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POLYTECHNIQUE MONTRÉAL

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Optimal treatment strategies to prevent and manage cyanobacteria and cyanotoxins in drinking water sludge

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Thèse présentée en vue de l'obtention du diplôme de *Philosophiæ Doctor*

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Optimal treatment strategies to prevent and manage cyanobacteria and cyanotoxins in drinking water sludge

présentée par **Farhad JALILI**

en vue de l'obtention du diplôme de *Philosophiæ Doctor*

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For in and out, above, about, below,

Tis nothing but a Magic Shadow- show,

Play'd in a Box whose Candle is the Sun,

Round which we Phantom Figures come and ago.

Omar Khayyam

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RÉSUMÉ

Ces dernières années, l'accumulation de cyanobactéries et leur cyanotoxines dans les boues générées par des procédés de traitement conventionnels (coagulation, floculation, décantation et filtration) ont suscité des inquiétudes liées à la santé dans les usines de filtration de l'eau potable. Le devenir des cyanobactéries et cyanotoxines dans les boues est complexe en raison de l'impact simultané de la composition des cyanobactéries de l'eau brute, des conditions environnementales et de divers phénomènes tels que la survie des cellules, la croissance, la lyse et le relâchement de cyanotoxines pendant le stockage des boues.

Cependant, peu d'informations est disponible sur i) la dynamique des changements de la composition de la communauté cyanobactérienne entre l'eau brute et les boues stockées, ainsi que le surnageant associé, ii) le devenir des cyanobactéries et cyanotoxines lors du stockage de boues et iii) le traitement des boues contenant des cyanobactéries et cyanotoxines.

L'objectif général de cette recherche est d'étudier le devenir des cyanobactéries et des cyanotoxines dans les usines de filtration de l'eau potable pendant le stockage et la manipulation. Les objectifs spécifiques sont de : i) Diagnostiquer les points critiques des usines de filtration des eaux où les cyanobactéries et leurs cyanotoxines associées s'accumulent, ii) Déterminer la relation entre les communautés de cyanobactéries dans l'eau brute, les boues et le surnageant de boues ; iii) Déterminer l'impact des éléments nutritifs sur les changements de la communauté cyanobactérienne dans l'eau brute, les boues et le surnageant des boues ; iv) Comparer les données taxonomiques avec les résultats du séquençage métagénomique shotgun ; v) Valider la croissance des cyanobactéries pendant le stockage des boues, vi) Étudier la dynamique des compositions cyanobactériennes dans les boues stockées sur des conditions contrôlées, vii) Enquêter sur les cyanobactéries le plus résistant et le plus sensible pendant le stockage des boues, viii) Étudier l'impact sur la santé des cyanobactéries survivantes lors du stockage des boues, ix) Étudier l'impact de l'oxydation sur les cyanobactéries et les cyanotoxines en laboratoire et à grande échelle, x) Déterminer l'efficacité de l'oxydation pendant le stockage des boues, xi) Évaluer l'impact de l'oxydation et de la stagnation sur les communautés microbiennes et cyanobactériennes, xii) Mener une revue critique sur le devenir des cyanobactéries et des cyanotoxines dans les boues des usines de filtration des eaux et durant le stockage des boues et, xiii) Développer un cadre décisionnel pour

déterminer les meilleures pratiques pour minimiser les risques associés aux cyanobactéries et présence de cyanotoxines dans les usines de filtration des eaux.

Les objectifs ont été étudiés en trois phases :

La première phase (chapitre 4) étudie l'impact de la composition microbienne/ cyanobactérienne de l'eau brute et des nutriments sur les communautés microbiennes/ cyanobactériennes des boues et leur surnageant associé. La nouveauté est d'utiliser deux méthodes dans la détermination de la composition des cyanobactéries: Les comptes taxonomiques au microscope et le séquençage shotgun métagénomique. Les procédés de traitement conventionnels ont éliminé plus de 92 % des cyanobactéries mais ont conduit à une accumulation des cyanobactéries dans les boues jusqu'à 31 fois plus élevée que dans l'eau brute. Ensemble floculation, coagulation et décantation ont permis d'éliminer sélectivement plus de 96 % de *Microcystis* et *Dolichospermum*. Les communautés de cyanobactéries ont varié de l'eau brute aux boues pendant le stockage (1 à 13 jours). Cette variation était due à l'élimination sélective par la coagulation/ sédimentation ainsi qu'à l'accumulation de cellules capturées au cours de la période de stockage. Cependant, la prédiction de la composition de la communauté cyanobactérienne dans le surnageant des boues restait complexe. Concernant la corrélation des nutriments avec la présence des cyanobactéries, il ressort que disponibilité des orthophosphates était liée au profil de la communauté dans l'eau brute, tandis que la communauté des boues était influencée par l'azote total, l'azote Kjeldahl, le phosphore total et particulaire, et le carbone organique total. Aucune tendance n'a été observée concernant l'impact des nutriments sur les communautés cyanobactériennes du surnageant. Cette étude a présenté de nouveaux défis sanitaires, environnementaux et techniques dans le traitement des eaux potables par les procédés conventionnels en raison du devenir complexe des cyanobactéries dans les boues et leur surnageant.

La deuxième phase (chapitre 5 et 6) a été menée pour étudier l'impact du stress oxydatif (stockage et oxydation) sur les communautés microbiennes/cyanobactériennes des boues en utilisant les comptes taxonomiques, le séquençage shotgun métagénomique (taxonomie et fonction), la quantification du gène *mcyD* et la quantification des microcystines. Une étude longitudinale a été menée pour mesurer l'impact de l'oxydation par $KMnO_4$ (5 et 10 mg/L) et H_2O_2 (10 et 20 mg/L) suivie d'une stagnation sur des boues contenant des cyanobactéries de l'environnement (des floraisons). En parallèle, deux séries d'oxydation ont été réalisés dans le bassin de stockage des

