To date, no studies have explored and compared H_2O_2 and $CuSO_4$ for effective cyanobacteria control in the field *via* mesocosm experiments with an emphasis on employing metagenomics shotgun sequencing to assess both the cyanobacterial taxonomic and functional profiles during such treatment. Therefore, the objective of this study was to evaluate the impact of H_2O_2 and $CuSO_4$ on the cell integrity of cyanobacteria, toxin production and release, DOC release and the cyanobacterial community composition among the whole microbial community structure and the cyanobacterial functional composition. Mesocosms were placed in bloom water sources in Quebec, Canada (Missisquoi Bay and Petit-Lac-Saint-François), to assess the advantages and disadvantages of these control and pre-treatment options.

3.2 Specific research objectives

1) Determine the impact of $CuSO_4$ and H_2O_2 application on cyanobacterial cell integrity.
2) Study the impact of $CuSO_4$ and H_2O_2 application on DOC concentration (release/treatment).
3) Evaluate the impact of oxidant addition on cyanotoxins concentration (toxin release and degradation).
4) Investigate the potential of cyanobacteria removal/damage caused by oxidation stress using microscopic cell count.
5) Asses the changes in cyanobacterial diversity and the structural composition after pre-oxidation using taxonomic cell counts and metagenomic shotgun sequencing.

3.3 Methodology

This research was conducted as a contribution to the Algal Blooms, Treatment, Risk Assessment, Prediction and Prevention Through Genomics (ATRAPP) project. The ATRAPP project, which was started in fall 2016, aims to prevent and control cyanobacteria propagation in waterbodies, especially during bloom seasons. The first two sampling events occurred during summer and fall 2019, using mesocosms installed in two lakes, Missisquoi Bay and Petit-Lac-Saint-François respectively, situated in Quebec, Canada (see Table 3.2 for details). Mesocosms filled with water

from the bloom were installed in the lake adjacent to the docks. During this experiment, the mesocosms were exposed to natural weather conditions such as sunlight, precipitation, wind, etc. Due to difficult sampling conditions, the last set of the experiments was conducted at Polytechnique Montreal in the lab using PLSF raw water. In this final set of experiments, 2L beakers were used as microcosms instead of plastic bags, which were put on the bench with sunlight exposure to simulate the previous conditions as possible. Subsequently, the desired dose of treatments was added to each mesocosm in order to evaluate their impact on cyanobacteria removal, community composition and toxin production. Each experiment was performed for 48h of exposure time. Samples were taken from each mesocosm before oxidant addition (T0) and 48 hours (T48) after oxidant/treatment addition. $CuSO_4$ and H_2O_2 were added as treatments. Samples were analyzed for DOC and cell integrity in the Polytechnique Montreal laboratory, for toxin concentration at Environmental Chemistry lab at Université de Montreal, for taxonomic cell count at Université du Quebec à Montreal, for metagenomic shotgun sequencing at Polytechnique Montreal in collaboration with the Université de Montréal and Génome Québec.

3.3.1 Sampling site characteristics

Two separate sampling sites, starting from August till the end of October 2019, were chosen for performing this study: Missisquoi Bay, which provides raw water for a drinking water treatment plant in the southern part of Quebec, Canada and the Petit-Lac-Saint-François (PLSF) lake, which is typically used for recreational purposes. Table 3.1 indicates these two lakes description.

Table 3.1 Sampling sites description

	Missisquoi Bay	***PLSF***	***Ref***
Classification	Eutrophic	Hypereutrophic	(McQuaid et al., 2011; Vermaire et al., 2017)
Utilization	Drinking water source, recreational site	Recreational site	(McQuaid et al., 2011; Vermaire et al., 2017)
Lake area (km^2)	77.5	0.87	(Gariépy, 2019; McQuaid et al., 2011)

3.3.2 Mesocosm experiments

Mesocosms were modeled after those developed by Wood and her team (Wood et al., 2012). As it can be seen in Figure 3.1, mesocosms were made of an impermeable transparent polyethylene bag, 610 mm depth and 508 mm width. To float the bags on the surface of the water, they were attached to a foam buoy. The capacity of each mesocosm was estimated to be 21L. Then, the mesocosms were installed close to each lakeshore where they could receive sufficient sunlight.

In total, 24 mesocosms and microcosms were deployed at Missisquoi Bay (5 mesocosms), Petit lac St-Francois (10 mesocosms) and CREDEAU laboratory (9 microcosms) in the summer of 2019 (August-October) during 3 different cyanobacteria bloom events. First experiment was performed at Missisquoi Bay and the other two at Petit-lac-Saint-Francois and the CREDEAU laboratory, respectively. Experimental units were randomly assigned to control and oxidant treatments. Water samples were taken at the beginning of the experiment before any treatment addition (time zero) and after addition of oxidants/algicides (48 hours). First experiment was performed using only H_2O_2 oxidant, however, the other two were performed by adding $CuSO_4$ and H_2O_2.

Initially, the water containing cyanobacteria and scum was transferred to a 200 L barrel and mixed evenly for the experiments in Missisquoi Bay and PLSF. Then the water was uniformly distributed in all mesocosms to reduce differences in each mesocosm's initial conditions. The same mixing procedure on a smaller scale (21L barrel) was done at Polytechnique's lab prior to distribution.

One mesocosm was considered to be a control, in which no oxidant was added, (control was duplicated in experiments at PLSF due to sufficient space and resource availability) and also other mesocosms were duplicated for oxidant addition. Water samples were collected at time zero and 48 hours for metagenomics, toxin (extra- and intra-cellular), taxonomic cell count, cell integrity and dissolved organic carbon (DOC) analysis.

Two different doses of H_2O_2 (20 mg/L and 10 mg/L) and $CuSO_4$ (5 mg/L and 2 mg/L) were added to the mesocosms. Phycocyanin, chlorophyll *a*, dissolved oxygen, pH, conductivity, and temperature were measured using in situ probe (brand: YSI, model: EXO2) at the site. H_2O_2 with the dose of 10 mg/L was chosen to have the opportunity to compare the results of natural bloom oxidation with the previous work done by Moradinejad et al. (2019) in the lab. Additionally, 20 mg/L was studied as the highest dose since this concentration was proved to be safe for aquatic

community (Zhou et al., 2013a). Based on the previous studies (Fan et al., 2014b; Zamyadi et al., 2020) dealing with copper residual will be challenging. Therefore, preliminary batch tests were done in the lab before the field test to ensure finding the $CuSO_4$ doses, with which copper will be used up within 48 hours.

Figure 3.1 Mesocosms installation at Missisquoi Bay, Summer 2019

Details of types and concentrations of the added oxidants to each mesocosm, manipulation dates and the number of the mesocosms are indicated in the following table (Table 3.2).

Table 3.2 Summary of the performed experiments

Lake	***Date***	***H_2O_2***	***$CuSO_4$***	***Control***	***Number of mesocosms***
Missisquoi Bay	12-14 Aug. 2019	10 mg/L, 20 mg/L	-	-	5 (H_2O_2 in duplicate)
PLSF	09–11 Oct. 2019	10 mg/L, 20 mg/L	2 mg/L, 5 mg/L	-	10 (all mesocosms in duplicate)
Lab experiment	21–23 Oct. 2019	10 mg/L, 20 mg/L	2 mg/L, 5 mg/L	-	9 (all microcosms in duplicate except control)

3.3.3 Preparation of chemicals and reagents

Hydrogen peroxide stock solution of 40 g/L was prepared using 30% w/w Sigma Aldrich hydrogen peroxide (H_2O_2) solution. During the experiment, a colorimetric test kit (Chemetrics K-5510, Midlands, VA, USA), (limit of detection: 0.1 mg/L), was used to quantify the H_2O_2 residual. After 48h, when the second set of the samples were taken, in case hydrogen peroxide residual was detected, sodium thiosulfate was added to quench the oxidation reaction. To stop further oxidation, 1.2 mg/L of sodium thiosulfate was added per each 1 mg/L H_2O_2.

$CuSO_4$ stock solution (15 g/L) was prepared by dissolving $CuSO_4.5H_2O$ (blue crystals) in ultrapure water. A palintest, SA1100 scanning analyzer (limit of detection: 0.1 mg/L) was utilized to monitor the copper residual during the experiment to be sure about not having residual at T48. As there was not enough evidence regarding the capability of sodium thiosulfate as a quench to stop $CuSO_4$ reaction, extended exposure time (48 hours) was given to the mesocosms to avoid dealing with copper residual (Zamyadi et al., 2020).

Both oxidant/algicide stock solutions were prepared on the same day as the experiment. Then the calculated volumes (using $C_1V_1 = C_2V_2$) of stock solution were respectively added to mesocosms with to achieve desired concentrations.

3.3.4 Sampling and analysis

Before oxidant addition in mesocosms, a sample of 1L of the mesocosms (20L) and 600ml of the microcosms (2L) was taken in a sterilized bottle, pre-rinsed with lake water, to perform taxonomy, DOC, metagenomics, cell integrity and toxin analysis. T0 sampling was done to ensure the homogenous spread of cyanobacteria between the mesocosm and gain information about the condition of the mesocosm before adding treatment. Each sampling and analysis procedure will be fully explained later in separate sections. Assigned treatments were added to each mesocosm. Another set of all the mentioned samples were taken 48h after oxidant addition and taken for analysis to discover the changes been made.

It is worth mentioning that all the analysis and samplings were completed based on ATRAPP project protocols in order to maintain consistency in results to facilitate data comparison among the group.

3.3.4.1 DOC analysis

For this set of analysis, a filtration process was required. A pre-rinsed 0.45µm membrane filter (0.45µm (pore size), 47m (GHP filter), PES PALL.) was used for vacuum gravity filtration. The filtered water was then transferred to a carbon-free vial and it was then kept at 4°C. Sievers 5310C Laborator TOC (total organic carbon) meter from SUEZ company was utilized to measure the DOC in each sample at Polytechnique Montreal Laboratory.

3.3.4.2 Cyanotoxin analysis

In order to determine the cyanotoxin content, 60ml of the sample, directly taken from mesocosms, was filtered using 0.45µm sterile membrane filter (45µm (pore size), 47m (GHP filter)) by employing vacuum gravity filtration again. The filtrate (filtered liquid) was then transferred into 60mL polyethylene terephthalate glycol modified (PETG) amber bottles. The filter and the filtrate were kept at -20°C for later measurement of intracellular and extracellular cyanotoxins,

respectively. This process was performed twice (duplicate sampling) for each mesocosm at T0 and T48 to provide duplicate results.

Cyanotoxin concentrations were obtained using on-line solid phase extraction coupled to ultra-high-performance liquid chromatography high resolution mass spectrometry (SPE−UHPLC–HRMS). According to Fayad et al. (2015) (2017 campaign) and Roy-Lachapelle et al. (2019) (2018-2119 campaign) work, a combination of these two methods enables Université de Montréal to identify the presence and the concentration of around seventeen cyanotoxins (12 microcystins (Microcystin-RR, YR, HtyR, LR, HilR, LA, LY, LW, LF, [Asp3Dhb7]MC-RR and [Asp3]MC-LR), anatoxin-a, homoanatoxin-a, cylindrospermopsin, anabaenopeptin A(AP-A) anabaenopeptin B (AP-B) and cyanopeptolin A).

3.3.4.3 DNA extraction and metagenomics analysis

For this section of the study, samples were first filtered in duplicate using 0.22µm (pore size) Polyethersulfone hydrophilic sterile filter. Then, the filters were kept in 5ml DNA-RNA free Eppendorf tubes at -80°C before performing DNA extraction. RNeasy PowerWater Kit (Qiagen Group, Germantwon, MD, USA) was utilized to extract total nucleic acid from filters and following the protocol described in Jalili et al. (2021). Extracted DNA were each resuspended in 60µL of nuclease-free water and later quantified with a Qubit v.2.0 fluorometer (Life Technologies, Burlington, ON, Canada). To perform pyrosequencing (Roche 454 FLX instrumentation with Titanium chemistry), 30µL of DNA was sent to Genome Quebec. For sequencing DNA libraries, an Illumina NovaSeq 6000 S4 was applied.

Community dynamics was also assessed using shotgun metagenomics levels of phylum, order, genus and species. The number of reads for taxonomic data was normalized by relative abundance. Bioinformatic analysis for quality trimming of raw reads, artificial duplicate removal based on screening of identical leading 20 bp and assessment of taxonomic affiliation of gene fragments encoding proteins were performed at Université de Montréal (Prof. Shapiro's team) as described in Jalili et al. (Jalili et al., 2021). Statistical analysis was then performed by R (3.6.2) at Polytechnique Montreal by Juan Francisco and Hana Trigui. Analysis of Bacterial communities at the phylum, order, and genus level were performed by phyloseq (1.28.0) (McMurdie and Holmes, 2013). Normalization of taxonomic data was conducted by centered log-ratio transformation using

easyCODA (0.31.1) (Graffelman, 2019). The cyanobacterial species community was described based on the first 25 most frequent species by heatmap (1.0.12) (https://CRAN.R-project.org/package=pheatmap). Diversity indexes (Chao and Shannon) were analyzed by phyloseq's estimate_richness function and by vegan package (2.5–6) (https://CRAN.R-project.org/package=vegan).

A principal component analysis (PCA) was performed to evaluate the impact of constrained variables on the taxonomy of samples, at >95% significance. The homogeneity of variances was validated before the model implementation. A model was defined by the ordistep function (Blanchet et al., 2008) to illustrate the impact of environmental parameters on the distribution of cyanobacterial communities at the order level within treated mesocosms with H_2O_2 and $CuSO_4$ and non-treated samples. The Envfit function was used to find similar scores and to scale the fitted vectors of variables based on the correlations. The permutation test (>95% significance) was applied to select significant variables. Differential heat trees and differential heat tree matrices were drawn using the Metacoder (0.3.3) (Foster et al., 2017). For functional profiling, the predicted functions of best hits through SEED Subsystems databases (Kanehisa et al., 2012; Tatusov et al., 2000) were retrieved to assess the function of protein fragments. PCA for functional structure using SEED Subsystems databases was used to investigate the relationship between the coordinates of samples on the PCA plot and oxidation with H_2O_2 or $CuSO_4$, according to the model of Blanchet et al. (Blanchet et al., 2008).

3.3.4.4 Taxonomic cell count

Lugol's Iodine was added to 45ml of the sample, taken directly from mesocosms, in order to count the cyanobacteria. The vials were then kept at 20-25°C until they were sent to the laboratories at Université du Québec à Montréal. Taxonomic count was then performed using inverted microscopy method developed by (Lund, 1959). Microscopic cell count is always subject to human error especially at low cell density (Buskey and Hyatt, 2006), however, as the count in this study was done by a single person (Irina Moukhina) with many years of experience, it is expected that the error was minimized to the maximum extent possible. In this section of the study only cyanobacteria cells were counted and the count of phytoplanktonic community was ignored.

3.3.4.5 Cell integrity

Samples with a volume of 45mL were taken directly at T0 and T48 from the mesocosms. Then the samples were kept at 20-25°C for less than 24 hours until the analysis with flow cytometry (FCM). Integrity of the cells was evaluated by flow cytometry (BD Accuri C6 Flow Cytometer, San Jose, CA, U.S). For total and dead/compromised cells quantification, flow cytometry was utilized with staining method using SYBR Green I (SG) and propidium iodide (PI), which only enters compromised cells, respectively (Buskey and Hyatt, 2006; Nebe-von-Caron et al., 2000; Wert et al., 2013). This fast quantification method has the capability to count more than 1000 cells s^{-1}. In this counting process, any cell without a membrane which is intact is considered as dead/compromised cell. These cells that lost their viability will then decompose in the environment (Foladori et al., 2010).

CHAPTER 4 RESULTS AND DISCUSSION

In this chapter, the results of the three performed experiments are shown and discussed. The results of samples taken in duplicate, are illustrated with error bars in figures.

4.1 Evaluation of the impact of H_2O_2 and $CuSO_4$ on cell count and integrity by flow cytometry

In this section, results of total cell count are presented by (event/ml) or (cell/ml), which is quantified by flow cytometry (FCM). Cell integrity percentage for each sampling point was calculated based on viable counts divided by the total counts (viable/total). The integrity percentage was compared before and after treatment addition to evaluate each oxidant impact on the cyanobacterial viability. As observed in the previous studies, initially, the oxidant damage the cell membrane and it results in a reduction in cells viability (Coral et al., 2013). Then, further cell membrane permeability will result in total cell lysis and a change in total cell count (Coral et al., 2013).

During the first event, sampling was performed in the field at Missisquoi Bay Aug. 12-14th 2019. Only H_2O_2 was applied as a treatment to the deployed mesocosms with doses of 10 mg/L and 20 mg/L. FCM for this event was performed without coloration (SYBER Green and PI); therefore, only total/direct cyanobacteria count is available. It is shown in the following figure (Figure 4.1) that the total cyanobacteria count is similar in all the mesocosms before treatment addition, which assures the homogeneous cell distribution. No significant change was observed in the total cell numbers in the control mesocosm. The total initial cell number was around $2.0x10^6$ per mL in all the mesocosms during this event. More than 1 log decrease in total cell counts (95%) was achieved 48h after applying 10 mg/L of H_2O_2 in both mesocosms. This change has been attributed to the complete lysis of the existing cyanobacterial cells in the sample (Wert et al., 2013). As expected, with an increase in treatment dose (20 mg/L), a more dramatic drop in the total cell number was observed. More than 2 logs were reduced (>99% reduction) from the original cell number, reaching a final cyanobacteria cell number under $1.0x10^4$. Therefore, the reduction of cell density progressed with increasing concentrations of H_2O_2. A decrease has been seen in the cyanobacteria cell density after 20 mg/L of H_2O_2 addition by others (Xu et al., 2019).