boues (full-scale) de l'usine de filtration en utilisant 10 mg/L de $KMnO_4$. Les boues non-oxydées et oxydées ont été laissées en stagnation pendant 7 à 38 jours dans l'obscurité. Les comptes taxonomiques des échantillons des boues avant la stagnation et l'oxydation ont varié de 0,7 x 10^6 à 5,8 x 10^6 cellules/mL et *Aphanothece clathrata brevis*, *Aphanocapsa delicatissima*, *Dolichospermum spiroides* et *Microcystis aeruginosa* ont été les espèces prédominantes. Le séquençage shotgun métagénomique a montré que *Synechococcus*, *Microcystis* et *Dolichospermum* étaient prédominants. Les microcystines totales dans les boues variaient de 63 à 7100 ng/L, dont 63 % et 37 % étaient intra- et extra- cellulaires, respectivement. Des essais sur les conditions contrôlées en laboratoire ont montré que le $KMnO_4$ et le H_2O_2 réduisaient les comptes taxonomiques jusqu'à 77 %. Dans 4 des 8 dates d'échantillonnage, la croissance des cyanobactéries a été observée par les comptes taxonomiques pendant une stagnation prolongée dans l'obscurité allant de 7 à 38 jours. La croissance des cyanobactéries était dominée par les producteurs potentiels de microcystines tels que *Microcystis*, *Aphanocapsa*, *Chroococcus* et *Dolichospermum*. L'abondance relative des protéobactéries a augmenté après oxydation, tandis que l'abondance relative des cyanobactéries a diminué. La réduction maximale de microcystines obtenue après l'oxydation était de 41 %. La stagnation a provoqué la croissance des cyanobactéries dans 9 des 22 échantillons oxydés. Cependant, l'augmentation des comptes taxonomiques et du nombre de copies du gène de *mcyD* pendant la stagnation ont révélé l'impact de l'oxydation/stagnation sur la croissance des cyanobactéries. Le séquençage shotgun métagénomique a révélé que le stress tel que l'oxydation et la stagnation changent les communautés cyanobactériennes de l'ordre sensible des Nostocales (*Dolichospermum*) vers des ordres moins compromis et des producteurs potentiels de microcystines tels que Chroococcales (*Microcystis*) et Synechococcales (*Synechococcus*).

La troisième phase de cette étude de recherche (chapitre 7) est l'élaboration d'un cadre pour minimiser les défis liés aux cyanobactéries et aux cyanotoxines dans les usines de filtration des eaux et leurs boues. Une stratégie peut consister à minimiser l'accumulation de cyanobactéries dans les boues. Le cadre décisionnel que nous proposons comprend trois étapes : i) évaluation du risque lié à l'eau de source, ii) évaluation et gestion de la percée du traitement et, iii) évaluation et gestion du risque lié aux boues et au surnageant. Selon ce cadre décisionnel, l'eau brute sera monitorée pendant les saisons des fleurs d'eau « blooms » afin de fournir des informations suffisantes pour la prise de décision concernant l'optimisation du traitement de l'eau. Parallèlement, les cyanobactéries

et les cyanotoxines seront contrôlées dans les usines par l'optimisation des procédés de traitement, tels que l'ajustement de la coagulation, la pré-oxydation ou l'utilisation du charbon actif. Par ailleurs, le surnageant des boues sera contrôlé avant le recyclage en tête de l’usine ou rejeté à la source. Les boues chargées de cyanobactéries (solides) seront traitées et l'épandage (land application) /enfouissement (landfill) sera réglementé.

Pour la première fois, les comptes taxonomiques, le séquençage shotgun métagénomique (taxonomie et fonction), la quantification du nombre de copie du gène *mcyD* et la mesure de la concentration des microcystines ainsi que le suivi des paramètres physico-chimiques ont été appliqués pour la compréhension des défis des cyanobactéries et cyanotoxines dans les boues des usines de filtration de l’eau potable.

ABSTRACT

Health-related concerns on accumulation of cyanobacteria and cyanotoxins in the sludge of conventional treatment processes (coagulation, flocculation, sedimentation, and filtration) have been raised in drinking water treatment plants (DWTPs) in recent years. The fate of cyanobacteria and cyanotoxins in the sludge is complex due to the simultaneous impact of cyanobacterial composition in the intake water, environmental conditions, and various phenomena such as cell survival, growth, lysis, and cyanotoxin release during sludge storage.

However, little information about the dynamics of cyanobacterial shifts from the intake water towards sludge and supernatant, the fate of cyanobacteria and cyanotoxins during sludge storage, and treatment of cyanobacteria-laden sludge is available.

The general objective of this research study is to investigate the fate of cyanobacteria and cyanotoxins in the sludge of DWTPs during storage and handling. The specific objectives are to: i) Diagnose critical points of DWTPs where cyanobacteria cells and their associated cyanotoxins accumulate, ii) Determine the relationship between cyanobacterial communities in the intake water, sludge, and sludge supernatant; iii) Determine the impact of nutrients on cyanobacterial community shifts in the intake water, sludge, and sludge supernatant; iv) Compare taxonomic cell counts with shotgun metagenomic sequencing results; v) Validate cyanobacterial cell growth during sludge storage, vi) Study the dynamics of the cyanobacterial compositions in the stored sludge under controlled conditions, vii) Investigate the most resistant and susceptible cyanobacterial genus during sludge storage, viii) Study the potential health impact (i.e. cyanotoxin release) of the genera surviving sludge storage, ix) Investigate the impact of oxidation on cyanobacteria and cyanotoxins in both lab- and full- scales, x) Determine the efficiency of oxidation during sludge storage, xi) Evaluate the impact of oxidation and stagnation on microbial and cyanobacterial communities, xii) Conduct a critical review on the fate of cyanobacteria and cyanotoxins in conventional treatment plants' sludge and during sludge storage and, xiii) Develop an operational decision framework to determine the best practice to minimize risks associated with cyanobacteria and cyanotoxin presence in DWTPs.

The objectives were studied in three phases:

The first phase of this research study (Chapter 4) contains the impact of microbial/ cyanobacterial composition of the intake water and nutrient parameters on microbial/ cyanobacterial communities of sludge and its supernatant. The novelty is using a combination of microscopy taxonomic cell counts and taxonomic shotgun metagenomic sequencing. In parallel, the impact of nutrients on cyanobacterial communities (prepared by shotgun metagenomic sequencing) is investigated. Conventional treatment processes removed more than 92% of cyanobacterial cells but led to cell accumulation in the sludge by up to 31 times higher than in the intake water. Coagulation/ sedimentation selectively removed more than 96% of *Microcystis* and *Dolichospermum*. Cyanobacterial communities varied from the intake water to sludge during storage (1–13 days). This variation was due to the selective removal of coagulation/ sedimentation as well as accumulation of captured cells over the period of storage time. However, the prediction of the cyanobacterial community composition in the sludge supernatant remained a challenge. Among nutrient parameters, orthophosphate availability was related to community profile in the intake water samples, whereas communities in sludge were influenced by total nitrogen (TN), Kjeldahl nitrogen, total (TP) and particulate (PP) phosphorus, and total organic carbon (TOC). No trend was observed on the impact of nutrients on sludge supernatant communities. This study profiled new health-related, environmental, and technical challenges for the production of drinking water due to the complex fate of cyanobacteria in cyanobacteria-laden sludge and supernatant.