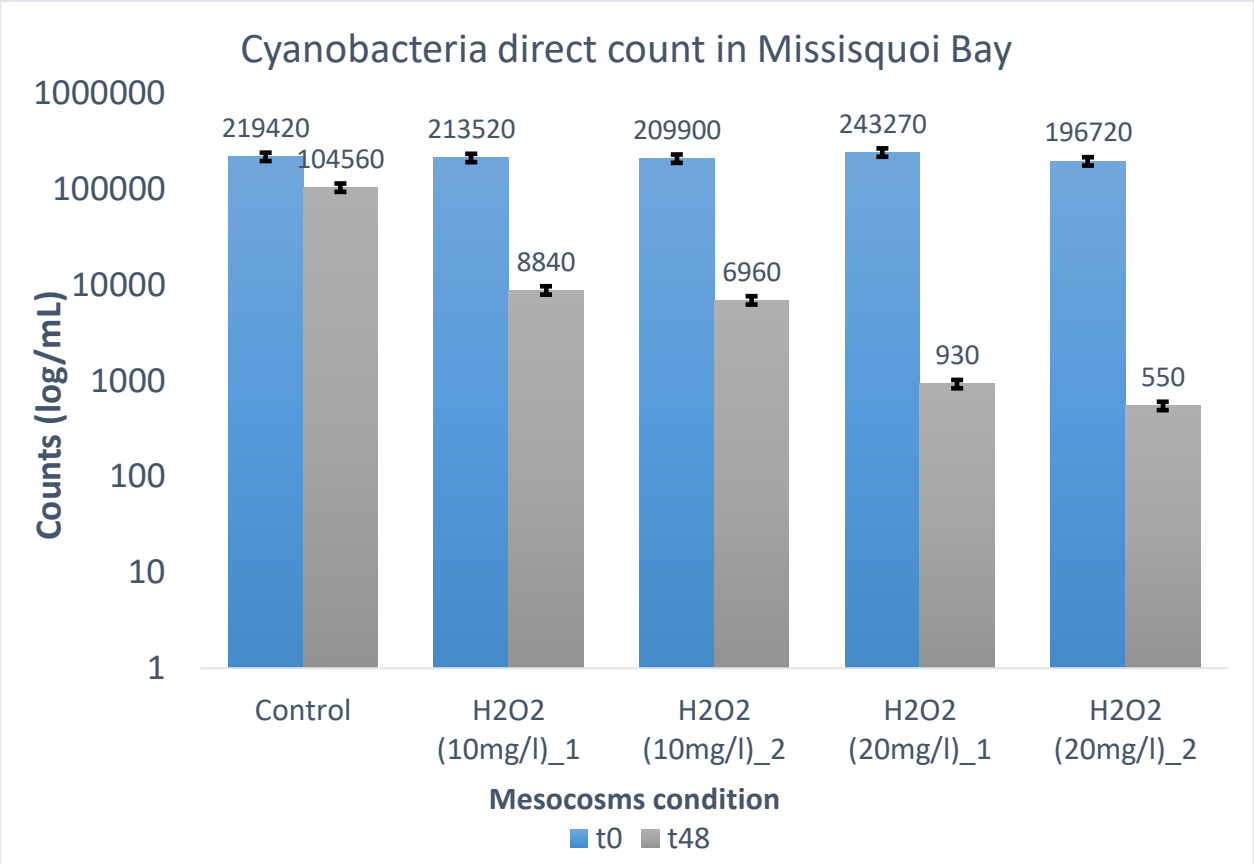

Figure 4.1 Total cyanobacteria count using FCM before and after adding H_2O_2 in Missisquoi Bay

In the second set of field experiments, at PLSF during 09–11 Oct. 2019, after performing the sampling at t0, H_2O_2 was immediately added with the mentioned doses (10 mg/L and 20 mg/L) and $CuSO_4$ was also added with doses of 2 mg/L and 5 mg/L. Cell viability was FCM with the addition of SYBER Green and PI.

Initially, all the samples had more than 70% intact cyanobacteria cells (Figure 4.2). With the addition of the lowest H_2O_2 dose (10 mg/L), a drop of 62% in cells viability can be seen. This amount increased to around 85% when the applied H_2O_2 dose was doubled. Interestingly, cyanobacteria cells exposed to both $CuSO_4$ doses almost totally lost their viability. The resulting cell integrity percentages in mesocosm containing $CuSO_4$ as the treatment dropped to less than 1% after 48 hours (Figure 4.2 A).

It was observed during the experiment that $CuSO_4$ decay is slower than H_2O_2, which is in accordance with the previous studies (Fan et al., 2013b). We assume the copper might be

accumulated in the forms of sediments at the bottom of the mesocosm when the dose is high (5mg/L). Additionally, due to the difference in the mode of action, $CuSO_4$ has a higher cell lysis rate at the added doses (2 mg/L and 5 mg/L) when compared to H_2O_2 (10 mg/L and 20 mg/L) (Xu et al., 2019). Due to these differences, $CuSO_4$ was more effective in compromising the cyanobacteria cells membrane. Consequently, $CuSO_4$ was more effective in reducing the cell integrity percentage in comparison with H_2O_2.

Regarding the total cyanobacteria count in this event (Figure 4.2 B), less than a 0.5log increase was observed in all mesocosms after 48 hours, except the mesocosm treated with $CuSO_4$. However, both $CuSO_4$ doses were strong enough to hinder the minor growth in the total counts. Unlike the last test performed in Missisquoi Bay, added treatments were unable to lyse the cells completely. This can be attributed to the fact that the initial cell density in this experiment was around $9.5x10^5$, which is almost five times higher than the previous experiment cell density in Missisquoi Bay. Additionally, the concentration of DOC could affect the oxidant performance, which is discussed later in a section 4.2. Therefore, it can be concluded that during this event, none of the treatments could impact the total cyanobacteria count due to possible higher cell density and background DOC concentration, which were studied before as well (Coral et al., 2013; Moradinejad et al., 2019). Not reaching the completed cell lysis situation is not a disadvantage since with complete cell lysis, more cyanotoxins release into the environment could be expected.

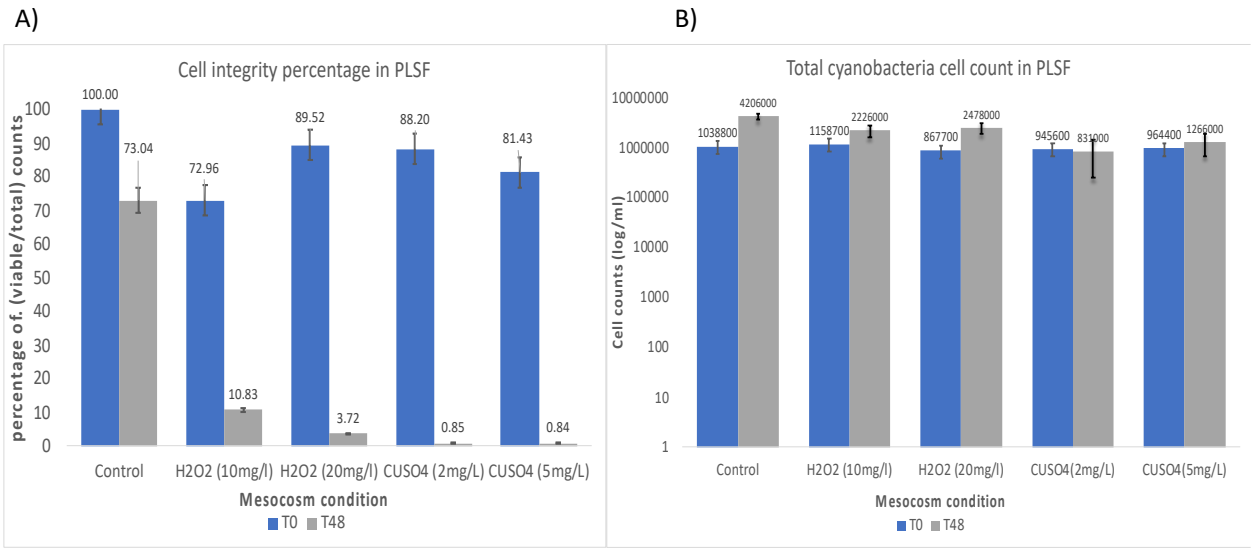

Figure 4.2 Summary of (A) Cyanobacteria cell integrity percentage (B)Total cyanobacteria cell count using FCM before and after adding H_2O_2 and $CuSO_4$ in PLSF

During the last set of experiments performed in the lab (21–23 Oct. 2019) as illustrated in Figure 4.3 A, a slight growth in the cells was evident in the control mesocosm (4.5%). Conversely, as was observed previously, the treatment was able to lower the cell integrity. Indeed, the addition of 10mg/L of H_2O_2 was capable of reducing the cell integrity percentage from 86% to 72%. At higher the H_2O_2 dose (20 mg/L), a greater reduction was observed; almost 50% of cells lost their viability. Comparing cell integrity results upon H_2O_2 oxidation of PLSF samples in the field and in the lab shows that more cyanobacteria cells lost their viability during field oxidation in PLSF. As mentioned before, this could be attributed to the fact that H_2O_2 in the field has more efficiency in impairing the cells than in the lab due to UV radiation (Fan et al., 2014b).

As observed in the previous event, among the added treatments, $CuSO_4$ was stronger in damaging the cell membrane as compared to H_2O_2. This resulted in having approximately 84% and 96% viability loss in mesocosms with 2 mg/L and 5 mg/L $CuSO_4$, respectively. Furthermore, the percentages of integrity loss obtained with both oxidants/algicides increased when the doses were higher as a result of a longer existence of H_2O_2/$CuSO_4$ residual in the field.

When looking at total cyanobacterial counts in Figure 4.3 B, it is evident that similar to the previous event in PLSF, total cell counts remained relatively stable following treatment. This illustrates the fact that in this set of experiments, total cell lysis did not occur. As the initial total cell count is

similar to the first event ($1.0x10^6$ cells), this stability of cell counts could be possibly attributed to the high DOC concentration in the background, which increases the oxidant demand. More information about the background DOC is available in the following section.

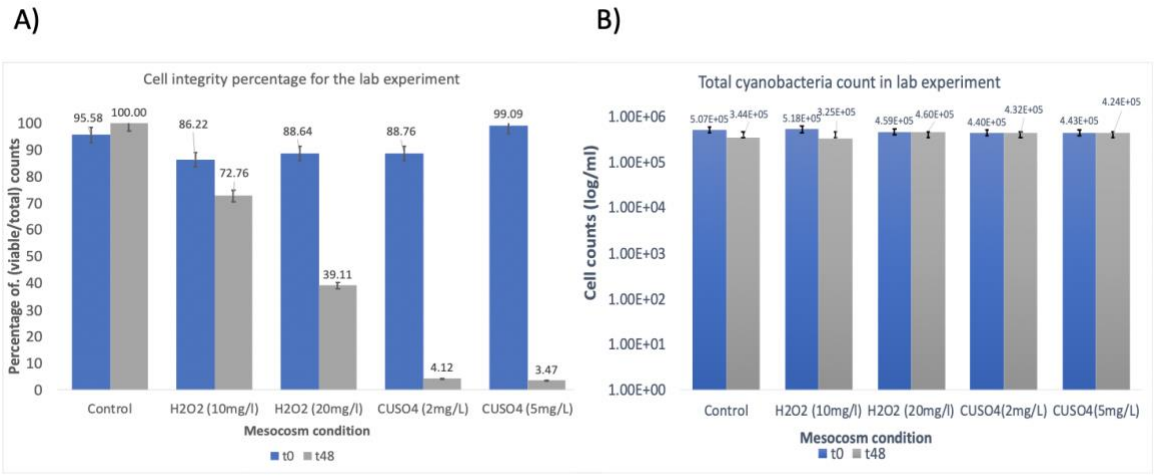

Figure 4.3 Summary of (A) Cyanobacteria cell integrity percentage (B) Total cyanobacteria cell count using FCM before and after adding H_2O_2 and $CuSO_4$ performed in the lab (raw water retrieved from PLSF)

4.2 Impact of oxidation on DOC

Dissolved organic carbon (DOC) was measured for collected samples of each mesocosm before adding treatment (T0) and 48hours after oxidant addition (T48). The difference in DOC concentration was monitored. As previously mentioned, oxidant addition will damage cell walls, resulting in the release of intracellular material. Thus, it is expected that with cell damage, the concentration of extracellular organic material will increase (Audenaert et al., 2013; Coral et al., 2013) unless, the oxidant dose is high enough to not only release the intracellular organic material but also able to break the organic chains and treat them.

During the first set of experiments performed in Missisquoi Bay, (Figure 4.4 A), the initial DOC concentration in all mesocosms varied between 5.6 mg/L to 6.5 mg/L. When comparing the DOC difference in the control mesocosm and the mesocosms containing 10 mg/L of H_2O_2, as expected, organic material was released and increased when oxidant was added. The resulting DOC

concentration was almost similar in mesocosms with 10 mg/L H_2O_2 addition (6.63 mg/L and 6.87 mg/L). When the highest dose of oxidant was added (20 mg/L), more damage was induced to the cell structure and more intracellular material was released. Thus, the final released DOC concentrations in mesocosms with 20 mg/L H_2O_2 were 7.98 mg/L and 8.25 mg/L (a release of 0.27 mg/L).

In the second set of experiments performed in PLSF, Figure 4.4 B, the initial DOC concentrations for all mesocosms were between 12-13 mg/L, which is more than twice of DOC concentration in the Missisquoi Bay samples initially (5-6 mg/L). When the initial background DOC concentration increases, the oxidant demand will subsequently grow (Matthijs et al., 2012a; Moradinejad et al., 2019). Therefore, the same oxidation dose may not be able to induce the same extent of cell damage as compared to the conditions with less background organic matter. This pattern correlates with the cell integrity results mentioned in the previous sections (4.1, 4.2).

Based on a previous study, in order to reduce by 90% the cell integrity, H_2O_2 should be added to the sample with the ratio of $4H_2O_2$: DOC (Zamyadi et al., 2020). This means H_2O_2 with a dose of around 55 mg/L is required. This explains the observed cell integrity loss percentages (62% and 86% for 10 mg/L and 20 mg/L of H_2O_2, respectively) mentioned in the previous section (Figure 4.2).

For PLSF mesocosms oxidized in the field, DOC markedly increased in all mesocosms (Figure 4.4 B), revealing an important DOC release after H_2O_2 or $CuSO_4$ addition as a consequence of cell membrane rupture. The resulting DOC concentrations in all mesocosms with treatments were almost similar. During this event, the mesocosms contained a large amount of scums, which could possibly justify the important DOC increase.

Looking at the DOC results related to the last set of experiments performed in the lab, Figure 4.4 C, the initial DOC concentration in all mesocosms were about 15 mg/L, which shows the desired distribution before the beginning of the experiment. In general, no significant change was observed in the DOC concentrations 48 hours after treatments addition. DOC values have showed a very slight increase in all mesocosms with treatments addition except in the mesocosm with 5 mg/L $CuSO_4$, which exhibit a very slight reduction as compared to the T0 mesocosm (Figure 4.4 C). Approximately 0.5 mg/L and 0.9 mg/L augmentation in DOC values in mesocosms with 10 mg/L

and 20 mg/L of H_2O_2 could respectively be attributed to intracellular material release after 48 hours. This statement can also be supported by the decrease observed in the cell integrity percentage in Figure 4.3. When the H_2O_2 dose increased, more cell damage was induced, and more intracellular organic material was released. When comparing the trend of DOC release in lab and the trend for DOC release using mesocosm in other experiments using H_2O_2, it is evident that more release was observed when mesocosms were deployed. This could be attributed to the fact that this oxidant (H_2O_2) has more destructive effect on cyanobacteria cells when it is exposed to UV radiation in natural light (Fan et al., 2014b). When 2 mg/L of $CuSO_4$ was added, a slight rise was seen within the DOC content after 48 hours due to cell rupture and release of intracellular material.

As was stated in previous section (cell integrity percentage), $CuSO_4$ with both doses almost completely damaged the cyanobacteria cells membrane. $CuSO_4$-5 mg/L were associated with a 10% decrease of DOC at T48 compared to its T0 concentration.

The extent of intracellular material release depends on several factors, in particular, the bacterial community and cells size (Coral et al., 2013; Wert et al., 2014). Besides water quality, bacterial community and cell sizes could be another reason for different DOC trends after field oxidant application (Bay Missisquoi and PLSF) and lab-scale oxidation. The dynamics within the cyanobacterial community composition after addition of H_2O_2 and $CuSO_4$ in PLSF mesocosms will be further discussed in the following sections of this thesis.

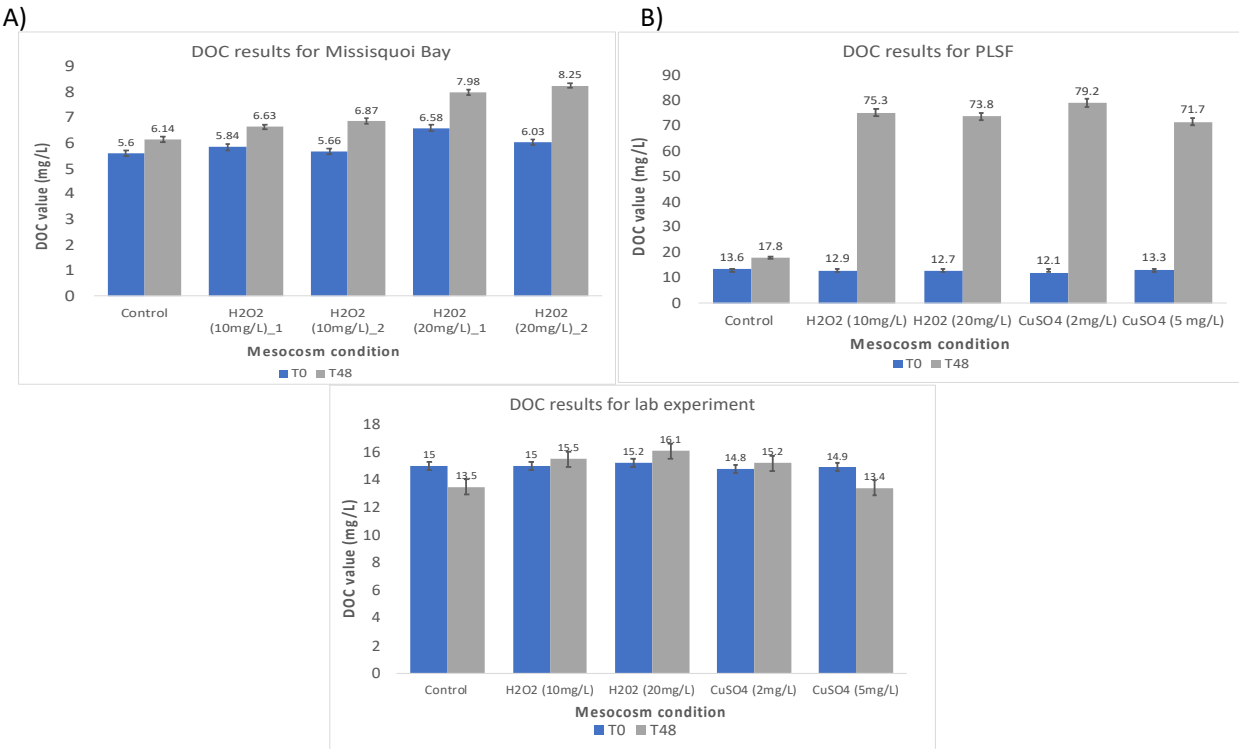

Figure 4.4 DOC concentrations in mg/L before and after oxidant addition measured in (A) Missisquoi Bay (B) PLSF (C) Laboratory using PLSF raw water

4.3 Impact of oxidation on cyanotoxin release and removal

For cyanotoxins, at each sampling point (T0 and T48), duplicate intracellular (filter) and extracellular (filtrate) samples were taken. The difference in intracellular and extracellular toxin concentrations, presented in µg/L, is discussed in this section before and after treatment.

Since microcystins (MCs) are considered the most frequently detected cyanotoxins and the fact that there are fixed regulations established, this section is focused on changes to MC concentrations. Cyanobacteria tend to release cyanotoxins under stress as a defense mechanism or as a result of cell rupture. As a result, an increase in extracellular MCs is expected along with DOC release (Latifi et al., 2009; Taton et al., 2020; Zamyadi et al., 2020).

In the experiment at Missisquoi Bay (Figure 4.5 A), the total concentrations of MCs were below the maximum acceptable concentration (MAC) established by the government of Canada (1.5µg/L). No evident change in the total concentration (intracellular and extracellular) of

microcystin in the control mesocosm was seen after 48 hours. However, when 10 mg/L of H_2O_2 was added, a remarkable decrease in the extent of the intracellular MCs was observed (decrease of more than 0.3µg/L), which can be attributed to cyanobacterial cell rupture (Zhou et al., 2013b). Even though an increase in the dissolved toxins is excepted consequent to the cell membrane damage, the concentration of the extracellular MCs increased slightly from 0.237µg/L to 0.286µg/L. This could be explained by the impact of H_2O_2 on oxidizing the released MCs in the lake when the dose is sufficient, especially in the presence of UV light (Crafton et al., 2019; Zamyadi et al., 2020). When the oxidant dose increased from 10 mg/L to 20 mg/L, the final concentration of intracellular (0.014µg/L) and extracellular (0.263µg/L) MCs were slightly lower at the end of the experiment.

Looking at the experiment in PLSF (Figure 4.5 B), in the control mesocosm, possibly due to cyanobacterial growth, the concentration of intracellular MCs increased after 48 hours. This resulted in reaching an intracellular toxin concentration of 3.34µg/L in T48 from 2.62µg/L at T0. When 10 mg/L of H_2O_2 was added, the concentration of extracellular MCs increased from 0.24µg/L to 0.77µg/L due to cell lysis and release of intracellular toxins. Simultaneously, a significant drop (around 3µg/L) is observed in the intracellular MCs concentration 48 hours after the addition of 10 mg/L of H_2O_2. Oxidation processes result in membrane damage and toxin release. Intracellular toxins were released, and they were further oxidized by H_2O_2 as was mentioned in the previous section. This resulted in having a lower sum of intracellular and extracellular MCs after 48 hours. When 20 mg/L of H_2O_2 was added, the extent of extracellular MCs followed the same pattern and decreased from 3.13µg/L to 1.28µg/L within 48 hours. The resulting extracellular toxins in both samples were below the established MAC (1.5µg/L). It can be concluded that H_2O_2 is capable of treating the toxins and decreasing the total concentration (the sum of the intracellular and extracellular toxin) after 48 hours. Additionally, as was mentioned in the literature review section, H_2O_2 can impair genes responsible for MC production and release (Mikula et al., 2012). This could be another reason for having a lower concentration of dissolved MCs at T48. Droplet digital PCR (ddPCR) monitoring the copy number of MCs synthesis gene *mcyD*, supports this hypothesis (Appendix A). In figure A.1, it can be seen that H_2O_2 addition decreased the number of mcyD genes after 48 hours. Indeed, it has been previously suggested that monitoring of *mcyD* gene, identified as a gene among ten *mcy* (A-J) genes, encoding MC synthesis (Baxa et al., 2010), could

be an appropriate approach to predict toxic cyanobacterial bloom (Davis et al., 2009; Fortin et al., 2010b; Rinta-Kanto et al., 2009). Pearson correlation analyses (p-value<0.05) for the *mcyD* gene copy numbers, assessed by ddPCR, and MCs concentrations in PLSF are presented in Appendix A. When H_2O_2 was added, copy number of *mcyD* gene correlated positively with cell integrity and intracellular MCs. No significant correlation was found between *mcyD* copy number and dissolved MCs.

Lastly, previous studies demonstrated that bacteria such as *Proteobacteria* in the community can contribute to MC degradation (Kormas and Lymperopoulou, 2013). The relative abundance of this phylum within the bacterial community will be discussed in this thesis's section regarding cyanobacteria structural composition dynamics after oxidation in PLSF.

On the other hand, when $CuSO_4$ was added as a treatment, an obvious decrease in the intracellular and an undeniable increase in the extracellular toxin concentrations are observed. This can be attributed to the cell damage provoked by this algicide. The same pattern was noted in previous studies (Matthijs et al., 2016; Zhou et al., 2013b). After 2 mg/L $CuSO_4$ addition, the concentration of extracellular MCs increased from 0.18µg/L to 2.63µg/L. When the $CuSO_4$ dose increased to 5 mg/L, the extent of dissolved MCs released increased as well until it reached the concentration of 3.08µg/L. This release could also happen as a result of the stress, induced by $CuSO_4$ addition, which resulted in the total release of intracellular metabolites. It can be concluded that cyanobacteria cells are more susceptible to $CuSO_4$; however $CuSO_4$ is unable to oxidize or break the released extracellular toxins (Zamyadi et al., 2020). The resulting extracellular toxin concentrations in mesocosms with $CuSO_4$ addition exceed the established MAC. This issue should be considered, as it would be problematic since several conventional treatment processes are not able to remove dissolved cyanotoxins (Henderson et al., 2008; Zamyadi et al., 2013) thereby increasing the chance of cyanotoxin breakthrough from the drinking water treatment plant.

Results of *mcyD* copy number and MC concentrations revealed a significant positive correlation (p-value<0.05) between *mcyD* copy number and dissolved MCs as well as $CuSO_4$ exposure time. A significant negative correlation (p-value<0.05) was obtained between *mcyD* copy number and cell integrity, supporting the negative impact of $CuSO_4$ on cell integrity that was found following 48 hours of $CuSO_4$ oxidation. ddPCR results monitoring *mcyD* gene copy number during $CuSO_4$

addition in PLSF corroborates with MCs release and cell integrity data. Performed ddPCR analysis during onsite treatment with either H_2O_2 or $CuSO_4$ suggested that the monitoring of *mcyD* gene could be an appropriate approach to predict treatment efficiency to cope with toxic cyanobacterial bloom.

Lab experiment results with PLSF raw water illustrate that all the total MCs were still below the MAC (1.5 μg/L) even after treatment addition (Figure 4.5 C). The concentration of total intracellular and extracellular MCs remained almost unchanged in the control mesocosm during the experiment, with a slight decrease of 0.06 μg/L in the extracellular toxin concentration 48 hours later. When the lowest H_2O_2 dose (10 mg/L) was added, the extracellular microcystin moderately increased from 0.358 μg/L to 0.495 μg/L, which can be attributed to mild cell damage and cell response oxidative stress. When the H_2O_2 dose doubled (20 mg/L), cyanobacteria released their toxins, resulting in around 0.4 μg/L more final dissolved toxin than the initial condition. This dose was also capable of slightly lowering the summation of intracellular and extracellular metabolites during the experiment.

As was observed in the previous event in PLSF (field), $CuSO_4$ with both doses severely attacked the cell membrane and resulted in a noticeable release of intracellular metabolites. With 2 mg/L and 5 mg/L $CuSO_4$ addition, MC concentration inside the cells underwent decreases of 0.36 μg/L and 0.5 μg/L respectively. After the induced stress, these intracellular metabolites released resulted in dissolved MC mesocosm concentrations in mesocosm concentration 0.73 μg/L (2 mg/L $CuSO_4$ dose) and 0.34 μg/L (5 mg/L $CuSO_4$ dose).

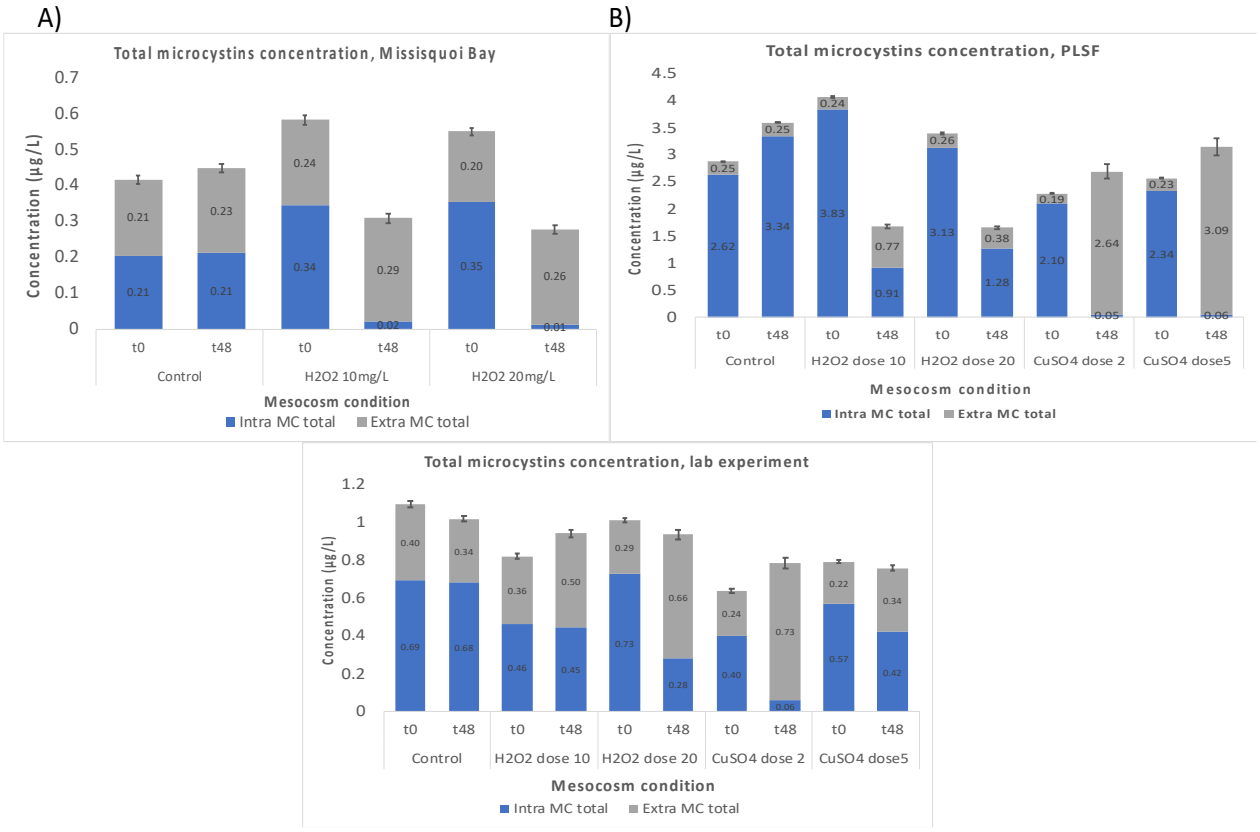

Figure 4.5 Concentrations of total intracellular and extracellular microcystin measured in (A) Missisquoi Bay before and after addition of H_2O_2 (B) PLSF (C) Lab before and after addition of H_2O_2 and $CuSO_4$.

4.4 Impact of oxidation on taxonomic cell count

Taxonomic cyanobacteria cell counts by microscopy in this section are presented in units of (cells/ml). Microscopic cell counts provide the absolute cyanobacteria cell count, in contrast to metagenomic sequencing methods, which provide the relative abundance of the cyanobacterial community (Moradinejad et al., 2020).

In this section, the ability of each oxidant/algicide for reducing the cyanobacterial cell density is assessed within 48 hours of the experiment. Cell count results illustrated at the species level are shown in Figure B.1 (Appendix B). In this section, cell counts are analysed at the genus level for the three events (oxidation in the field in Missisquoi Bay and PLSF and Lab oxidation of PLSF samples).

As is illustrated in Figure 4.6 A (Missisquoi Bay experiment), total cell counts in all mesocosms at T0 are similar and equal to $1.7x10^5$. The most abundant genus in the experiment, on 12^{th}-14^{th}Aug. 2019, is *Dolichospermum (Anabaena)*, followed by *Aphanizomenon*. Except for the control mesocosm, the cyanobacteria cell density decreased in all mesocosms with oxidant addition.

When 10 mg/L of H_2O_2 was added, a reduction of 1log (90%) in cyanobacteria cell number was observed in both duplicates after 48 hours. This reduction increased to slightly less than 2logs (94%) when 20 mg/L of this oxidant (H_2O_2) was added. Therefore, as expected, more cyanobacteria cells were removed with a higher dose of the oxidant due to higher exposure and contact time.

Figure 4.6 A shows that even after oxidation (T48), *Dolichospermum (Anabaena)* remained the dominant genus. At the same time, the most evident decrease in cells number with oxidant addition was seen within this genus compared to the other existing genera after 48 hours.

As seen in Figure 4.6 B (PLSF experiment), according to the taxonomic cyanobacteria cell counts, similar to the previous experiment, *Dolichospermum (Anabaena)* was again the most abundant taxa. As also observed in metagenomics results provided in the following section (Figure 4.12). No evident difference was observed in the cyanobacteria cell count reduction pattern when the H_2O_2 oxidant concentration increased from 10 mg/L to 20 mg/L. With both doses, a slightly more than 1log decrease in the counts were observed at T48.

When 2 mg/L of $CuSO_4$ was added in the mesocosm, no significant drop was observed at T48 (induced lowest reduction in cyanobacteria cell counts compared to other treatments). This could be an indication of insufficient concentration added. However, in mesocosm with 5 mg/L $CuSO_4$, the steepest drop (almost 2logs or 99%) among all the mesocosms in the cell count was observed. Therefore, it can be concluded that the cyanobacterial community was more susceptible to $CuSO_4$-5 mg/L.

Finally, based on the microscopic count, *Dolichospermum (Anabaena)* remained by far the most abundant taxa even after the addition of both treatments. However, it was the taxa that was most affected by treatment addition. In this set of experiment, $CuSO_4$-5 mg/L had the most impact on cyanobacteria cell reduction, followed by H_2O_2.

It is evident in Figure 4.6 C that again the experiment performed in the lab using PLSF raw water, *Dolichospermum (Anabaena)* is still the most found taxa under the microscope, followed by

Aphanothece and then *Aphanocapsa* genera. Similar to the previously seen pattern regarding the addition of 10 mg/L and 20 mg/L of H_2O_2 in the previous experiment (Figure 4.6 B), no noticeable difference (2%) was seen when the concentration of this oxidant doubled. Both of which were capable of removing slightly more than 1log (95%) of the total existing cyanobacteria cells after 48 hours. It seems like in both mesocosms with H_2O_2 as oxidant, *Aphanothece* taxa appeared to be slightly more resilient to oxidation compared to *Dolichospermum (Anabaena)*.

Like the previous event (Figure 4.6 B), $CuSO_4$-2 mg/L was demonstrated to be the weakest treatment among treatments tested by inducing around 0.5log (65%) of total cyanobacteria cell reduction at T48. Conversely, when the $CuSO_4$ dose increased to 5 mg/L, total cyanobacteria cell count decrease reached 1log (90%).

Consequently, H_2O_2 with both doses and $CuSO_4$-5 mg/L induced similar effects on taxonomic cell count reduction after 48 hours, and $CuSO_4$-2 mg/L proved to be the weakest treatment.