The second phase of this research study (Chapter 5 and Chapter 6) was conducted to investigate the impact of oxidative stress (storage and oxidation) on microbial/ cyanobacterial communities of sludge. The novelty is implication of microscopy taxonomic cell counts, taxonomic and function metagenomics, *mcyD* and MC qualification. A longitudinal study was conducted to measure the impact of oxidation with $KMnO_4$ (5 and 10 mg/L) and H_2O_2 (10 and 20 mg/L) followed by stagnation on sludge containing natural cyanobacterial cells (blooms). In parallel, two on-site shock oxidation treatments were performed in the DWTP's sludge holding tank using 10 mg/L of $KMnO_4$. Non-oxidized and oxidized sludge were left to stagnate for 7 to 38 days in the dark. In the sludge samples before stagnation and oxidation, taxonomic cell counts varied from 0.7×10^6 to 5.8×10^6 cells/mL and A*phanothece clathrata brevis*, *Aphanocapsa delicatissima*, *Dolichospermum spiroides*, and *Microcystis aeruginosa* were the predominant species. Shotgun metagenomic sequencing showed that *Synechococcus*, *Microcystis*, and *Dolichospermum* were predominant.

Total MCs in the sludge varied from 63 to 7100 ng/L, 63% and 37% of which were cell-bound and dissolved, respectively. Controlled laboratory trials showed that $KMnO_4$ and H_2O_2 decreased taxonomic cell counts up to 77%. For 4 out of 8 sampling dates, cyanobacterial cell growth was observed by total taxonomic cell counts during extended stagnation in the dark ranging from 7 to 38 days. Growth of cells was dominated by potential MC producers such as *Microcystis*, *Aphanocapsa*, *Chroococcus*, and *Dolichospermum*. The relative abundance of Proteobacteria increased after oxidation, while the relative abundance of Cyanobacteria decreased. The maximum MC level reduction achieved after oxidation was 41%. Interestingly, stagnation caused cell growth in 9 out of 22 oxidized samples. The increase of cell counts and *mcyD* gene copy numbers during stagnation revealed the impact of oxidation/ stagnation on cell growth. Shotgun metagenomic sequencing unveiled that stress conditions such as oxidation and stagnation shift cyanobacterial communities from the sensitive Nostocales (*Dolichospermum*) order towards less compromised orders and potential MC producers such as Chroococcales (*Microcystis*) and Synechococcales (*Synechococcus*).

The third phase of this research study (Chapter 7) is development of a decision framework to minimize challenges related to cyanobacteria and cyanotoxins in DWTPs and sludge. One strategy can be minimizing cyanobacterial accumulation in the sludge. Our suggested decision framework includes three steps as: i) Source water risk assessment, ii) Treatment breakthrough assessment and management and, iii) Sludge and supernatant risk assessment and management. According to this decision framework, intake water will be monitored during cyanobacterial seasons to provide sufficient information for decision about water treatment optimization. Meanwhile, cyanobacteria and cyanotoxins will be controlled in DWTPs by treatment process optimization including coagulation adjustment or pre-oxidation or activated carbon implication. Furthermore, sludge supernatant will be monitored before recycling to the head of DWTPs or discharging into the source. Cyanobacteria-laden sludge (solids) will be treated and land application/ filling will be regulated.

This research study provides new insights into i) The dynamics and the fate of cyanobacteria and cyanotoxins in the sludge and its associated supernatant during storage, ii) The efficiency of oxidation as a potential treatment approach of cyanobacteria-laden sludge, and iii) Development of a strategy for handling of cyanobacteria-laden sludge. For the first time, microscopy taxonomic

cell counts, taxonomic/ function metagenomic sequencing, *mcyD* gene copy, cyanotoxin quantification, and physico-chemical parameter measurement were applied for a better-understanding of cyanobacteria and cyanotoxin challenges in the DWTP's sludge.

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$AlCl_3$	Aluminum chloride
BMAA	β-N-methylamino-L-alanine
aka	Also known as
AOM	Algal organic matter
ATX-a	Anatoxin-a
BAC	Biological activated carbon
BMAA	beta-methylamino-L-alanine
CAPEX	Capital expenditures
Chla	Chlorophyll-a
CW	Clarified water
CYN	Cylindrospermopsin
DAF	Dissolved air flotation
DL	Detection limit
DN	Dissolved nitrogen
DO	Dissolved oxygen
DOM	Dissolved organic matter
DON	Dissolved organic nitrogen
DOP	Dissolved organic phosphorus
DP	Dissolved phosphorus
DWTP	Drinking water treatment plant
ELISA	Enzyme-linked immunosorbent assays

$FeCl_3$	Ferric chloride
FW	Filtered water
GAC	Granular activated carbon
GC	Gas chromatography
HPLC	High performance liquid chromatography
LC	Liquid chromatography
MAC	Maximum allowable concentrations
MCs	Microcystins
MC-LR	Microcystin-LR
MC-YR	Microcystin-YR
MC-RR	Microcystin-RR
MC-LA	Microcystin-LA
MC-LY	Microcystin-LY
NO_2/NO_3	Nitrite/Nitrate
OP	Orthophosphate
OPEX	Operating expenses
PAC	Powdered activated carbon
PAFC	Polyaluminium ferric chloride
PC	Phycocyanin
PP	Particulate phosphorus
PPIA	Phosphatase Inhibition Assay
R	Pearson correlation
RW	Raw water- intake water

SEM	Scanning electron microscope
SST	Sludge holding tank supernatant
ST	Sludge holding tank
STX	Saxitoxin
TN	Total Nitrogen
TP	Total Phosphorus
TW	Treated water, finished water, chlorinated water
US EPA	United States Environmental Protection Agency
WHO	World Health Organization
WWTP	Wastewater treatment plant

CHAPTER 1 INTRODUCTION

Cyanobacteria (aka blue-green algae) are a group of photosynthetic prokaryotes able to fix nitrogen and to produce oxygen (having chla) (Dodds et al., 1999; Hamilton et al., 2016). Cyanobacteria can also produce cyanotoxins, which are harmful for human, and animals (Ibelings et al., 2014). It is reported that around 40 species of cyanobacteria can produce cyanotoxins. Five groups of cyanotoxins as microcystins (MCs), cylindrospermopsin (CYN), anatoxin-a (ATX-a), saxitoxins (STXs) and β-N-methylamino-L-alanine (BMAA) are common in fresh water resources (Wang et al., 2022; Westrick et al., 2010).