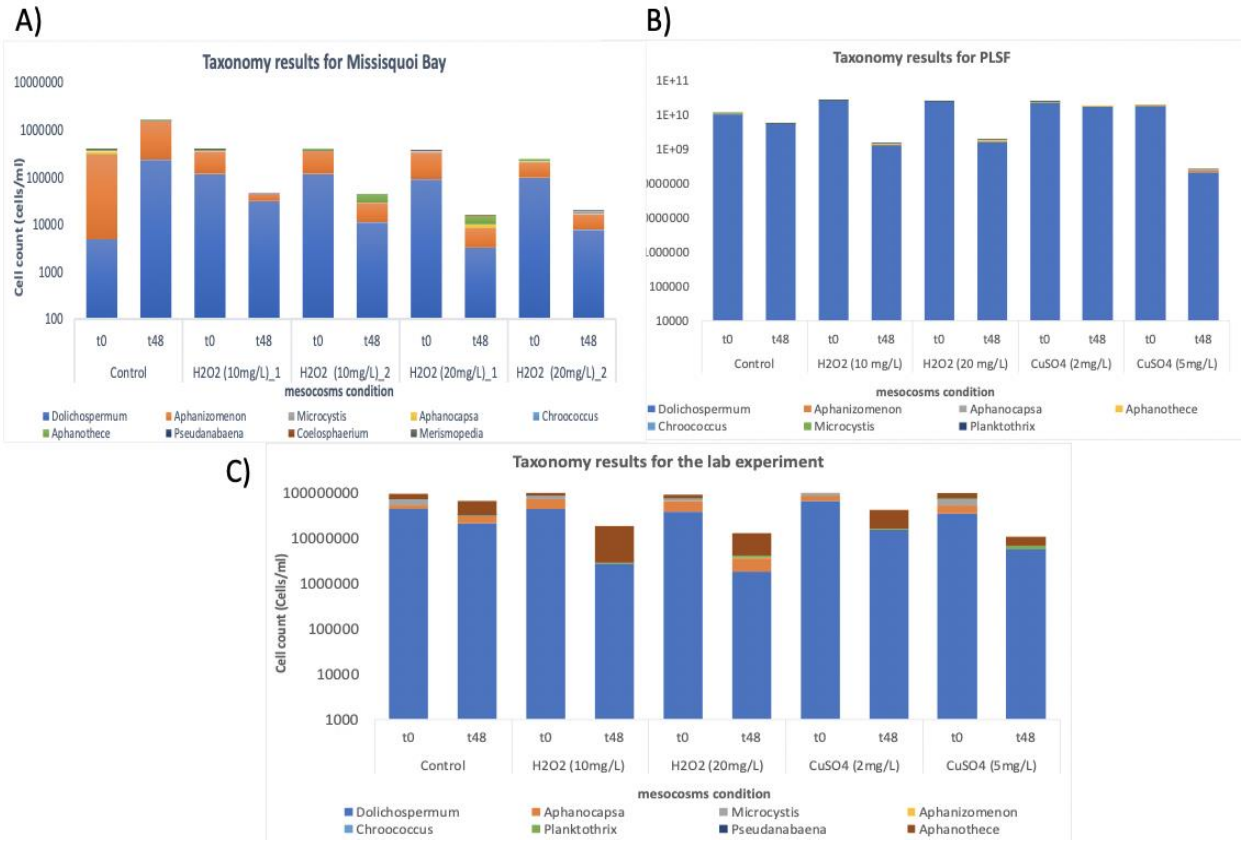

Figure 4.6 Cyanobacteria microscopic taxonomic cell count presented in logarithmic scale at genus level in (A) Missisquoi Bay (B) PLSF (C) the Lab experiment

4.5 Impact of oxidation on cyanobacterial diversity within mesocosms assessed by shotgun metagenomic sequencing: PLSF

4.5.1 Cyanobacterial composition

Diversity, community variation and composition of cyanobacteria were assessed in mesocosm samples retrieved from PLSF following 48hours of on-site treatment with $CuSO_4$ (2 mg/L, 5 mg/L) and H_2O_2 (20 mg/L), using comparative shotgun metagenome sequencing at the phylum, order and genus level. The number of reads for taxonomic data was normalized by relative abundance. Metagenomes of control mesocosms, that were not oxidized, were also analyzed at T0 (immediately before oxidation) and T48 (48h following oxidation). As described in the methodology, two samples were taken from each mesocosm in all conditions ($CuSO_4$ oxidation, H_2O_2 oxidation and control) at both T0 and T48. Due to the low quality and/or quantity of either extracted DNA or DNA sequencing, one of the duplicates of T48-oxidized sample with 2 mg/L of $CuSO_4$ and all samples from mesocosm with 10 mg/L H_2O_2 addition and one of the duplicates of T0-sample of all tested conditions ($CuSO_4$, H_2O_2 oxidation and control (without oxidation)) were not statistically analyzed after metagenomic shotgun sequencing. Shotgun metagenomic sequencing was assessed for PLSF only in this section, and samples for other events are on the waitlist to be analyzed.

Analysis of relative abundance of the bacterial community at the phylum level for all samples (oxidized and non-oxidized at T0 and T48) showed that *Cyanobacteria*, *Bacteroidetes*, *Proteobacteria* were the three most abundant phyla followed by *Actinobacteria*, *Firmicutes* and *Verrucomicrobia* (Figure 4.7 A). Control mesocosm samples (non-oxidized) had similar taxonomic profiles at T0 and T48. Cyanobacteria were by far the most abundant phylum in control condition (both T0 and T48). It also remained the predominant phylum within oxidized samples at both T0 and T48, but at a lower extent when treatments (H_2O_2 and $CuSO_4$) were added (T48). After 48h of H_2O_2 exposure, the fraction reduction in the relative abundance of cyanobacteria is less than what was observed after $CuSO_4$ exposure with both doses (2 mg/L and 5 mg/L). Indeed, the relative abundance of cyanobacteria decreased markedly from T0 to T48 (from 84% to 47% after $CuSO_4$ addition (2 mg/L), from 87% to 63% after $CuSO_4$ addition (5 mg/L) and from 59% to 50% after

H_2O_2 oxidation (20 mg/L)). The changes in the cyanobacterial community structure suggest that cyanobacteria are sensitive to both doses of $CuSO_4$ (2 mg/L and 5 mg/L) and H_2O_2 (20 mg/L) with lower tolerance toward H_2O_2 compared to $CuSO_4$.

Parallel to the decrease of the relative abundance of cyanobacteria after 48h in mesocosm with treatments added, both *Bacteroidetes* followed by *Proteobacteria* relative abundance increased after $CuSO_4$ addition (2 mg/L and 5 mg/L). Indeed, *Bacteroidetes* and *Proteobacteria* account for less than that 5% of total relative abundance assigned to the phylum level for all T0 samples (control and oxidized samples). After 48h of $CuSO_4$ oxidation (2 mg/L), *Bacteroidetes* and *Proteobacteria* relative abundance is five times higher (around 25%) than that observed at T0. Indeed, at a higher dose with stricter treatment, $CuSO_4$ (5 mg/L) and after 48h of exposure, *Bacteroidetes* account for 20% in both duplicates, while *Proteobacteria* account for 7% and 10% in T48 duplicates #1 and #2, respectively. As for the oxidation with H_2O_2 (20 mg/L), *Bacteroidetes* does not show any change in its relative abundance between T0 and T48 samples, while the relative abundance of *Proteobacteria* has shown an opposite pattern as compared to what was observed for cyanobacteria. In fact, *Proteobacteria* relative abundance increased in H_2O_2 mesocosm from 20% at T0 to 30% and 35% at T48 in duplicates #1 and #2, respectively. Previous studies have also reported that several phylogenetically diverse heterotrophic bacteria within Proteobacteria have displayed a competitive advantage compared to cyanobacteria following oxidation (Daft and Stewart, 1971; Wright and Thompson, 1985). In addition, the increase of *Proteobacteria* composition within the bacterial community upon H_2O_2 oxidation has been observed by Wang et al. (2017) after advanced oxidation processes on subsequent biological water treatment. Altogether, it suggests that *Proteobacteria* may be better adapted to chemical oxidation stress induced by H_2O_2 than other phyla and that included oxidation response mechanism during H_2O_2 exposure.

Figure 4.7 B and 4.7 C show the cyanobacterial community composition changes at order and genus level, respectively. At the order level, the cyanobacterial community was mainly dominated by members of the *Nostocales* followed by *Oscillatoriales*, *Chroococcales*, *Stigonematales* and *Pleurocapsales*. At the genus level, the proportion of unclassified/unidentified sequences are the most abundant proportion with more than 65%. Among the identified genera, the cyanobacterial community was primarily represented by *Nostoc*, followed by *Fischerella*, *Calothrix*, *Microcystis*, *Cyanothece* and *Anabaena (Dolichospermum)*. No change has been detected between T0 and T48

taxonomic profiles for all samples (oxidized and control (non-oxidized)), except an evident composition change at the order level for the oxidized replicate #2 with H_2O_2 (20 mg/L) at T48, when compared with replicate #1 and its corresponding T0 profile. Indeed, we observed a decrease within *Nostocales versus* a slight increase within *Oscillatoriales* and *Stigonematales*. At the order level, the stability within cyanobacterial community relative abundance before and after oxidation might be attributed to the fact that there is an equal chance for all phylum to be affected by the added treatment. Therefore, the change within the order level could be visible if the absolute abundance was available instead.

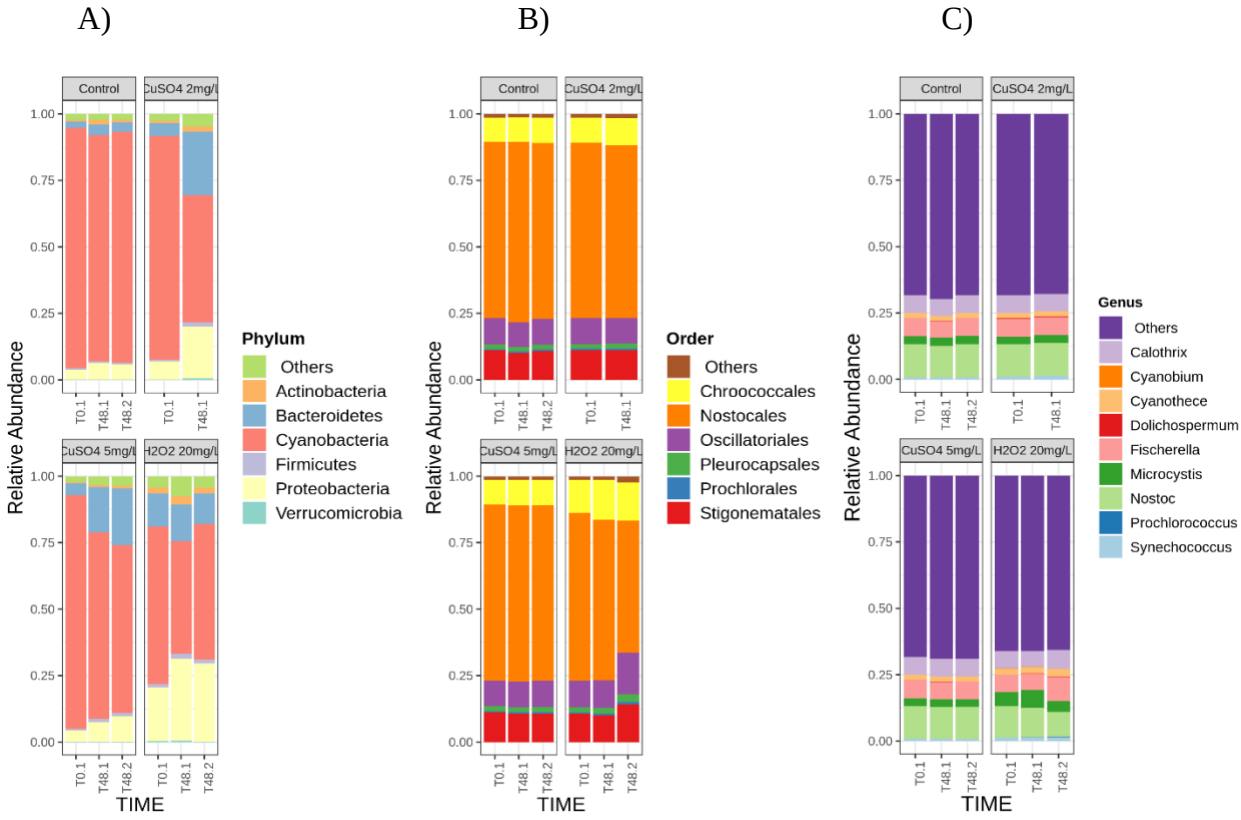

Figure 4.7 Identity of major detected bloom-associated cyanobacterial community members at T0 and T48 of oxidation with $CuSO_4$ (2 mg/L and 5 mg/L) and H_2O_2 (20 mg/L): (A) relative abundance of the different phylum, (B) the relative abundance of orders within cyanobacterial phylum, (C) relative abundance of genera belonging to the *Nostocales*, *Chrooccocales* and *Oscillatoriales* orders.

To better survey cyanobacterial community composition after oxidation with $CuSO_4$ and H_2O_2, we used a differential heat tree matrix, which allows for a better understanding of community structure

than stacked bar charts. Indeed, the intensity color of nodes and edges are correlated with the abundance of taxa in each community. The intensity of the color is relative to the log-2 ratio of the difference in median proportions (Foster et al., 2017). The green color indicates that the taxa is more abundant in the sample stated on the ordinate (T0) than the sample stated on the abscissa (T48), while the brown color indicates the opposite. Figure 4.8 illustrates differential heat tree matrices that allows examining the impact of the three treatments at once, by showing pairwise comparisons of the cyanobacterial community after 48h oxidation with H_2O_2-20 mg/L, $CuSO_4$-2 mg/L and $CuSO_4$-5 mg/L. The gray tree on the lower left serves as a key for the smaller unlabeled trees. Figure 4.8 depicted the same trend of change for the most abundant order and genus already described above in Figure 4.7. In addition, it displayed differences in the abundance of each taxon, some of them were not revealed by stacked bar charts.

Interestingly, the matrix of $CuSO_4$-2 mg/L-T48 and $CuSO_4$-2 mg/L-T0 shows that MCs producers *Dolichospermum (Anabaena)* and *Anathece* are significantly the most enriched representative genera after 48h of $CuSO_4$-2 mg/L oxidation (dark brown nodes, red circular frame), followed by *Prochlorococcus Cyanobium* and *Cylindrospermopsis* (clear brown nodes) (red circular frame, Figure 4.8 A). *Dolichospermum (Anabaena)* was also found to be the most abundant genus by taxonomic cell count for all treatments (Figure 4.6). For $CuSO_4$-2 mg/L, it exhibited the lowest cell count decrease within *Dolichospermum (Anabaena)* after 48h.

Comparing $CuSO_4$-5 mg/L-T48 with $CuSO_4$-5 mg/L-T0 cyanobacterial relative abundance, the matrix shows that the three most enriched genera are the cytotoxic compound producers, *Jaaginema*, *Symploca* and *Scytonematopsis* (red circular frame, Figure 4.8 B). To less extent, *Thermosynechoccus*, *Gloecapsopsis* and *Gloeothece* are also more enriched $CuSO_4$-5 mg/L-T48.

H_2O_2-20 mg/L-T48 *versus* H_2O_2-20 mg/L-T0 matrix shows a noticeable dominance of the brown color within cyanobacterial clades, especially those affiliated to *Oscillatoriales* and *Stigonematales*, confirming the persistence of their affiliated taxa during H_2O_2 oxidation (red circular frame, Figure 4.8 C).

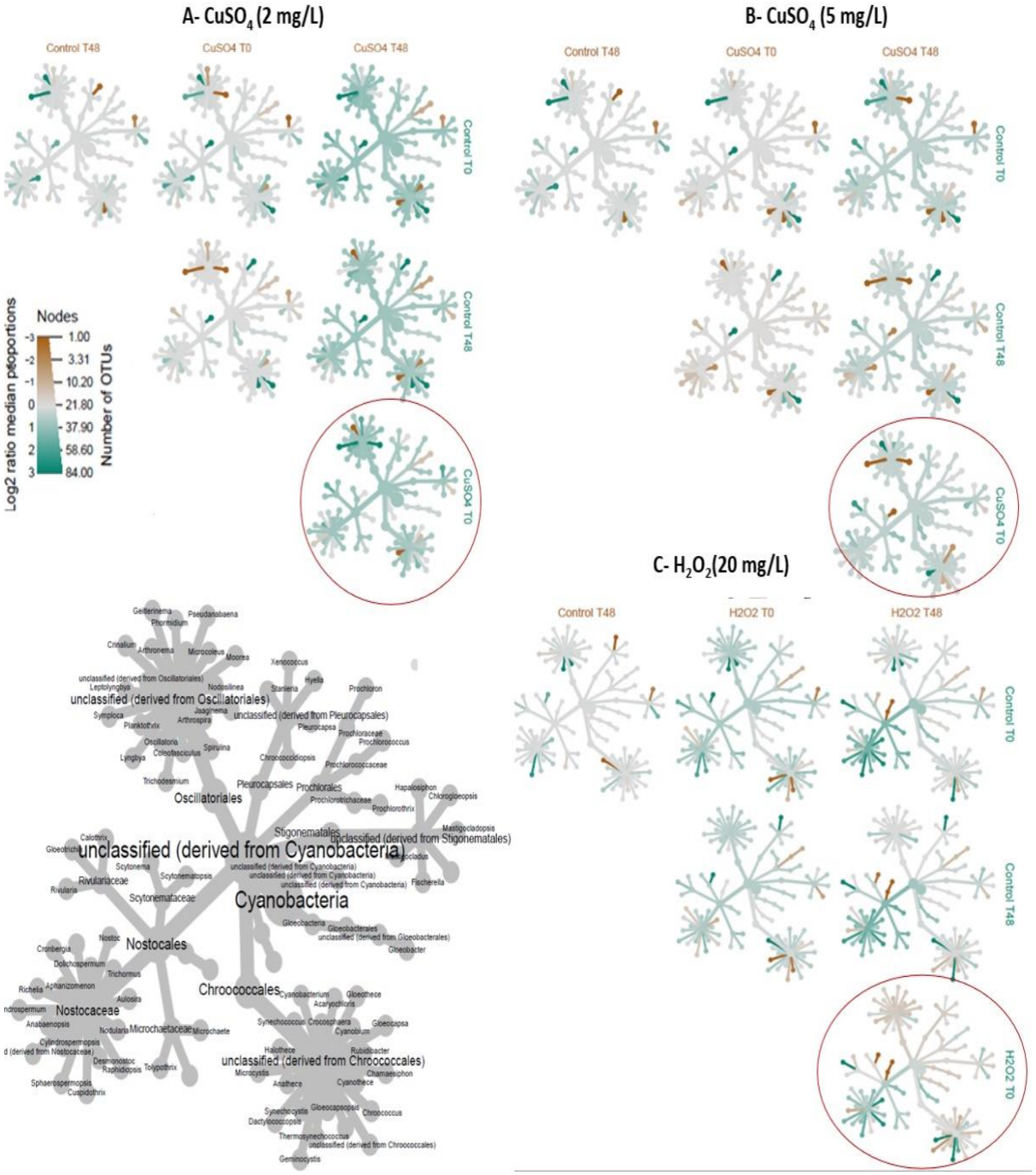

Figure 4.8 Heat trees matrix showing pairwise comparisons of communities across oxidation with H_2O_2-20 mg/L, $CuSO_4$-2 mg/L and $CuSO_4$-5 mg/L

What is intriguing in the metagenomics profiling after $CuSO_4$ addition is the unexpected higher relative abundance of cyanobacteria with $CuSO_4$-5 mg/L addition compared to $CuSO_4$-2 mg/L (Figure 4.7). It was not expected because cell count results have shown that cyanobacterial cells

treated with $CuSO_4$-2 mg/L for 48hours were less compromised than those treated with higher dose ($CuSO_4$-5 mg/L). For that, we sought to explore the structural composition of the bacterial and cyanobacterial community before and after oxidation. Dynamics of the composition structure of the neighboring and/or associated microbial heterotrophic species after oxidation, may be behind this disproportional relative abundance of cyanobacteria towards $CuSO_4$ addition. Indeed, cyanobacteria provide microenvironments for interactions with associated microorganisms that shelter in the cyanobacterial mucilage (Abed et al., 2018). Cyanobacterial tolerance to oxidant may be affected by direct and /or indirect changes in the taxonomic structure of these associated communities. According to our result, taxa assigned to *Bacteroidetes*, *Proteobacteria*, *Actinobacteria* and *Firmicutes* are frequently detected to share the ecosystem of cyanobacteria in freshwater sites and to be dominant heterotrophic phyla associated with the micro-environment of cyanobacteria (Ding et al., 2015; Zwart et al., 2002). In the following section, we broadly survey the impact of oxidation on the composition changes of the most abundant heterotrophic phyla that were represented in Figure 4.9: *Bacteroidetes*, *Proteobacteria*, *Actinobacteria* and *Firmicutes.*

4.5.2 Composition of bacterial communities surrounding cyanobacteria

Figure 4.7 shows that in both $CuSO_4$ containing mesocosms (2 mg/L and 5 mg/L), *Bacteroidetes* and *Proteobacteria* relative abundance were higher at T48 mesocosms, compared to the non-oxidized mesocosms (T0), whereas cyanobacteria exhibited the reverse trend. Indeed, after 48h of $CuSO_4$-2 mg/L and $CuSO_4$-5 mg/L addition, *Bacteroidetes* relative abundance was 6 and 5 times higher, respectively than those observed at corresponding T0 samples, whereas *Proteobacteria* tripled and doubled, respectively.

For the oxidation with H_2O_2 (20 mg/L), the trend is different from the trend mentioned for $CuSO_4$. While the *Bacteroidetes* community does not show an important change in its relative abundance between T0 and T48 samples, *Proteobacteria* relative abundance increased from 20% at T0 to around 30% at 48h. Our findings suggest that *Bacteroidetes* and *Proteobacteria* better tolerate $CuSO_4$ addition (2 mg/L and 5 mg/L) and H_2O_2 (20 mg/L) than cyanobacteria.

Interestingly, differential heat trees (Figure 4.9), which demonstrates the changes in bacterial taxonomic profiles after 48h of each oxidant, relative to the corresponding non-oxidized samples (T0/T48), shows that Natranaerobiales within the order of Firmicutes is more abundant at T0 rather

than T48 after oxidation with $CuSO_4$-5 mg/L and $CuSO_4$-2 mg/L, displaying almost the same level of enrichment in both doses of $CuSO_4$. Like cyanobacteria, Natranaerobiales, which encompasses fermentative heterotrophs, seem to be sensitive to $CuSO_4$ (2 mg/L and 5 mg/L). No difference in the relative abundance of Natranaerobiales between T0 and T48 after H_2O_2-20 mg/L oxidation has been observed, suggesting that this group of Firmicutes better tolerates H_2O_2-20 mg/L than $CuSO_4$ (2 mg/L and 5 mg/L).

For *Actinobacter*, the differential heat tree notably shows that *Actinobacter* nodes are dark brown after $CuSO_4$-5 mg/L oxidation, meaning that clades of *Actinobacter* are more abundant in the non-oxidized sample, suggesting susceptibility of Actinobacterial taxa to $CuSO_4$-5 mg/L. This result was not obtained for H_2O_2-20 mg/L nor $CuSO_4$-2 mg/L, suggesting that the treatment with higher dose of $CuSO_4$ (5 mg/L) may be directly and/or indirectly behind this taxonomic profile response of *Actinobacter* and consequently the higher relative abundance of cyanobacteria after $CuSO_4$-5 mg/L compared to 2 mg/L of $CuSO_4$ (Figure 4.7).

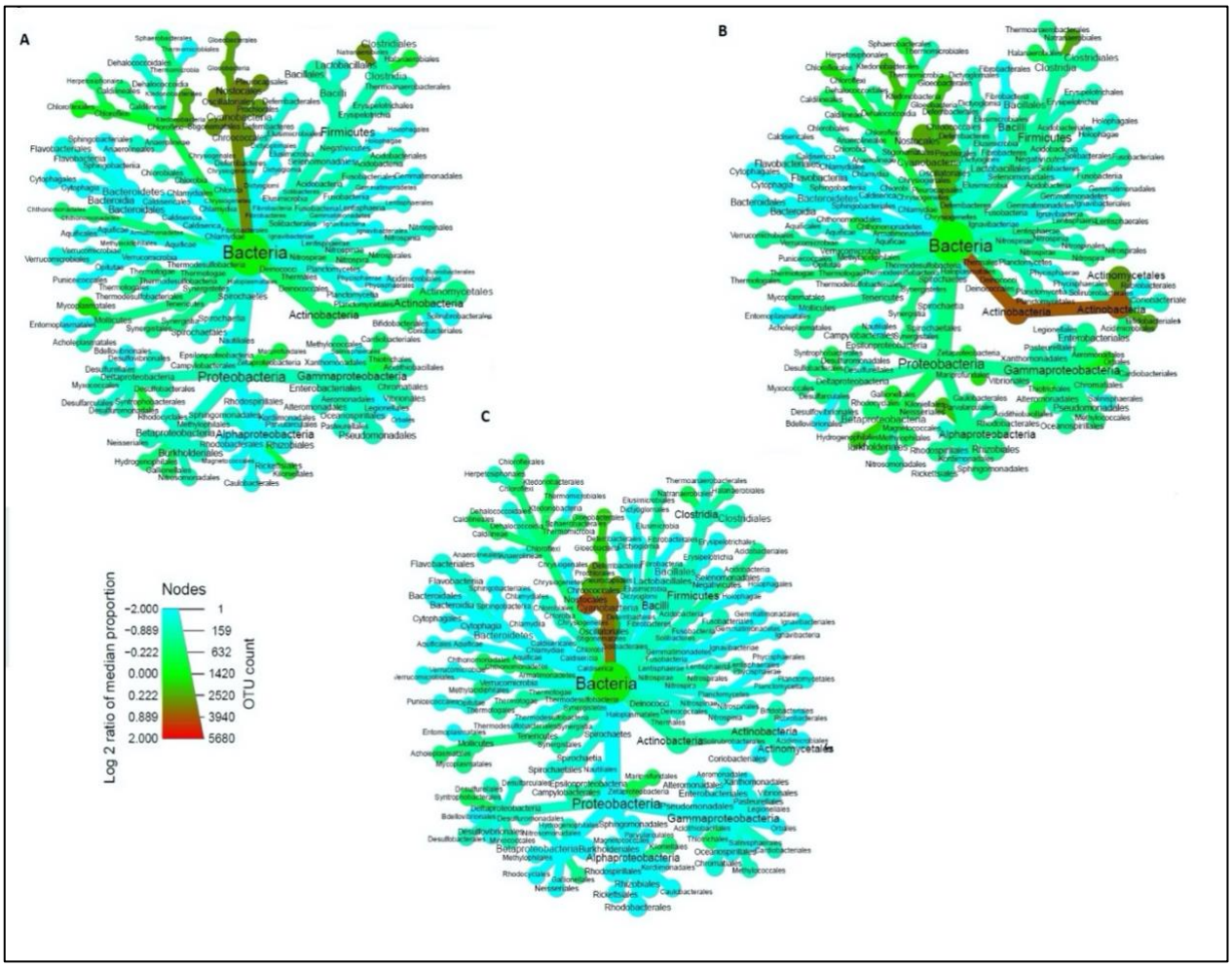

Figure 4.9 Differential heat tree demonstrating changes in taxonomic profiles the class level of mesocosms placed in PLSF oxidized for 48h with (A) $CuSO_4$-2 mg/L (B) $CuSO_4$-5 mg/L and (C) H_2O_2-20 mg/L at T0/T48, T0=control, T48= after 48h contact time.

4.5.3 Principal Component Analysis of the microbial community in PLSF

Principal Component Analysis (PCA), Figure 4.10 A, shows the dissimilarity among the bacterial communities following oxidation with $CuSO_4$ at two different doses (2 mg/L and 5 mg/L) and H_2O_2 (20 mg/L). Principal axis one and principal axis two for PCA represent 12.4% and 75.8% of the variation among the samples, respectively. Samples that appear more closely together within the PCA are assumed to be more similar in bacterial and cyanobacterial composition.

Three cluster groups are distinguished in PCA results (Figure 4.10). The first one (framed in blue) includes the control condition samples at T0 and T48 and non-oxidized T0-samples for $CuSO_4$ and

H_2O_2 conditions tested. The community structure of this mesocosm group correlates with the production of total intracellular microcystins (MCs). This means that at the initial condition (T0) and the control mesocosm, the highest values of the intracellular MCs were detected, which is attributed to having intact cyanobacteria cells. Figure 4.10 B and 4.10 C show that the taxonomic profile of this group is associated with cyanobacteria phylum and, more precisely, with *Nostocale*, *Prochlorales*, *Osciallatoriales*, *Stigonematales* within the known orders of cyanobacterial taxa.

$CuSO_4$ oxidation at two different doses, 2 mg/L (one replicate) and 5 mg/L (duplicate #1 and #2)), are grouped in a distinct second cluster (framed in green), revealing close microbial assemblage between both $CuSO_4$ oxidation conditions. Interestingly, PCA shows also that total dissolved MCs and DOC correlate with this cluster. That finding corroborates with cell integrity and MCs assessments after $CuSO_4$ addition (2 mg/L and 5 mg/L) (figures 4.2 and 4.5 B), where the highest concentration of total MCs, mainly composed of extracellular MCs, and the lowest percentage of intact cells were obtained. The considerable distance between $CuSO_4$ exposure at T0 and T48 in PCA shows that $CuSO_4$ (2 mg/L and 5 mg/L) induced large shifts in the corresponding taxonomic profiles, supporting the impact of $CuSO_4$ (2 mg/L and 5 mg/L) on the microbial community structure. The microbial assemblage shown in Figure 4.10 B reveals that *Bacteriodetes* relative abundance is associated with $CuSO_4$ addition.

The third cluster (framed in black) encompasses H_2O_2 (20 mg/L) oxidized samples at T48 and the control non-oxidized at T0. The shift between the taxonomic profile at T0 and T48 is smaller than what was observed for $CuSO_4$. H_2O_2 oxidation is correlated with *Proteobacteria* within phylum level and *Chloroccocales* (clear blue) within cyanobacteria (Figure 4.10 C).

Our results confirmed that when water with the same quality is treated with $CuSO_4$, the microbial composition is significantly different from the microbial composition after H_2O_2 addition. These differences between the observed trends for these oxidants were expected because of different mechanisms of actions and kinetics, persistence and selectivity (Drábková et al., 2007; Elder and Horne, 1978; Kay et al., 1982; Padovesi-Fonseca and Philomeno, 2004).

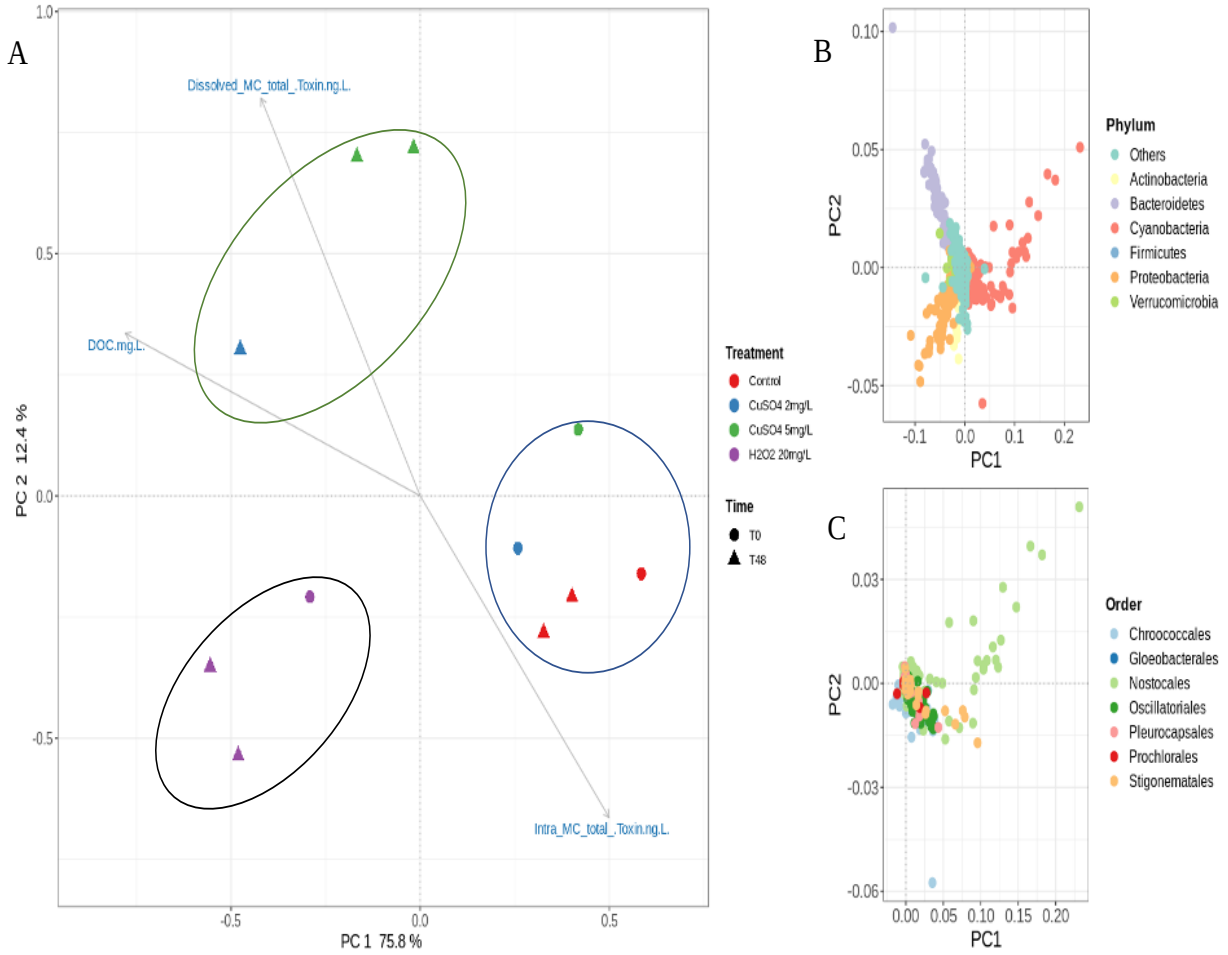

Figure 4.10 Principal components analysis (PCA) of (A) cyanobacterial communities with respect to DOC and toxin concentration in oxidized and non-oxidized samples; (B) microbial distribution at the phylum level; (C) cyanobacterial distribution at the order level.

4.5.4 Effect of oxidation on cyanobacterial community richness and diversity

Selected alpha diversity parameters (Shannon Index and Chao1) are presented in Figure 4.11.

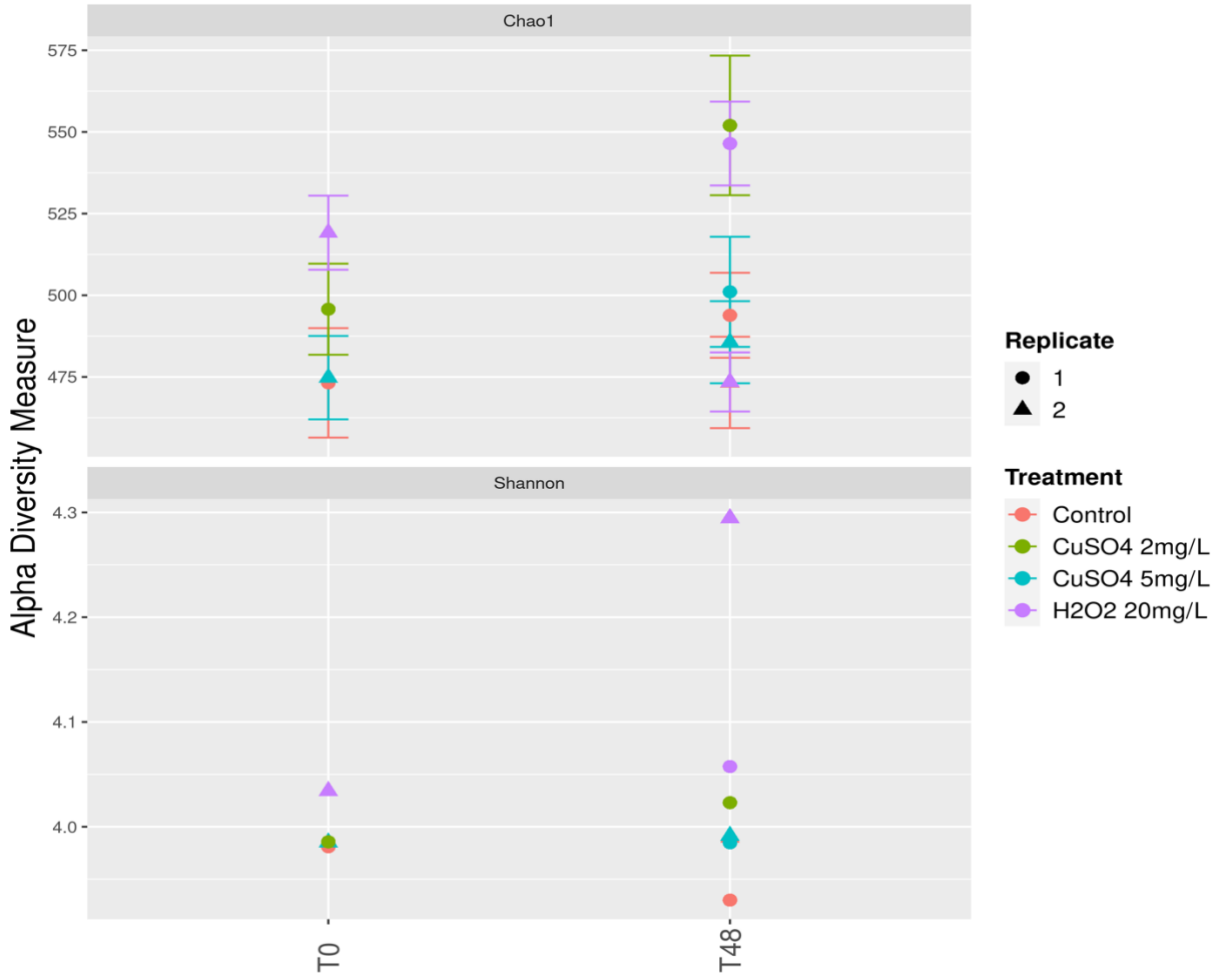

Figure 4.11 Alpha diversity measures of the cyanobacterial communities following oxidation with $CuSO_4$ (2 and 5 mg/L) and H_2O_2 (20 mg/L)

At the cyanobacterial level, unlike H_2O_2 oxidation, where Chao1 index decreased, $CuSO_4$ (2 mg/L and 5 mg/L) addition increased Chao1, especially for $CuSO_4$-2 mg/L (Figure 4.11).