Cyanobacteria and cyanotoxins are considered as a challenge in water resources and DWTPs worldwide (Ho and Michalak, 2019; Ho et al., 2019; Kimambo et al., 2019; Pick, 2016; Winter et al., 2011). Several human and animal death has been reported due to cyanotoxin presence in water resources (Giannuzzi et al., 2012; Griffiths and Saker, 2003; Gugger et al., 2005; Jochimsen et al., 1998; Kotak and Zurawell, 2007; Onodera et al., 1997; Svrcek and Smith, 2004). Moreover, cyanobacteria and cyanotoxins can cause: i) Negative aesthetic impacts on taste, odor, and color of water; ii) extra water treatment requirements such as ozonation and membrane filtration; and iii) increase in consumption of coagulants, flocculants, and activated carbon (Shang et al., 2018; Westrick et al., 2010; Zamyadi et al., 2013a).

Conventional water treatment processes including coagulation, flocculation, sedimentation, and filtration are common approaches to remove cyanobacteria and cell-bound cyanotoxins in DWTPs (Drikas et al., 2001; Westrick et al., 2010; Zamyadi et al., 2013a). However, the constraints and limitations of these processes such as: i) Inefficiency in removing of dissolved cyanotoxins (Newcombe and Nicholson, 2004), ii) Cell/ trichomes lysis/ damage leading to cell-bound cyanotoxin release throughout the treatment chain (Pestana et al., 2019; Pietsch et al., 2002), and iii) Huge accumulation of cyanobacteria over the surface of clarifier and filters and the clarifier's sludge (Drikas et al., 2001; Ho et al., 2012; Zamyadi et al., 2013a; Zamyadi et al., 2012c) should be considered during operation. It is reported that cyanobacterial cells can be accumulated by up to 100 times higher in the sludge in comparison with the intake water, even in DWTPs with low cyanobacterial flux (<1000 cells/mL) (Almuhtaram et al., 2018; Zamyadi et al., 2013b). Furthermore, toxic cyanobacterial breakthrough has been reported in conventional processes and even post-oxidation step (Zamyadi et al., 2012c).

Several studies highlighted that toxic cyanobacterial cells such as *Microcystis aeruginosa*, *Dolichospermum circinale*, *Oscillatoria sp*., and *Cylindrospermopsis raciborskii* can survive in the stored sludge within 2-12 days. Then, they can be lysed and release cyanotoxins. (Drikas et al., 2001; Ho et al., 2012; Li et al., 2018; Sun et al., 2013; Sun et al., 2012; Sun et al., 2018; Xu et al., 2016). Furthermore, the probability of cell growth in the stored sludge has been highlighted in recent studies. However, the authors of these studies hypothesized either cell growth or i) underestimation of the cell quota, ii) increase in metabolite production during cyanobacteria-laden storage, and iii) additional cell settlement from the supernatant to the sludge during storage (Dreyfus et al., 2016; Pestana et al., 2016; Water Research Foundation (WRF) and Water Research Australia, 2015). Therefore, the fate of cyanobacteria and cyanotoxins during sludge storage is not still well-understood. Additionally, all the previous investigations have been performed on the sludge generated by jar test (laboratory-based) on spiked cultured cyanobacteria (mostly *M. aeruginosa*) into raw water. Therefore, there is no data about the fate of natural cyanobacteria (blooms) in the stored sludge of full scale DWTPs.

Accumulation of cyanobacteria in the sludge can lead to health challenges and technical issues when the sludge supernatant is recycled to the head of DWTPs. It is reported that recycling of cyanobacteria-laden sludge supernatant to the head of the DWTP may increase cyanobacterial cell counts by up to 40% in the intake water (Zamyadi et al., 2019). Thus, a practical approach against cyanobacteria-laden sludge is required.

Some studies suggest that cyanobacteria-laden sludge should be disposed prior to 4 days to minimize risks associated with metabolite release (Pei et al., 2017; Sun et al., 2015). However, these studies solely focused on cell lysis and cell-bound cyanotoxin release and not on cell survival during sludge storage. While oxidation is a common approach to control cyanobacteria and cyanotoxins in water (Fan et al., 2013a; Fan et al., 2013b; Fan et al., 2014b; Lusty and Gobler, 2020; Matthijs et al., 2012; Moradinejad et al., 2020; Piezer et al., 2020b; Zamyadi et al., 2010; Zamyadi et al., 2012c), there is only one study conducted on the treatment of thickened sludge using $KMnO_4$ and powdered activated carbon (PAC) in a full-scale DWTP (Zamyadi et al., 2016b). Therefore, more study on oxidation of cyanobacteria-laden sludge is essential.

Next generation sequencing (NGS) is a powerful tool for prediction of cyanobacterial blooms (Berry et al., 2017; Tromas et al., 2017) and cyanotoxin prediction (Fortin et al., 2010). Recently, these techniques have been applied to investigate the fate of microbial/ cyanobacterial communities throughout water and wastewater treatment chains (Fortin et al., 2015 ; Hou et al., 2018 ; Li et al., 2017 ; Lira et al., 2020; Lorenzi et al., 2019 ; Lusty and Gobler, 2020; Ma et al., 2020 ; Moradinejad et al., 2020; Pinto et al., 2012 ; Quince et al., 2009 ; Zamyadi et al., 2019) and metabolic functions (Moradinejad et al., 2021a). However, there are few studies applied NGS for investigation of microbial and cyanobacterial communities in the DWTP's sludge (Pei et al., 2017; Xu et al., 2018). The authors of Xu et al. (2018) observed similar bacterial communities in sludge samples collected from six different DWTPs with the same treatment processes. They suggested that bacterial communities in sludge are shaped by the intake water communities, however they did not compare the bacterial composition in sludge with that in the intake water. Thus, dynamics of cyanobacteria from intake water to sludge and supernatant has not been investigated. The authors of Pei et al. (2017) studied the impact of different coagulants on bacterial communities and metabolite release in the sludge. They demonstrated that the relative abundance of *Microcystis* decreased after 4 days, reflecting cell damage and the subsequent release of extracellular MCs and organic matter. They suggested that the sludge should be treated or disposed within 4 days to control concentrations of cyanotoxin and organic matter. However, cell survival (or cell growth) has not been investigated in this study.