The Shannon index is constant between T0 and T48 for $CuSO_4$-5 mg/L and shows a slight increase for $CuSO_4$-2 mg/L at T48. Compared to the trend observed for $CuSO_4$-2 mg/L, a steep increase in Shannon index was seen with H_2O_2-20 mg/L oxidation at T48. Altogether, low Chao1 and high Shannon following H_2O_2-20 mg/L oxidation unveiled that dominant cyanobacterial species could be compromised by H_2O_2, which allowed having a better distribution of cyanobacteria after H_2O_2-20 mg/L oxidation.

Additionally, the heatmap graph (Figure 4.12), demonstrates the Shannon index variation and shows that the relative abundance of the majority of the most abundant species decreased at T48 of H_2O_2-20 mg/L oxidation the first in the list being *Anabaena (Dolichospermum 90)*. The same trend was also observed for $CuSO_4$-2 mg/L, where Shannon shows a slight increase at T48.

In conclusion, H_2O_2-20 mg/L showed more impact on the cyanobacterial and microbial distribution within the community than $CuSO_4$.

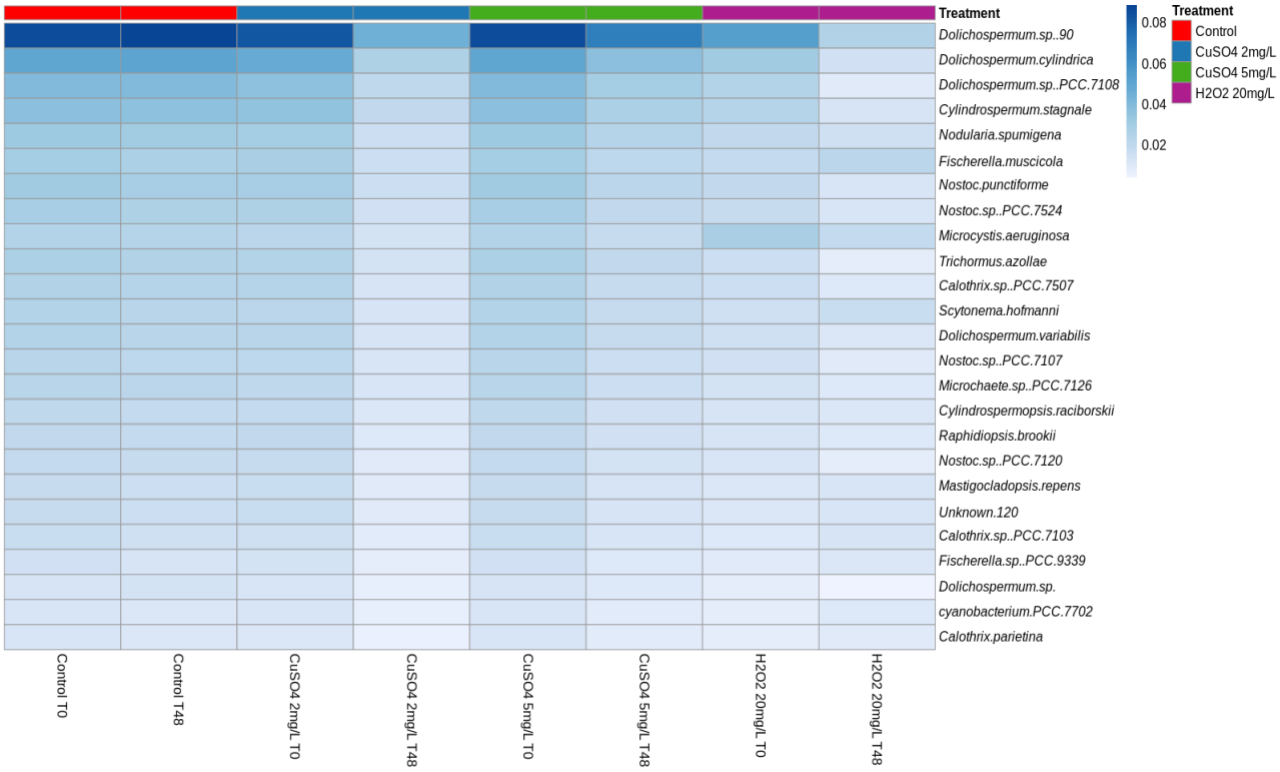

Figure 4.12 Cyanobacterial species heat map of the relative abundance of the top 25 major abundant species in mesocosm of cyanobacterial bloom water, oxidized by $CuSO_4$ (2 and 5 mg/L) and H_2O_2 (20 mg/L)

4.5.5 Oxidation impact on the bacterial functional response

In the current study, the functional profiles of oxidized and non-oxidized samples (control) were explored to correlate them with the taxonomic pattern described in Section 4.5. Figure 4.13 shows the 29 most abundant categories based on the relative abundance of functional genes composition assigned to subsystem groups (level 1) for non-oxidized and oxidized samples with $CuSO_4$ (2 mg/L

and 5 mg/L) and H_2O_2 (20 mg/L) for 48h. To better understand the shifts within the functional gene composition following oxidation, the oxidant type and dose-related variation in the gene's composition is shown for each oxidant. The relative abundance of the genes related to "Carbohydrate'', "RNA metabolism", "Amino acids and derivatives", "Membrane transport", "Motility and chemotaxis" increased after oxidation with $CuSO_4$ and H_2O_2. The relative abundance of "DNA metabolism" is slightly higher for mesocosms exposed to $CuSO_4$-5 mg/L, when compared to $CuSO_4$-2 mg/L. These gene composition profiles corroborate with several studies showing that bacteria must synthesize stress response-related factors or metabolize excess nutrients in the environment to cope with stressful conditions (Cheriaa et al., 2012; Polek and Godočíková, 2012). This means that bacteria must increase protein synthesis and, in consequence, increase RNA and DNA synthesis (Humayun, 1998; Krämer, 2010). Figure 4.13 illustrates that oxidized metagenomes do not show a decrease of housekeeping genes (protein, DNA and RNA metabolisms) at $CuSO_4$ and H_2O_2 compared to non-oxidized condition, but rather shows an increase for RNA metabolism after H_2O_2-20 mg/L and $CuSO_4$ (2 mg/L and 5 mg/L), and for DNA metabolism only after 48h of $CuSO_4$-5 mg/L.

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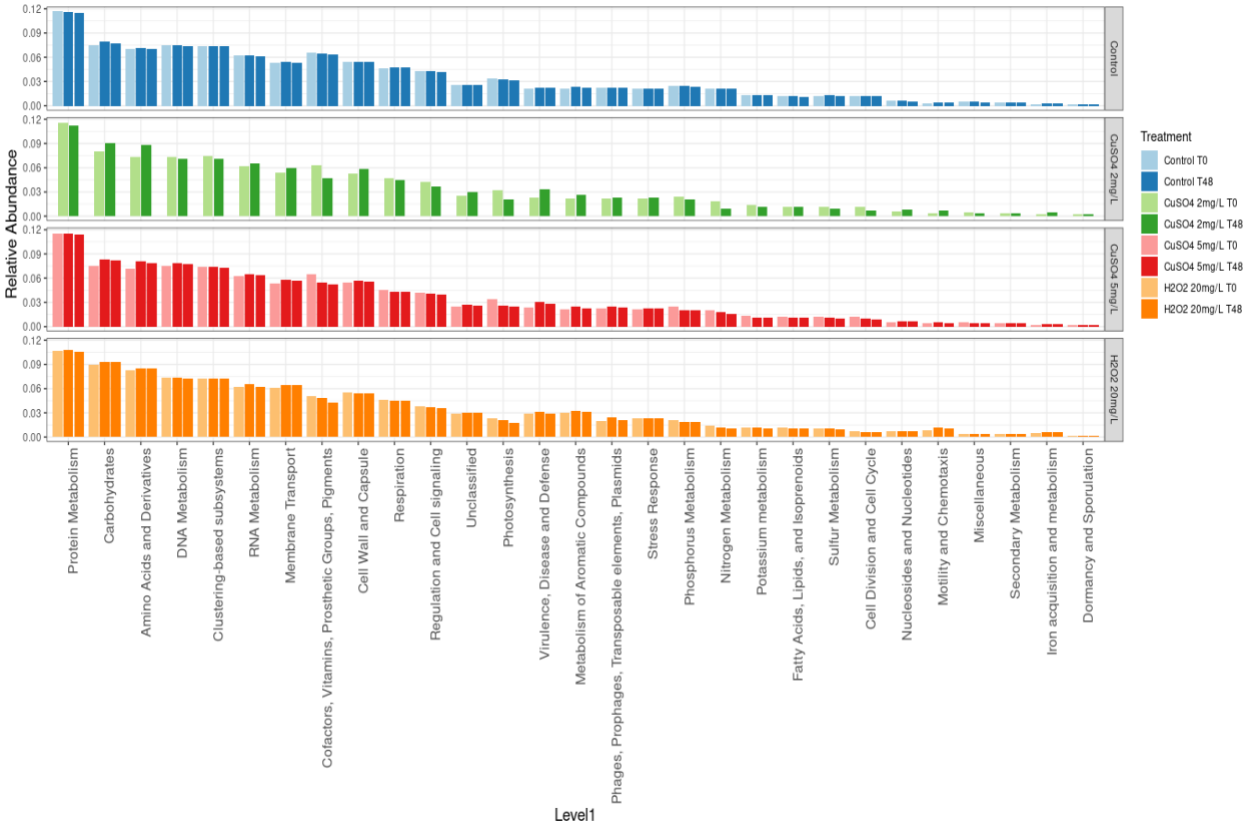

Figure 4.13 Functional response of the cyanobacterial bloom during $CuSO_4$ and H_2O_2 oxidation. The analysis was generated from the metagenomics dataset

To assess the impact of chemical oxidation on the functional composition dynamics, PCA was conducted on mesocosms of cyanobacterial bloom before and after oxidation (Figure 4.14). The PCA of functional response following oxidation with $CuSO_4$ showed different functional profiles compared to H_2O_2. Three cluster groups were detected. The first group encompassed control conditions at T0 and T48h (red symbols) and the initial condition of the oxidized mesocosms, T0, (blue, purple and green circles). The cluster is grouped with functional gene categories related to "Respiration", "Photosynthesis", "Sulfur metabolism", "Potassium metabolism", "Phosphorus metabolism'', "Protein metabolism'', "Cell division and cell cycle'', "Regulation and cell cycling'', "Fatty acids, lipids and Isopernoids", "Miscellanous'' related gene categories. These gene-related categories correspond to the pattern of bacterial adaptation for survival in aquatic systems, encompassing various regulation and cell signaling pathways for nutrient acquisition (Song et al., 2017).

The second cluster group, which corresponds to oxidized mesocosm samples with H_2O_2 (20 mg/L) at T0 and T48, is clustered with "Carbohydrates", "Iron acquisition and metabolism", "Metabolism of aromatic compounds", "Motility and Chemotaxis" and "Membrane transport" related gene categories. It seems that H_2O_2 oxidation of the cyanobacterial bloom water mesocosm did not cause a change in the relative abundance of the genes associated with acquiring and sequestering nutrient sources such as carbohydrates. Oxidation with H_2O_2 resulted in distinct shifts in the functional related gene categories (Moradinejad et al., 2020).

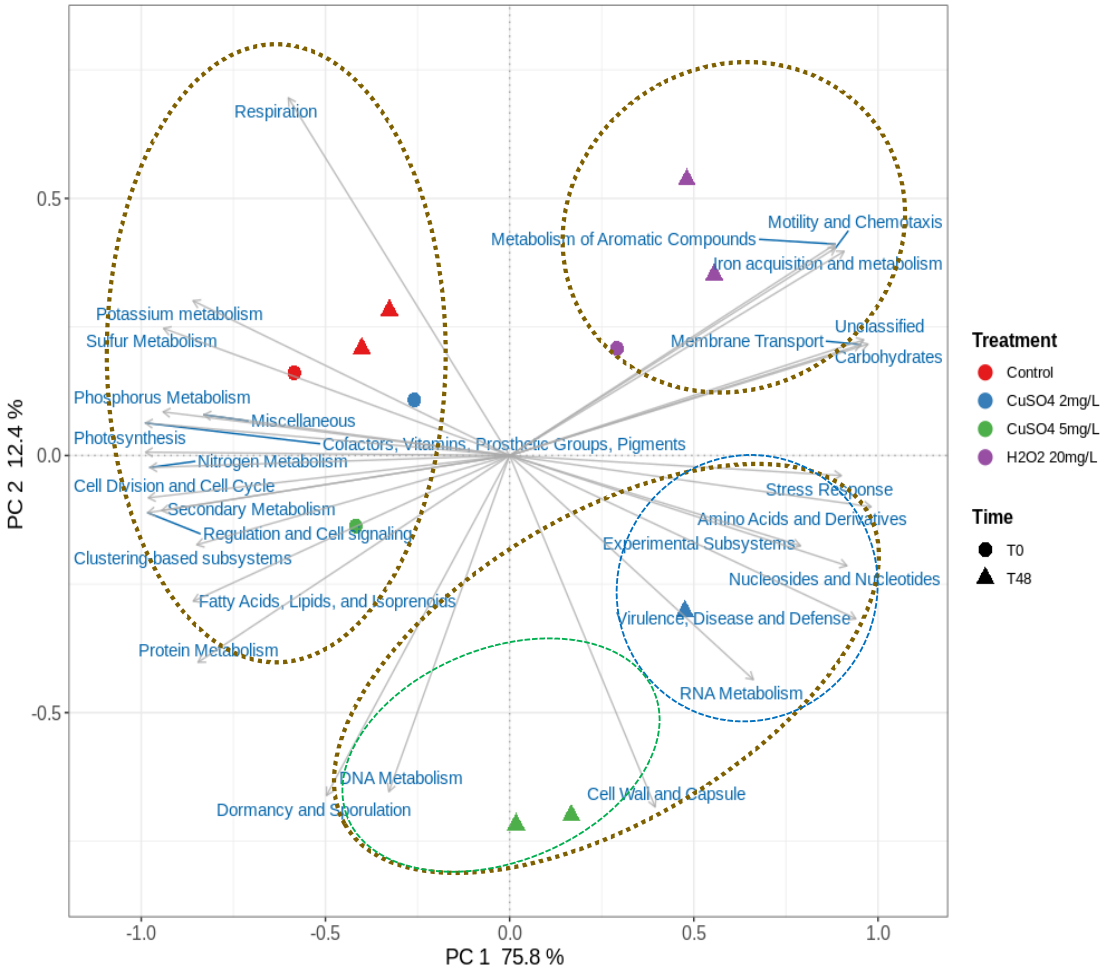

Figure 4.14 PCA of the functional structure following $CuSO_4$ and H_2O_2 oxidation

The third cluster group encompassed treated samples with $CuSO_4$ (2 and 5 mg/L) for 48h with gene categories related to "Amino acids and derivatives "Nucleosides and Nucleotides", "Stress

response", "DNA metabolism", "RNA metabolism", "virulence disease and defense'', "Cell wall and capsule'' and "Dormancy and sporulation''. This implies that bacterial persistence after $CuSO_4$ oxidant exposure was associated with the presence of stress response genes.

Close observation in this group cluster distinguishes two sub-clusters. The green framed sub-cluster encompasses $CuSO_4$-5 mg/L with "Cell wall and capsule'', "DNA metabolism'' and "Dormancy and sporulation'', the second sub-cluster that associates $CuSO_4$-2 mg/L with the rest of the functional groups (framed in blue). Interestingly, opposite to the $CuSO_4$-2 mg/L sub-cluster, which includes the "Amino acid derivatives'' category, $CuSO_4$-5 mg/L sub-cluster does not include any gene categories related to metabolism or nutrients related functions, revealing less permissive condition for $CuSO_4$-5 mg/L. This finding supports what was observed about the close association of DOC availability with $CusO_4$-2 mg/L in the PCA in Figure 4.10, displaying the correlation of the taxonomic profile and environmental parameters. Furthermore, the association of $CuSO_4$-2 mg/L with the "Nucleosides and Nucleotides" reflects differences in reactivity towards nucleic acids when compared to H_2O_2-20 mg/L and $CuSO_4$-5 mg/L.

The increase in "DNA metabolism" and "RNA metabolism" indicates that bacteria have entered the survival mode in harsh environmental conditions (Cheriaa et al., 2012; Polek and Godočíková, 2012). Furthermore, the association of $CuSO_4$ with the "Nucleosides and Nucleotides" reflecting differences in reactivity towards nucleic acids when compared to H_2O_2.

Altogether, as shown by the PCA (Figure 4.14), the differential taxonomic structure of mesocosms oxidized with either $CuSO_4$ or H_2O_2 were associated with distinct microbial functional profiles after oxidation, signifying different functional structure associated with each treatment method (Sharma et al., 2020). It can be concluded that each algicide/oxidant has its own mode of action.

4.6 Oxidation impact on selected cyanobacterial biomarkers

To better understand the functional cyanobacteria strategy in response to oxidation, three cyanobacterial biomarkers (level 3 subsystem) where selected: "Cyanobacterial circadian clock", "Heterocyst formation in Cyanobacteria" and "Transcription factors cyanobacterial *rpoD*-Like sigma factors", (referred in the rest of text as *rpoD*-Like) (Figure 4.15).

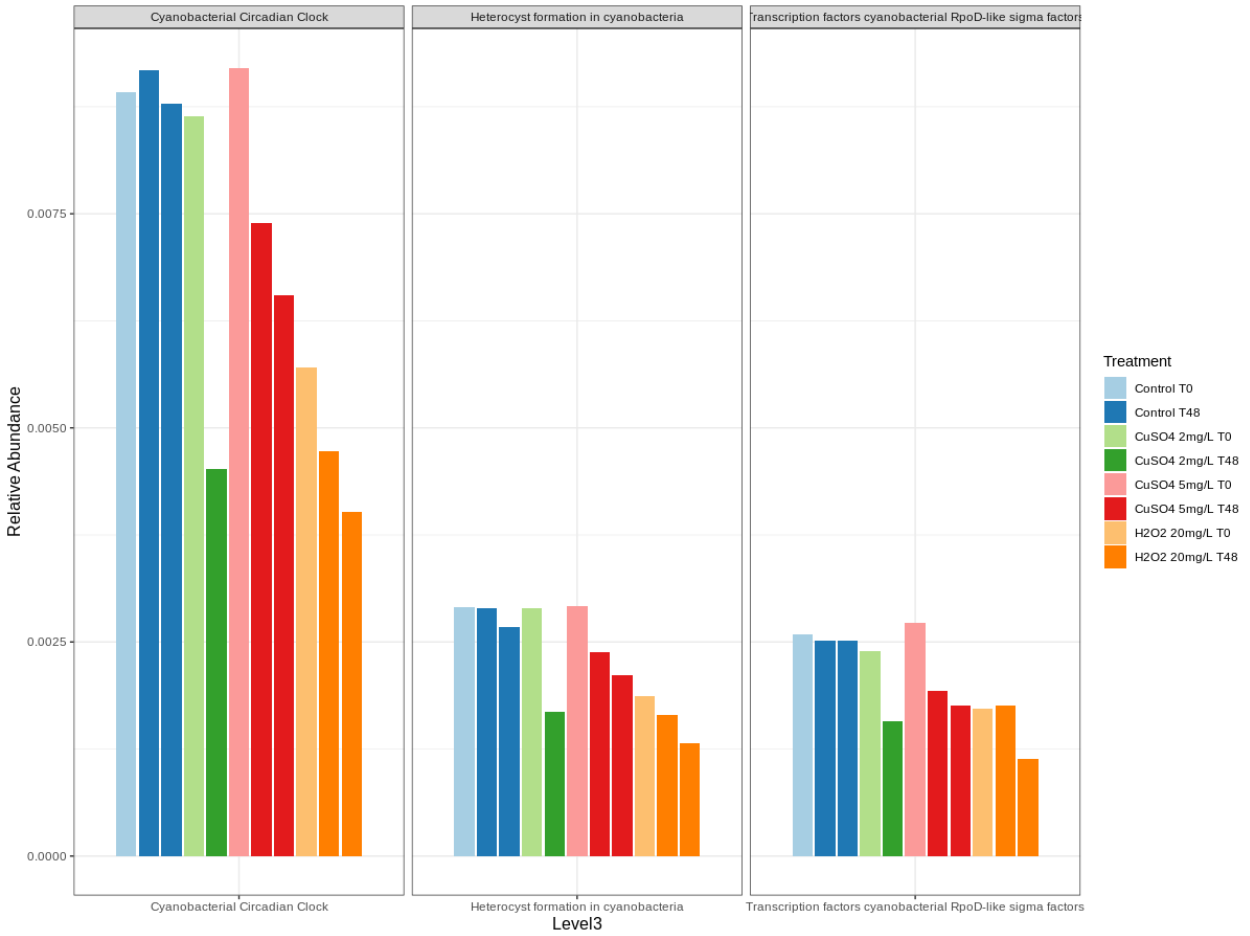

Figure 4.15 Illustrates the impact of $CuSO_4$ and H_2O_2 oxidation on selected cyanobacterial biomarkers related to cell division (Cyanobacterial Circadian Clock), cell cycle subsystems (Heterocyst formation in cyanobacteria) and RNA metabolism (Cyanobacterial *RpoD*-like sigma factors)

Cyanobacterial circadian clock and Heterocyst formation are two well-studied cyanobacterial biomarkers (Bale et al., 2019; Taton et al., 2020; Xing et al., 2020). Circadian clock systems have evolved as an essential component of microbial adaptation to a wide range of stress responses and adaptability in competitive environments where cellular functions are linked to photochemical processes (Hörnlein et al., 2020). Heterocyst formation has been related to a wide range of roles in cyanobacteria, such as nitrogen regulation (especially during N-limited condition), cell cycle, cell size and cell division regulation (Xing et al., 2020; Zhang et al., 2006). The *RpoD*-Like factor has evolved as a biomarker and a regulator to acclimate cyanobacteria and allow them to cope with

chemical oxidative stress (Srivastava et al., 2020). Previous work demonstrated that *RpoD* factor is upregulated in high light intensity, singlet oxygen and H_2O_2 stresses, and overproduction of the *RpoD* factor in the mutant strain lacking other *RpoB*, *RpoC* and *RpoE* (ΔsigBCE) leads to a higher ability of growth of this mutant in those stress conditions (Hakkila et al., 2019).

Oxidation with $CuSO_4$ and H_2O_2 showed that the relative abundance of these three biomarkers decreased markedly. The decrease was more evident in mesocosms containing 2 mg/L $CuSO_4$ as treatment compared to other oxidation conditions. These findings are supported by the decline of relative abundance of cyanobacterial phylum after $CuSO_4$ and H_2O_2 oxidation observed in Figure 4.7.

CHAPTER 5 GENERAL DISCUSSION

This chapter includes the main findings of this research and indicates the limitations of this study. The main objective of this study was to evaluate the fate of cyanobacteria during pre-oxidation with H_2O_2 (with 10 mg/L and 20 mg/L) and $CuSO_4$ (with 2 mg/L and 5 mg/L) in mesocosms (deployed in Missisquoi Bay, PLSF, and in the laboratory using PLSF raw water, respectively). With this in mind, the following was studied within 48 hours:

1) Cyanobacteria cell integrity and their morphological response to each added treatment.
2) Influence of pre-oxidation on DOC and toxins (microcystins) release.
3) The alteration in community composition following pre-oxidation.
4) Cyanobacteria functional and taxonomic response to each treatment (in PLSF).

5.1 Cyanobacteria cell integrity and their morphological response to each added treatment

Previously, several studies have evaluated cyanobacteria morphological changes in a qualitative manner by using digital flow cytometry (Daly et al., 2007; Wert et al., 2013). Depending on each treatment mode of action (which is mentioned in the literature review), their impact on cyanobacteria morphological aspects will differ.

The fluorometric analysis by FCM have shown that across all mesocosms, the application of H_2O_2 or $CuSO_4$ for 48h resulted in an important decrease in viable cells (cell integrity) of the cyanobacterial community without fully eliminating them after treatment addition (except the first experiment in Missisquoi Bay, where a reduction in total cell count was observed as well). As was expected, each treatment induced more damage to cell membrane when the dose increased (in H_2O_2 from 10 mg/L to 20 mg/L and in $CuSO_4$ from 2 mg/L to 5 mg/L). However, when comparing each oxidant/algicide impact separately, more cyanobacteria lost their integrity with both doses of $CuSO_4$ (2 mg/L and 5 mg/L) addition as compared to when H_2O_2 was added. Fewer than 5% of cells remained viable in all performed experiments after $CuSO_4$ addition. As a result, it could be concluded that cyanobacteria membrane integrity was more affected by $CuSO_4$ than H_2O_2. This

difference in the impact of each treatment was later on confirmed when the extent of metabolite and DOC release in each mesocosm was investigated.

Altogether, results of cell integrity of cyanobacteria retrieved from oxidized mesocosms in the field (Bay Missisquoi, PLSF) and in the lab (raw water from PLSF) indicate that cyanobacterial community are susceptible to both H_2O_2 and $CuSO_4$. However, cyanobacteria viability appeared to be more vulnerable when exposed $CuSO_4$ possibly due to the mode of action of these oxidants/algicides. This trend can be confirmed by the previously performed studies as well (Matthijs et al., 2016; Zamyadi et al., 2020). The drastic reduction of cyanobacteria viable cells without completely eliminating them warrants some caution. The remaining cyanobacteria after oxidation are dominated by the filamentous cyanobacteria, *Dolichospermum* (*Anabaena)*, which could play the role of "seed'' population to trigger their resurgence.

However, further investigation with more doses of oxidants/algicides is required to determine the optimum added dose and studying the relationship between cyanobacteria morphological change and the extent of metabolites and DOC release.

5.2 Influence of pre-oxidation on dissolved organic compounds (DOC) and toxins (MCs) release

5.2.1 Pre-oxidation influence on DOC

The results of this study showed that in most cases, after treatment addition, DOC concentration increased due to cell rupture and release of intracellular organic matter. Many factors such as community composition, cell size, initial DOC concentration and the dose of added treatment impact the final DOC concentration (Coral et al., 2013; Wert et al., 2014). This could be the main reason for the previously seen difference in DOC trends for performed experiments. Additionally, the released DOC may hinder the efficient extracellular toxins (metabolites) oxidation/treatment as it creates a competitive environment for consuming the available oxidant (Laszakovits and MacKay, 2019). Further investigation is required to fully comprehend the organic matters oxidant consumption kinetics and its influence on treatment efficiency.

Additionally, when comparing the mesocosm study in the two first events and the last event performed in the lab, it could be concluded that when H_2O_2 is exposed to natural light (UV radiation), more cyanobacteria cell damage was observed. This resulted in having a higher final DOC concentration in the lake at T48 as compared to the lab test.

As a final note, it should be considered that an increase in DOC concentration is problematic for the water treatment procedures. DOC can contribute to disinfection by-product formation when entering the treatment chain (Jo et al., 2011; Xu et al., 2019; Zamyadi et al., 2012). In addition, DOC could potentially be used by the bacterial community as a substrate for further growth (Peterson et al., 1995). As a consequence, it is crucial to consider control and limit DOC release when applying chemical oxidants as a pre-oxidation treatment method.

5.2.2 Pre-oxidation influence on toxins (MCs)

At first, after oxidant/algicide addition, intracellular toxins are released due to cell damage, and subsequently, the remaining available oxidant will oxidize the released toxin. Therefore, an inadequate oxidant dose would result in partial toxin removal (Fan, 2013).

During the experiment performed in Bay Missisquoi and PLSF, total MCs concentration dropped more than 50% after the addition of both doses of H_2O_2 (10 mg/L and 20 mg/L). A slight decrease in total MCs concentrations was also observed with 20 mg/L of H_2O_2 in the lab experiment. When H_2O_2 was added (regardless of the dose), the extent of extracellular toxin increased at T48 compared to extracellular MCs at T0. In all cases, H_2O_2 was capable of oxidizing the released metabolites and resulted in having total MCs concentration below the established threshold for drinking water (1.5µg/L) (Health Canada, 2016). This ability shows the potential of this oxidant to be used as a pre-oxidant to prevent problems associated with toxin accumulation in treatment chains (Matthijs et al., 2012b).

Further studies could be beneficial to investigate the optimal H_2O_2 dose to ascertain desired oxidation of released metabolites from cyanobacteria. It is understood that highly variable source water conditions could be challenging for establishing an optimal dose, therefore, effective ranges of doses could be helpful based on source water characteristics.

In contrast to results for H_2O_2 oxidation, the concentration of total MCs following $CuSO_4$ (2 mg/L and 5 mg/L) slightly increased at T48. After $CuSO_4$ addition, MCs were mainly composed of extracellular MCs, revealing severe damage of cyanobacteria cell and intracellular metabolite release. Based on the results, $CuSO_4$ was not capable of removing the released MCs, which is unfavorable and problematic for further conventional treatment processes (Henderson et al., 2008; Zamyadi et al., 2013). As a result, $CuSO_4$, especially with high dose (5 mg/L), seems not to be the preferred choice for source treatment when considering the extent of released metabolites.

5.3 The alteration in community composition following pre-oxidation

Taxonomic cell count using microscopy is one way to study the impact of oxidation on community composition (Fan et al., 2013b; Fan et al., 2014a; Zamyadi et al., 2019). This method has a few drawbacks, such as being time-consuming, requiring a qualified person and miscount due to morphological similarity among species (Casero et al., 2019). Oxidation may also change the morphological formation of the species and result in miscounting. Still, this method is beneficial to achieve the absolute cyanobacteria cell count.

According to microscopic taxonomic cell count, the reductions of cyanobacteria varied between genera. All treatments could reduce cyanobacteria cell numbers; however, no evident change was observed in the cell number reduction when the H_2O_2 dose increased from 10 mg/L to 20 mg/L. Conversely, $CuSO_4$ dose augmentation from 2 mg/L to 5 mg/L reduced cyanobacteria cell counts, which is in accordance with previously performed study by Crafton et al. (2021).

Microscopy demonstrated that the taxonomic structure was composed mainly of *Dolichospermum* (*Anabaena),* followed by *Aphanothece* and to less extent *Planktothrix*. *Microcystis* was not detected after oxidation, and that corroborated with previous studies, where *Microcystis* have shown a heightened sensitivity to H_2O_2 relative to other genera. The lack of typical cyanobacterial catalases in some strains may be among the causes explaining this observed reduction (Bernroitner et al., 2009).

Cyanobacteria do not usually synthesize ascorbate peroxidases, but they are able to synthesize several other anti-oxidant enzymes to cope with H_2O_2, including catalases, peroxidases, and peroxiredoxins (Bernroitner et al., 2009). These enzymes are variable within the different

cyanobacterial taxa, and this variability may contribute to the change in relative abundance observed by metagenomics or taxonomic cell counts.

After $CuSO_4$, similar to H_2O_2 oxidation, taxonomic cell count showed that *Dolichospermum* (*Anabaena)* remains the most abundant genus. *Aphanizomenon* and *Aphanothece* made up a smaller portion of the cyanobacterial detected community. Interestingly, in the lab experiment, both genera *Aphanizomenon* and *Planktothrix* increased with increasing $CuSO_4$ dose (5 mg/L), meaning that they are not susceptible to $CuSO_4$-5 mg/L.

Little is known about the diversity, interaction and functional response of cyanobacteria and their surrounding bacteria. For more accurate taxonomic identification, metagenomics shotgun sequencing was used to assess the effects of H_2O_2 and $CuSO_4$ for 48h on the taxonomic and functional profiling of cyanobacteria and the most abundant co-occurring heterotrophic bacterial phyla within PLSF cyanobacterial bloom water (*Proteobacteria, Bacteriodetes, Actinobacteria and Firmicutes*).

When cyanobacteria are concerned, in all mesocosms, analysis of relative abundance at the phylum demonstrated that cyanobacteria were the most abundant phyla even after treatment addition. Species heat map (figure 4.13) illustrated that *Anabaena (Dolichospermum)* was the dominant taxa which is in accordance with the microscopic cell count.

The collective response of Metagenomics analysis data (diversity indexes, PCA, differential heat trees) leads to evidence to support that H_2O_2 oxidation reduced the most dominant species. Consistent with Lusty and Glober's proposal, this study suggests that the dominant taxa are "*suppliers of an organic surface area for the H_2O_2 to react with, allowing taxa at lower relative abundances to fill the niche left open by the formerly*'' (Lusty and Gobler, 2020). This phenomenon was also observed for $CuSO_4$-2 mg/L.

When cyanobacteria are studied considering their reaction with the surrounding bacteria, it is observed that for all treatments, opposite to the relative abundance of cyanobacteria trend, *Proteobacteria* relative abundance increased after the addition of $CuSO_4$ and H_2O_2. This contradictory bacterial composition trend between *Proteobacteria* and cyanobacteria following oxidation has also been noticed after 10 min of Cl_2-0.2 mg/L oxidation of cyanobacterial bloom raw water samples retrieved from the same lake (Moradinejad et al., 2020). Previous studies have

also reported that several phylogenetically diverse heterotrophic bacteria within *Proteobacteria* have displayed antagonistic activities against various cyanobacteria (Wright and Thompson, 1985). In addition, the increase of *Proteobacteria* composition within the bacterial community upon H_2O_2 oxidation has been observed by Wang et al. (2017) after advanced oxidation processes on subsequent biological water treatment. Altogether, it suggests that *Proteobacteria* may be better adapted to chemical oxidation stress induced by H_2O_2 than other phyla and that included oxidation response mechanism during H_2O_2 exposure. The relatively short generation time and the ability to metabolize complex organic compounds seen in *Proteobacterial* taxa allow them to cope rapidly with stress (DeLong et al., 1993; Newton et al., 2011; Nold and Zwart, 1998). Certainly, *Proteobacteria* benefit from catalase-peroxidases and manganese catalases to shelter from oxidative stress (Zámocký et al., 2012). Additionally, it was observed by Maghsoudi (2015) that this strain (*Proteobacteria*) is capable of degrading existing MCs in the water.

Based on PCA analysis (Figure 4.10), *Bacteroidetes* relative abundance is associated with the availability of DOC and $CuSO_4$ addition. This finding is supported by previous results stating that *Bacteroidetes* showed higher abundance when DOC is high (Zámocký et al., 2012). *Bacteroidetes* are also associated with low abundance or degradation of *Microcystis* (Shao et al., 2013). In this study, for all treatments, *Microcystis* relative abundance assessed by metagenomics or taxonomic cell count represented a small fraction or were absent, respectively.

Differential relative abundance within the *Actinobacter* community was more apparent following $CuSO_4$ addition compared to H_2O_2. Comparing $CuSO_4$-2 mg/L and $CuSO_4$-5 mg/L taxonomic profiles through differential heat trees unveiled that *Actinobacter* relative abundance is much lower after $CuSO_4$-5 mg/L addition, cyanobacteria relative abundance is higher, demonstrating that strains of *Actinobacteria* are susceptible to higher dose of $CuSO_4$ (5 mg/L). It has been shown previously that deep metagenomic sequencing to the microbial community of a freshwater reservoir has shown that abundances of *Actinobacteria* correlate inversely to cyanobacteria (Ghai et al., 2014). *Actinobacteria* have a negative impact on the prevalence of cyanobacteria and that by producing metabolites inhibiting bloom-forming cyanobacteria (Sun et al., 2018). It has also been reported that *Actinobacteria* relies on the availability of dissolved organic matter in the environment, serving as a provider of carbohydrates and other carbon sources (Buck et al., 2009; Pérez and Sommaruga, 2006). The slow growth rate may partly explain the susceptibility of

Actinobacteria to $CuSO_4$-5 mg/L since it will not allow them to recover the community that was initially reduced by $CuSO_4$-5 mg/L. PCA (Figure 4.11), showed that DOC is more correlated with $CuSO_4$-2 mg/L, where *Actinobacteria* is less susceptible than at $CuSO_4$-5 mg/L.