We believe that combination of microscopy taxonomic cell counts, NGS techniques and cyanotoxin measurement can be helpful for better understanding the fate of cyanobacteria-laden sludge during treatment (oxidation) and storage.

This thesis is comprised of ten chapters. Chapter 1 summarizes a short introduction on cyanobacteria and cyanotoxin, their impacts on sludge and the importance of cyanobacteria-laden sludge treatment. Chapter 2 presents a literature review on cyanobacteria and cyanotoxin principles, their occurrence, methods of detection and measurement, related regulations, critical points of DWTPs where cyanobacteria and cyanotoxins are accumulated, the fate of cyanobacteria and cyanotoxins during sludge storage, cyanobacteria and cyanotoxin treatment and gaps of knowledge. Chapter 3 explains the research objectives, hypotheses and methodology of this study. Chapter 4 is the first article published in the journal of *Toxins*. Chapter 5 contains the 2nd article submitted in

the journal of *Toxins*. Chapter 6 is the 3rd article published in the journal of *Water*. Chapter 6 is the 4th article published in the journal of *Toxins*. Chapter 8 presents general discussions. Chapter 9 presents conclusions and recommendations. Supplementary information is presented in 0.

CHAPTER 2 LITERATURE REVIEW

2.1 Overview and Definition

Cyanobacteria, also commonly named blue-green algae, are a group of photosynthetic prokaryotes able to fix nitrogen (convert N_2 to ammonium) and produce oxygen (having chlorophyll a). Even if "blue-green algae" is commonly used, it is a wrong appellation because cyanobacteria are prokaryotes, while algae are eukaryotes (Dodds et al., 1999; Hamilton et al., 2016).

Cyanobacteria have two accessories pigments belonging to the phycobiliprotein family that serves for light harvesting: 1) Phycocyanin (PC): blue pigment which is found in fresh and brackish water; as a fluorescent pigment, absorbs orange and red light in around 620 nm and emits it at 650 nm, 2) Phycoerythrin (PE): normally found in brackish water, a red protein that absorbs light at 530 nm and emits it at around 570 nm (Glazer, 1989; Pires, 2010). The harvested light is transmitted to only one type of chlorophyll present, namely chlorophyll a (Chla).

Climate change and its related factors such as water temperature changes, population increase, and phosphorus-rich nutrients discharging into water sources, will increase the likelihood of harmful cyanobacterial blooms (HCBs) proliferation (Delpla et al., 2009; Jasim and Saththasivam, 2017; Nour et al., 2006; Zamyadi et al., 2012b).

It is reported that around 40 species of cyanobacteria can produce cyanotoxins due to secondary metabolite release (Westrick et al. 2010). Five groups of cyanotoxins as microcystins (MCs), cylindrospermopsin (CYN), anatoxin-a (ATX-a), saxitoxins (STXs), and β-N-methylamino-L-alanine (BMAA) are more common in freshwater resources. In Table 2-1, the most common cyanobacteria, released cyanotoxins, and their relative toxicity are summarized.

Table 2-1 Major toxic cyanobacteria, cyanotoxins, associated toxicity and metabolites

Metabolite	LD50 (i.p. mouse) mcg/kg	Cyanobacterial producers (genus)	Impact on human organ	Reference
MCs	45-1000	*Microcystis, Planktothrix, Oscillatoria, Nostoc, Dolichospermum, Anabaenopsis, Hapalosiphon*	Liver	(Beagle Bioproducts Inc., 2013; Chorus and Bartram, 1999a; Chorus et al., 2000; Gupta et al., 2003; Schmidt et al., 2014; U.S. National Library of Medicine; Watanabe et al., 1988; Westrick et al., 2010)
MC-LR	25-158			
MC-YR	68-110.6			
MC-RR	235.4- 600			
MC-LA	39-50			
MC-LY	91			
ATX-a	250-375	*Dolichospermum, Oscillatoria, Aphanizomenon, Cylindrospermum, Microcystis, Planktothrix, Raphidiopsis, Arthrospira, Phormidium, Nostoc, Oscillatoria*	Nervous system	(Chorus et al., 2000; van der Merwe, 2014; Westrick et al., 2010)

Table 2-1 Major toxic cyanobacteria, cyanotoxins, associated toxicity and metabolites (continue)

Metabolite	**LD50 (i.p. mouse) mcg/kg**	**Cyanobacterial producers (genus)**	**Impact on human organ**	**Reference**
ATX-a (s)	20-40	*Dolichospermum*	Nervous system	(Chorus et al., 2000; United States National Library of Medecine (NLM))
STXs GTX2, GTX3, dc-GTX2, dc-GTX3, dc-STX, C1, C2 and GTX5	3.4-10	*Aphanizomenon, Anabaena, Lyngbya, Cylindrospermopsis*	Nervous and respiratory system	(Faber, 2012; van der Merwe, 2014; Westrick et al., 2010)
CYN	200-375	*Cylindrospermopsis, Anabaena, Umezakia, Aphanizomenon*	Kidney and liver	(Banker et al., 1997; Banker et al., 2001; Chorus et al., 2000; Westrick et al., 2010)
7-epicilyndrospermopsin	Non-toxic			
7-deoxycylindrospermopsin			-	(Banker et al., 2001; Merel et al., 2010a)
BMAA	3000	*Produced by all cyanobacteria.*	Nervous system	(Al-Sammak et al., 2014; Cox et al., 2005; Merel et al., 2010b)

2.2 Occurrence and Impact of Cyanobacteria and Cyanotoxins in Sources

Over the past decades, global warming and climate change resulting in water temperature increases, raising of nutrients (nitrogen and phosphorus) in water bodies due to the utilization of fertilizers or wastewater discharge increased the occurrence of cyanobacterial bloom frequency (Cremona et al., 2018; Kosten et al., 2012; Paerl and Paul, 2012a; Scholz et al., 2017; Sukharevich and Polyak, 2021).