It was suggested by Huston (1997) and then confirmed by Jaillard et al. (2014) that the interactions of a community members can shape members' relative abundances and establish the community assembly rules (like the dominance hierarchy: subordinate, dominant and super dominant species). Building on this theory, the unexpected higher relative abundance of cyanobacteria following $CuSO_4$-5 mg/L, compared to that at the lower dose ($CuSO_4$-2 mg/L) seems to depend on the response of other taxa, such as *Actinobacteri*a. The taxonomic profile after applying $CuSO_4$-5 mg/L favors a beneficial assembly effect for cyanobacteria distribution. This outcome is precisely the reverse of what this study aims to achieve, which is cyanobacteria control.

5.4 Cyanobacteria functional response to each treatment

In this section, some traits that may shed light on the potential functional responses of these communities to cope with oxidation are discussed. In particular, those associated with cyanobacteria. These traits may not characterize all the taxa within the phylum, especially the diverse ones (i.e *Proteobacteria*). No studies up until now have focused on the functional response of the bacterial community to chemical treatments such as H_2O_2 and $CuSO_4$.

Most importantly, the metagenomic functional data of this study adds another dimension to our understanding of the potential interactions that ultimately shape the biological network in the oxidized mesocosms leading up to cyanobacterial mitigation. Although $CuSO_4$-5 mg/L treatment promotes the relative abundance of cyanobacteria within the microbial community, PCA of the functional structure following $CuSO_4$ and H_2O_2 showed that $CuSO_4$-5 mg/L is globally the most stringent applied treatment for the microbial community. Indeed, the functional profile shifted to stress function categories: “DNA metabolism’’, “Dormancy and sporulation’’ and “Cell wall and capsule’’. After $CuSO_4$-2 mg/L, the functional profile was also associated with stress response-related categories (“Nucleosides and Nucleotides” “Stress response”, “RNA metabolism”, “virulence disease and defense’’) and also to “amino acid derivatives’’, demonstrating that this condition seemed to be ecologically more permissive to the whole microbial community assembly, since it might be able to degrade carbon substrates, such as amino acid derivatives’’. Our

hypothesis is that some heterotrophic bacteria taxa are susceptible to $CuSO_4$-5 mg/L, promoting the expansion of cyanobacteria at the expense of other phyla.

H_2O_2-20 mg/L treatment encompasses functional categories that are associated with energy metabolic pathways (“Carbohydrates”, “Iron acquisition and metabolism”, “Metabolism of aromatic compounds”, “Motility and Chemotaxis” and “Membrane transport”), indicating that microbial communities present in this oxidized mesocosm are well adapted to degrade carbon substrates such as soluble carbohydrates or polysaccharides. Our findings suggest that during H_2O_2-20 mg/L, the microbial community is less compromised as compared to $CuSO_4$-2 mg/L and $CuSO_4$-5 mg/L.

CHAPTER 6 CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

This project was performed to investigate the fate of cyanobacteria during source oxidation focusing on using high throughput sequencing technology (shotgun metagenomics sequencing). A mesocosm-based study was used to imitate large-scale field pre-oxidation. Mesocosms were deployed in Missisquoi Bay, PLSF, and laboratory using PLSF raw water. The effects of two different treatments, H_2O_2 (10 mg/L and 20 mg/L) and $CuSO_4$ (2 mg/L and 5 mg/L) on cyanobacteria fate were evaluated. A combination of microscopy, flow cytometry, ddPCR and high throughput DNA sequencing were used to assess the effects on cyanobacterial and bacterial communities. Results of the performed project led to following conclusions:

(A) Cyanobacteria cell integrity response to each added treatment.

- Both treatments impacted the viability of the cells without fully eliminating them. $CuSO_4$ (2 mg/L) was capable of impairing more cells membrane compared to the highest H_2O_2 dose (20 mg/L).

(B) Impact of pre-oxidation on cell material release:

- H_2O_2, specifically with the highest dose (20 mg/L), reduced the total MCs concentration after 48hours. However, the addition of $CuSO_4$ increased the release of dissolved MCs, which is problematic for the following water treatment with a conventional treatment process.
- Monitoring of *mcyD* gene for PLSF samples using H_2O_2 oxidation illustrated that *mcyD* gene copy number was positively correlated with cyanobacterial cell integrity and total intracellular MCs (p-value<0.05). It was also negatively correlated with oxidant exposure time, confirming the negative impact of H_2O_2 and MCs genes synthesis and MCs release. It suggests this treatment ability to hinder MCs production after oxidation When $CuSO_4$ was added, *mcyD* gene copy numbers positively correlated with the oxidant exposure time and total dissolved MCs. Therefore, when cyanobacteria are exposed to $CuSO_4$, *mcyD* gene copy number rises, and consequently, MCs are released (p-value<0.05). Which means we can predict potential toxin release with this treatment.

A negative correlation between *mcyD* copy number and cell integrity was found, supporting the results regarding dissolved MCs increase at T48 (p-value<0.05).

(C) Influence of pre-oxidation on community composition:

- Dominant species within the bacterial and cyanobacterial community were more impacted by H_2O_2 compared to $CuSO_4$, resulting in a better community distribution following H_2O_2 oxidation.
- Shotgun metagenomic sequencing revealed that all added treatments decreased cyanobacteria relative abundance, preventing cyanobacteria entrance in following water treatment processes.
- Surprisingly, cyanobacteria relative abundance was lower with 2 mg/L of $CuSO_4$ addition compared to 5 mg/L of $CuSO_4$. However, the absolute cyanobacteria count showed the reverse trend. We hypothesize that 5 mg/L of $CuSO_4$ further impacted other bacterial communities other than cyanobacteria, such as *Actinobacteria,* enabling cyanobacteria to take advantage of this condition and increase its abundance within the microbial assembly.

(D) Cyanobacterial functional response:

- When H_2O_2 is added, the cyanobacterial bloom community seemed to be well adapted to the oxidation due to developing functional responses associated with energy metabolic pathways. However, the community showed different functional responses to $CuSO_4$ addition; they were associated with stress functions such as "DNA metabolism'', "Dormancy and sporulation'' and "Cell wall and capsule". $CuSO_4$-5 mg/L treatment may cause the stringent condition unfavorable for some taxa such as *Actinobacter*. This condition seems to be more favorable to increase the relative abundance of cyanobacteria among the microbial community.

(E) Comparison of both added treatments

Choosing the most efficient yet safe treatment for source pre-oxidation is associated with many elements. Firstly, one of the essential factors is the cost of each added

treatment. As mentioned before, $CuSO_4$ costs much less (2-5$/ton) than H_2O_2 (500-2000$/ton). This makes H_2O_2 addition a relatively expensive source treatment method.

Secondly, based on the results in the metagenomics section, it was observed that $CuSO_4$ with high doses (5mg/L) could impact the non-targeted microbial community (not selective toward cyanobacteria). Also, copper is a metal, and its frequent addition in high doses will be detrimental to the environment and may result in Cu accumulation in sediments. However, H_2O_2 frequent addition results in no significant damage to the environment or to the lakes since it is environmentally safe due to its dissociation to water and oxygen molecules.

Additionally, the other important factor is releasing or having the lowest possible concentration of cyanotoxins in the source after the treatment addition as consequent treatment processes in the water treatment plant will face challenges in removing the dissolved toxins. Since $CuSO_4$ increased the *mcyD* gene copy numbers after 48hr, it could increase the possibility of MCs production and release into the environment. However, H_2O_2 decreased *mcyD* gene copy numbers after 48hr which lowers the possibility of MCs production and release. Additionally, cyanotoxin results (Figure 4.5) showed the final MCs concentration after H_2O_2 addition was much less than $CuSO_4$ due to the aforementioned reasons in the results section.

Consequently, based on the mentioned concerns, $CuSO_4$ addition in lakes, especially in high doses (5mg/L), is not recommended. However, since H_2O_2 is a relatively expensive treatment, it could be a reasonable solution for specific situations to be added in the localized areas.

6.2 Recommendations

Based on the mentioned findings, recommendations for further investigation are:

- Optimizing pre-oxidation by studying the effects of more varied doses of each oxidant/algicide to find the optimum dose (lowest final dissolved toxins concentration yet being safe for the environment) by considering cyanobacterial community composition and intracellular material release.
- Consider different nutrient (N and P) availability and limitations during the investigation of the impact of oxidation on cyanobacteria community composition.
- Investigate the fate of cyanobacteria using meta-transcriptomic analysis to obtain cyanobacteria gene expression under oxidative stress.
- Evaluate the long-term pre-oxidation method in mesocosms to determine the adaptability of cyanobacteria to a certain control method.
- Investigate the acceptability of using pre-oxidation in water bodies whose primary use is the production of drinking water as a short-term solution for cyanobacterial control.

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APPENDIX A A Supplementary Information, *mcyD* Gene Copy Numbers Results for PLSF Field Test

Figure A.1 illustrates *mcyD* gene copy numbers before and after treatment addition during the PLSF field experiment. As is observed, compared to the controls (T0), the number of *mcyD* copies obviously increased following $CuSO_4$ addition. This trend was reversed following H_2O_2 oxidation. The increase in *mcyD* copy numbers progress with the increase of $CuSO_4$ dose from 2 mg/L to 5 mg/L.

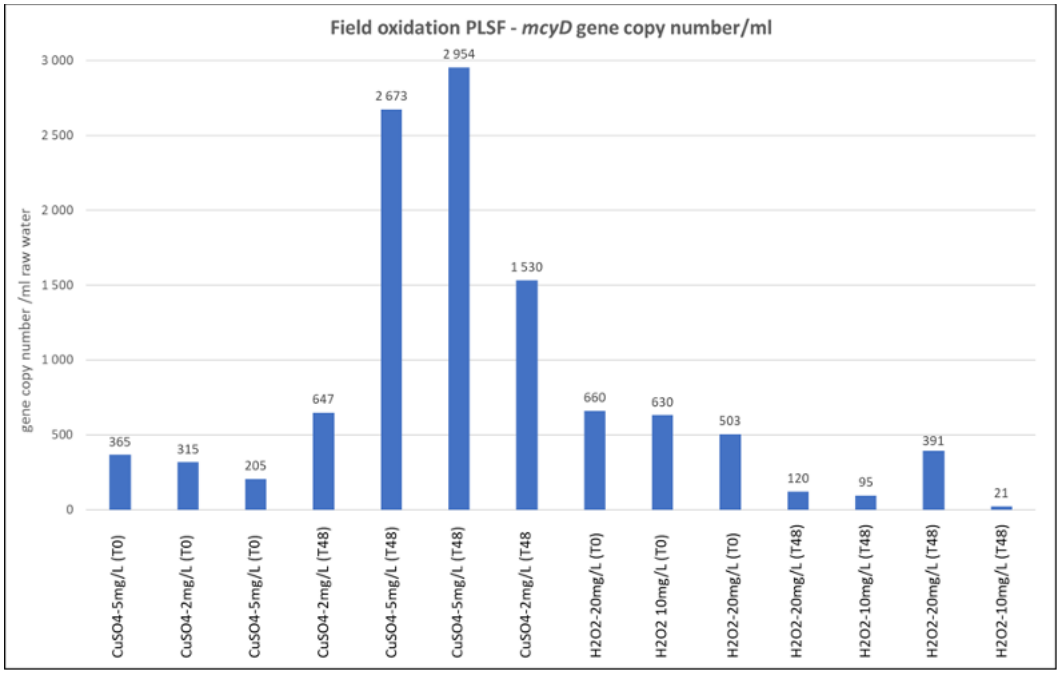

Figure 6.1 *mcyD* copy number in samples treated onsite with $CuSO_4$ and H_2O_2 in PLSF

Figure A.2 demonstrates the correlation analyses between oxidant dosage (mg/L), exposure time, DOC concentration (mg/L), cell integrity percentage, extracellular and intracellular MCs and *mcyD* gene copy numbers for the performed experiment in PLSF. Interestingly, when H_2O_2 was added, as the oxidation contact time increased, the copy numbers of *mcyD* gene, cell integrity percentage and the concentration of intracellular MCs decreased. However, in the case of $CuSO_4$ oxidation *mcyD* gene copy number correlates with the oxidant exposure time and intracellular MCs concentrations. A negative correlation is obtained between *mcyD* gene copy number and cell integrity, revealing the higher production of dissolved MCs concentrations when *mcyD* gene copy number increases.

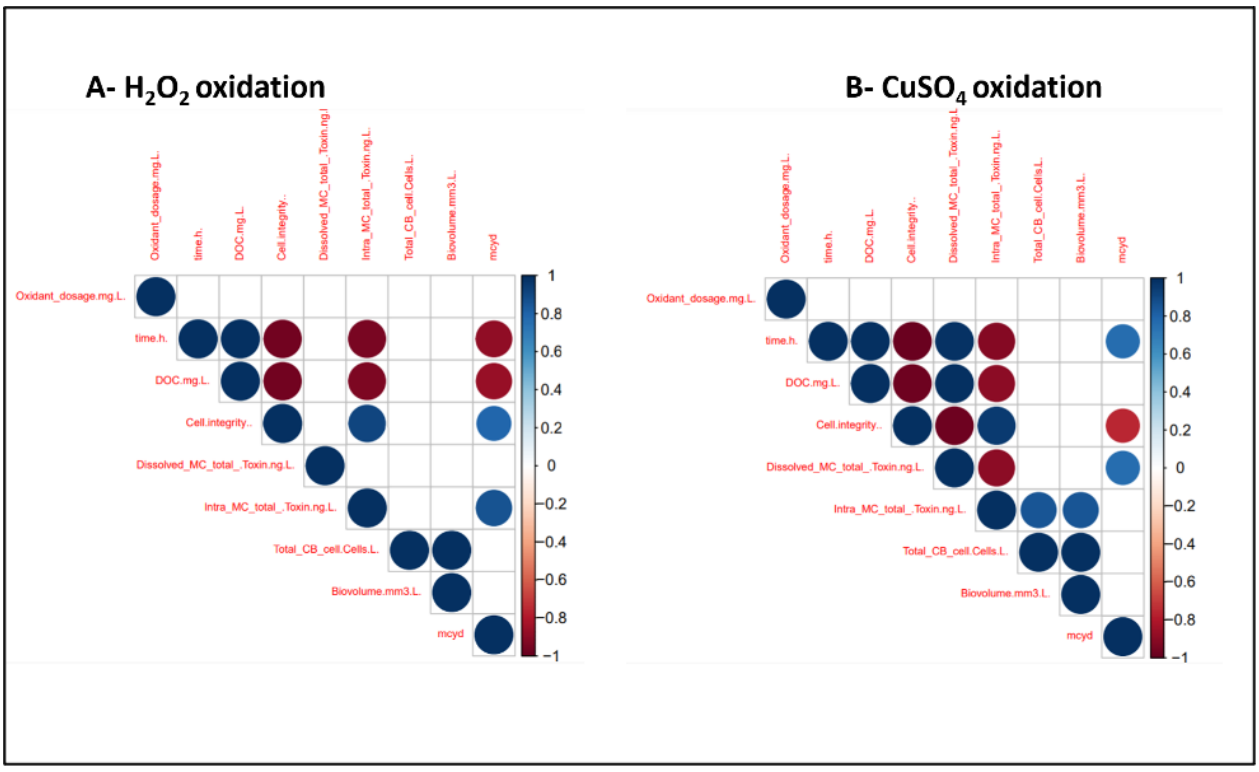

Figure A.2 Correlation analyses of the of *mcyD* gene copy numbers with MCs concentrations before and after onsite oxidation with (A) H_2O_2 and (B) $CuSO_4$ in PLSF. Colors represent the strength of correlations with their corresponding correlation coefficients.

APPENDIX B A Supplementary Information, Microscopic Taxonomic Cell COUNT

The microscopic taxonomic cell count observed in the results section was drawn based on genus level count, however, the following figure (Figure B.1) illustrates the cell count in the species level for all events.

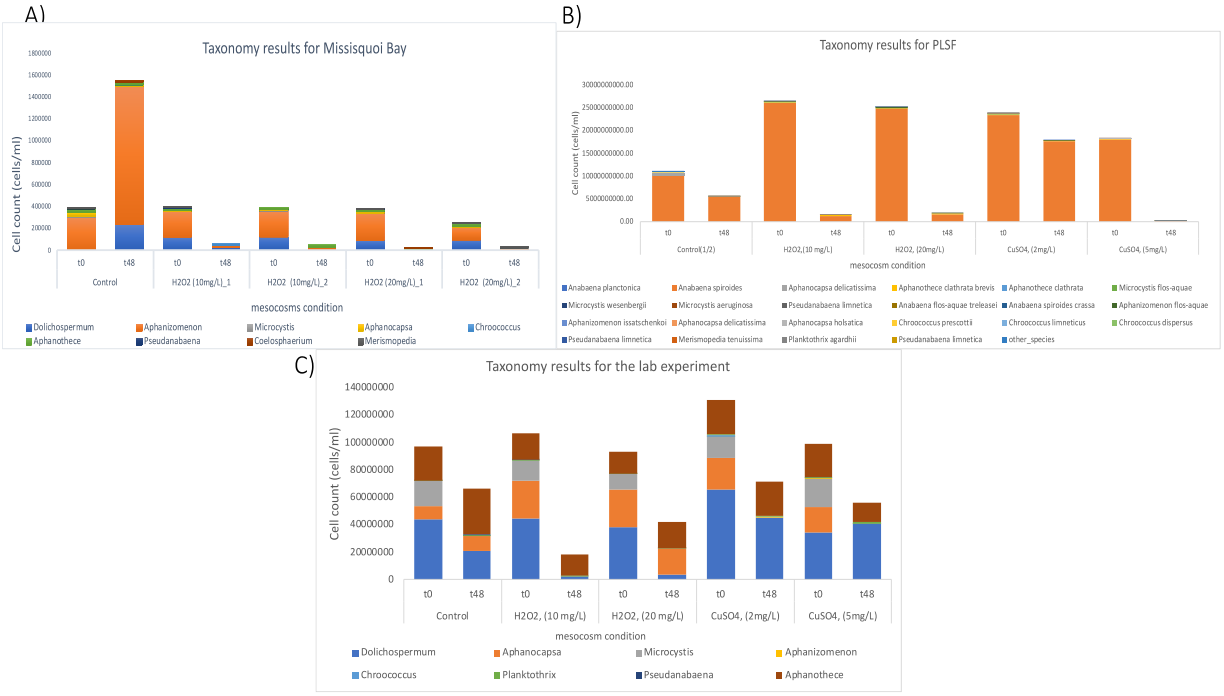

Figure B.1 Microscopic taxonomic cell count at the species level for (A) Missisquoi Bay (B) PLSF (C) The lab experiment