Cyanobacteria are considered as a crucial problem due to several reasons (Agence Française de Sécurité Sanitaire des Aliments (AFSSA) and Agence Française de Sécurité Sanitaire de l'Environnement et du Travail (AFSSET), 2006b; Carmichael, 1992; Gupta et al., 2003; Health Canada, 2012; Health Canada and Federal-Provincial-Territorial Commitee on Drinking Water, 2016; Zamyadi et al., 2016a):

- Health problems and even mortality in humans and other mammals as well as birds and fish due to cyanotoxin,
- Change the taste, odor, color, and produce scum on the water surface, all negatively impact water qualities and increase public complaints,
- High concentration of cyanobacteria increases water turbidity that may undermine mandatory efficient solid removals in DWTPs,
- Cyanobacteria proliferation affects the biodiversity of water resources in different ways. For instance, since cyanobacteria is rarely predated by zooplankton; its population remains fairly constant in comparison with other photosynthetic microorganisms; or, cyanobacterial blooms may cause a lack of light and nutrients for other fauna.
- Blooms cause negative financial impacts due to: i) Extra treatment requirements such as increasing the consumption of coagulant, flocculants, and activated carbon (for cyanotoxins) amount, or, implementation of advanced treatment processes (eg. ozonation and membrane filtration), and, ii) Recreational and tourist sites closing during bloom events.

Several reports documented fatal impacts of cyanotoxins on humans as well as on livestock and wildlife animals around the world (Giannuzzi et al., 2012; Gugger et al., 2005; Jochimsen et al., 1998; Kotak and Zurawell, 2007; Onodera et al., 1997). The first fatal incident of cyanobacteria on livestock was reported in 1878 in Australia (Svrcek and Smith, 2004). In 1979, extracellular CYN released from a *C. raciborskii* bloom lysed by copper sulfate poisoned 120 people in Queensland, Australia (Griffiths and Saker, 2003). In South America, water contaminated with MCs caused the death of 26 patients in a hemodialysis center in Caruaru, Brazil (Jochimsen et al., 1998). The occurrence of cyanobacterial in 2011 caused 34 heath-related reports including five cases of human illness and dog death in Kansas, USA (Trevino-Garrison et al., 2015).

Cyanobacteria occurrence and resulting cyanotoxins release are considered a widespread problem in many parts of the World (Churro et al., 2012; Kosten et al., 2012). Several reports documented the presence of toxic cyanobacterial blooms around the world, for instance, in Europe (Kahru et al., 2007), Africa (Verschuren et al., 2002), Asia (Duan et al., 2009), North and South America (Dorr et al., 2010; Stumpf et al., 2012), Australia (Al-Tebrineh et al., 2012) and even Antarctica (Hitzfeld et al., 2000). One investigation reported that *Microcystis* were detected in 108 of 257 countries and territories in the World (Harke et al., 2016). One intense and widespread bloom has been reported on the American side of Lake Erie in 2014 affected the drinking water supply of 400,000 residents of Toledo (Bullerjahn et al., 2016).

In Canada, cyanobacterial blooms have been documented since the early 1900s (Estepp and Reavie, 2015). Nowadays, cyanobacterial blooms occur very often especially, in important Canadian drinking water sources such as Lake Champlain, Lake Ontario, Lake Erie, Lake of the Woods, and Lake Winnipeg (Pick, 2016). In Quebec, bloom frequency increased by up to 20% from 2014 (Ministère du Développement durable, 2015). Since 2015, around 500 lakes have been impacted by cyanobacterial bloom in Quebec (Ministère du Développement durable, 2018).

2.3 Measurement and Detection

2.3.1 Measurement and Identification of Cyanobacteria

Identification and measurement of cyanobacterial cells is the first step in cyanobacterial management. An ideal cyanobacterial cell measurement method must be easily available,

affordable, not require high levels of skill, preferably applicable in situ, and sufficiently reliable. Additionally, a suitable cyanobacterial cell measurement technique should provide a quick response to enable a timely and appropriate response to increasing cyanobacteria in water resources or within DWTPs (Zamyadi et al., 2016a).

Visual observation is the first approach to monitoring cyanobacterial presence recommended to be done weekly or biweekly during high-risk seasons (Chorus and Bartram, 1999a; Newcombe, 2009; Newcombe et al., 2010). Visual observation is easy and has a low cost; however, it is not accurate to verify the presence of a cyanobacterial bloom in water resources (Bartram et al., 1999).

The methods of cyanobacterial cell measurements can be categorized into three groups:

1) Taxonomic cell counts;

2) Biomass estimation;

3) Viable biomass estimation.

These measurement methods are detailed in Table 2-2.

The microscopy taxonomic cell count technique is considered the classic measurement technique to enumerate cyanobacterial cells. It has been widely applied to identify and measure cells in water resources and DWTPs and in both water and sludge samples (Almuhtaram et al., 2018; Dreyfus et al., 2016; McQuaid et al., 2011; Pestana et al., 2016; Pinkanjananavee et al., 2021; Zamyadi et al., 2013a; Zamyadi et al., 2013b; Zamyadi et al., 2012c; Zamyadi et al., 2019). Microscopy taxonomic cell counts provide absolute values of different cyanobacterial species which is truly useful to i) Perform analysis of risk, and ii) Evaluate treatment efficiency throughout the water treatment chain.

Table 2-2 Techniques to measure cyanobacterial cells

Adapted from (Arar and Collins, 1997; Becker et al., 2002; Breuer et al., 2016; Cagnard et al., 2006; Chorus and Bartram, 1999a; Dziallas et al., 2011; Fortin et al., 2010; Gregor and Marsalek, 2004; Li et al., 2002; Nakamura and Watanabe, 1998; Richardson et al., 2010; Sobiechowska-Sasim et al., 2014; Thrane et al., 2015; Van Nevel et al., 2017; WU, 2014; Zamyadi et al., 2016a; Zamyadi and Prévost, 2007; Zhou et al., 2012).

Taxonomic Identification and Enumeration			
Measurement technique	**Description**	**Advantages**	**Disadvantages**
Taxonomic cell counts	Cell counts based on morphology of cells by inverted microscope (phase contrast)	Routine reference method Direct technique Affordable Only low amount of samples is required Provides absolute values	Time-consuming and expert analyst only In some cases, it is not easy to distinguish the cells. Especially, when the cells are lysed or broken. Due to the agglomeration of cells, cell numbers can be underestimated. Samples are preserved by Lugol's Iodine which must be re-added every 6 months or, by formaldehyde or glutaraldehyde which are hazardous and can distort cells. Significant standard deviation in some enumeration cases. No data about the toxicity of species is provided
Molecular	Based on the analysis of DNA or RNA	Accurate Cost effective Sensitive and specific Detection of cells growth and bloom in the early phase	Needs experienced technicians and advanced molecular methods. Not still considered a standard method. Subject to interferences (extraction). Results still need to be validated with taxonomic cell counts. Tedious Relative (abundance) results

Table 2-2 Techniques to measure cyanobacterial cells (continue)

Total Biomass Estimation			
Measurement technique	**Description**	**Advantages**	**Disadvantages**
Flow cytometry	Measurement based on fluorescence emission of chla and phycobilin pigments	Rapid quantification Accurate	Agglomeration of cells and multicellular cells cannot be enumerated with accuracy.
Combined flow cytometry and microscopy	Cells are recognized by size and fluorescence characterization	Accurate	Time-consuming Need experienced technicians
Chla content	Pigment analysis to measure chla concentration based on the cyanobacterial biomass using spectrophotometer or HPLC	Accurate Rapid in comparison to microscopic methods. Provide detailed information about the phytoplankton composition.	Not specific to cyanobacteria. Need a series of instruments and equipment. Need experienced technician. Expensive.
PC content	PC concentrations are measured based on its pigments using spectrophotometry or HPLC	Inexpensive and rapid in comparison to HPLC techniques.	A solvent such as acetone is required its risk must be considered. Need enough experience. Expensive

Table 2-2 Techniques to measure cyanobacterial cells (continue)

Viable Biomass Estimation			
Measurement technique	**Description**	**Advantages**	**Disadvantages**
Fluorescence combined with staining	Staining cells before measurement to distinguish alive and dead cells	Evaluation of cell integrity/ viability. Useful in low concentrations of cells to enumerate viable cells. Challenging to use for mixed cyanobacteria or in natural samples with other bacteria	Need experienced technician. Tedious Difficult in multicellular cultures or blooms.
In vivo fluorometry	Direct measurement of chla or phycobilin pigments fluorescence in viable cells	Rapid Large amount of data can be gathered. Suitable for real time measurement. Useful for monitoring a bloom in water resources or in the plants. Easy to be used Can be used continuously.	Periodic calibration is required The air bubbles and water turbidity can make interference with measurement. Light can interfere with fluorescent pigments of chla Measurement can be complicated in the presence of chlorine. Cannot identify and differentiate cyanobacterial species.

Despite the advantages of the taxonomic cell count technique, it cannot be applied in situ. Moreover, it can only be performed by experienced trained technicians, sample preservation in Lugol's Iodine may affect cyanobacteria biovolume, and the presence of debris in the sample or agglomeration of the cells may interfere with the results. In addition, there may be a considerable time between sampling and preparation of data, which may alter sample composition. More importantly, taxonomic cell counts cannot distinguish toxic cyanobacterial species from non-toxic ones (America Water Works Association (AWWA), 2010; Hawkins et al., 2005; Lawton et al., 1999; Sanseverino et al., 2017; Zamyadi et al., 2019).

Viable and dead cells can be distinguished using the flow cytometry technique combined with staining the cells using SYTO 9 and propidium iodide (PI) (Zhu and Xu, 2013). Although this technique provides a valuable overview of cell viability, it is still complicated and needs experienced technicians (Jin et al., 2018).

Although chla and PC measurement using fluorometric probes can be applied in situ, they need validation for each water resource. Also, in situ fluorometric techniques are subjected to encounter various interferences which negatively impact the results (America Water Works Association (AWWA), 2010; Cagnard et al., 2006; Gregor and Marsalek, 2004; Richardson et al., 2010; Zamyadi et al., 2016a).

2.3.2 Next Generation Sequencing (NGS)

Over 25 years, NGS has been introduced as a robust tool to study microbial communities, the interaction of species, and the impact of biotic and abiotic parameters on various communities such as the human gut, plant, soil, and water (Großkopf and Soyer, 2014; Raes and Bork, 2008). Accordingly, over the past decade, NGS has been widely applied to evaluate microbial, bacterial, and cyanobacterial communities in water resources, DWTPs, DWTP sludge, and WWTPs (Table 2-3). NGS is helpful i) To predict cyanobacterial blooms by studying bacterial shifts in water resources (Berry et al., 2017; Tromas et al., 2017), ii) For a better understanding of the impact of the water treatment chain on microbial and bacterial communities (Li et al., 2017; Ma et al., 2020; Zamyadi et al., 2019) and to investigate cyanobacteria evolution in the sludge (Pei et al., 2017; Xu et al., 2018). Recently, NGS has been applied for monitoring microbial and cyanobacterial shifts during oxidation in water resources (Lusty and Gobler, 2020; Moradinejad et al., 2020).

Table 2-3 NGS investigations on resource water, DWTP, sludge and WWTPs

NGS techniques	Targeted community	Sampling point	Location	Reference
16S rDNA	Microbial	Lake	-	(Quince et al., 2009)
16S rRNA	Bacterial	Recreational water	Beijing- China	(Cui et al., 2017)
16S rRNA	Cyanobacterial	Resource water and recreational site	Lake Champlain- Canada	(Fortin et al., 2015)
16S-23S rRNA	Cyanobacterial	Reservoirs of a DWTP	Ingazeira and Mundaú- Brazil	(Lorenzi et al., 2019)
16S rRNA	Cyanobacteria	Lake (a resource water and recreational site)	Lake Champlain- Canada	(Tromas et al., 2017)
16S rRNA	Cyanobacterial	Pond and lake- to study the impact of H_2O_2 on cyanobacterial communities	Georgica Pond, Mill Pond, Lake Agawam and Roth Pond- USA	(Lusty and Gobler, 2020)
16S rRNA	Cyanobacterial	Lake Erie	Lake Erie- Canada	(Berry et al., 2017)
16S rRNA	Cyanobacterial	Upper Klamath Lake	Oregan- USA	(Eldridge and Wood, 2019)
Shotgun Metagenomics	Microbial	Baltic Sea	-	(Ininbergs et al., 2015)
Shotgun Metagenomics	Bacterial, cyanobacterial	Resource water and recreational site, to study the impact of oxidation on bacterial and cyanobacterial communities	Lake Champlain- Canada	(Moradinejad et al., 2020)
Shotgun Metagenomics	Cyanobacterial	Resource water	Campina Grande reservoirs- Brazil	(Walter et al., 2018)
Shotgun Metagenomics	Cyanobacterial	River and sediment	North Han River- South Korea	(Kim et al., 2018a)

Table 2-3 NGS investigations on resource water, DWTP, sludge and WWTPs (continue)

NGS techniques	Targeted community	Sampling point	Location	Reference
Shotgun metagenomics	Cyanobacterial	Lake	Bahia, Brazil	(Affe et al., 2018)
Shotgun metagenomics	Cyanobacterial	Irrigation and recreational reservoir	Rosarito reservoir, Spain	(Casero et al., 2019)
Shotgun metagenomics	Cyanobacterial	Coral reef benthic	Bonaire, Caribbean Netherlands	(Cissell and McCoy, 2020)
16S rRNA	Microbial, bacterial	DWTP	Guangdong- China	(Hou et al., 2018)
16S rRNA	Microbial	DWTP	Wujiang- China	(Li et al., 2017)
16S rRNA	Bacterial	DWTP	Michigan, USA	(Pinto et al., 2012)
16S rRNA	Bacterial	DWTP (filters)	Zurich, Switzerland	(Lautenschlager et al., 2014)
16S rRNA	Bacterial	DWTP (4 plants)	Beijing Hangzhou, Huaishu river- China	(Ma et al., 2020)
16S rRNA	Bacterial	DWTP, distribution system and biofilm	The Netherlands	(Liu et al., 2014a)
16S rRNA	Cyanobacterial	DWTP	Australia	(Zamyadi et al., 2019)
Shotgun metagenomics	Microbial	DWTP	Pearl River Delta- China	(Chao et al., 2013)
Shotgun metagenomics	Microbial	DWTP and distribution system	-	(Zhang et al., 2017b)
Shotgun metagenomics	Microbial	DWTP	Zurich- Switzerland	(Oh et al., 2018)
16S rRNA	Microbial	Cyanobacteria-laden sludge (Artificial sludge made by coagulation in Jar test)	Raw water of Queshan Reservoir- China	(Pei et al., 2017)
16S rRNA	Bacterial and cyanobacterial	Cyanobacteria-laden sludge (sludge of clarifier)	6 DWTPs- China	(Xu et al., 2018)
Shotgun metagenomics	Bacterial	Hospital WWTP	Germany	(Schneider et al., 2020)
Shotgun metagenomics	Bacterial	Municipal WWTP	Portugal	(Lira et al., 2020)

Targeted amplicon sequencing and shotgun metagenomic sequencing are two common NGS techniques (Bharti and Grimm, 2021).

In targeted amplicon sequencing, genes are amplified by PCR and based on the selected regions (V1-V9). 16S rRNA is the most common marker applied to profile bacterial taxonomy (Baker et al., 2003; Costa and Weese, 2019; Pel et al., 2018). 16S rRNA is cost-effective and widely applied in different studies. However, the result is based on the attribution of the 16S rRNA gene with the operational taxonomic unit (OTU). Since OTU is generated at the phylum and genus level, the results are not accurate at the species level (Ranjan et al., 2016).

In shotgun metagenomic sequencing, untargeted DNA fragments are sequenced (directly and randomly) providing a picture of whole genomes that exist in a sample. Shotgun metagenomic sequencings can also provide data about functions (Costa and Weese, 2019; Romanis et al., 2021) to show ensemble biodiversity and what they can do in the sample (Sharpton, 2014). Shotgun metagenomic analysis is not dependent on the culture. In fact, this technique illustrates all culturable, unculturable, and even unknown taxa (Escobar-Zepeda et al., 2015; Gilbert and Dupont, 2011). Also, shotgun metagenomic sequencing has a better taxonomic resolution and diversity due to applying various genes instead of unimodal 16S rRNA (Bharti and Grimm, 2021; Quince et al., 2017; Riesenfeld et al., 2004; Romanis et al., 2021). Metagenomics access directly the functional genes of organisms and elucidate more information than polygenetic methods, which only analyze 16S rRNA (D'Agostino et al., 2016; Thomas et al., 2012). In brief, the metagenomic analysis includes the following steps: i) Sampling and processing in which DNA is extracted, ii) Shotgun sequencing to amplify DNA fragments, iii) Assembly to make longer cotigs in uncultured organisms and full-length coding sequences (CDs), iv) Binning DNA sequences, v) Annotation, to locate genes in one genome, vi) Statistical analysis, and vii) Storage and data sharing as metadata (Thomas et al., 2012). This information can be completed by metatranscriptomics, which establishes the activities of genes. Metagenomics can be effective in providing a comprehensive dataset about the diversity and the behavior of toxic gene expressions in a natural bloom (Segata et al., 2013; Steffen et al., 2015). There is growing recent information on the use of metagenomics and metatranscriptomics for cyanobacteria research.

Despite all benefits and achievements of NGS, bias should be considered during the usage of the results. Firstly, NGS does not provide absolute values (the results are relative). Then, analytical

bias during extraction and amplification can affect the results. Additionally, there is still a shortage in the usage of a standard pipeline for all species (Bag et al., 2016; Costa and Weese, 2019; Gevers et al., 2012; Kuczynski et al., 2011; Teeling and Glockner, 2012; Walters et al., 2016).

2.3.3 Cyanotoxin Measurement

The main measurement techniques for the quantification and assessment of toxicity of major cyanotoxins, MCs, ATX-a, CYN, and STXs, are presented in Table 2-3.

Animal bioassay techniques are considered as a non-specific detection method of cyanotoxins but are used to assess toxicity. LD_{50} on a specific animal (eg. mouse) is determined and the endpoint of this method is the death of the animal used as a model (Massey et al., 2020).

Immunochemical techniques based on the interaction of antibodies and antigens have been developed to measure cyanotoxins in environmental samples. In this category, Enzyme-Linked Immunosorbent Assays (ELISA) is an easy, rapid, and low-cost detection technique that is industrially applied to measure MCs, NODs, ATX-a, STX, CYN, and BMAA in water and sludge samples (Almuhtaram et al., 2018; Picardo et al., 2019; Sanseverino et al., 2017). ELISA has been certified by USEPA for MC/NOD measurement in drinking and ambient waters (United States Environmental Protection Agency (USEPA), 2016). Detection limits (DLs) depend on the extraction techniques and may vary for each cyanotoxin. For instance, DLs reported vary: for MCs: 0.05 μg/L, MC-LR: 0.1 ng/L, and NODs: 0.1 μg/L (Dixit et al., 2017; Sanseverino et al., 2017; Sedda et al., 2016). However, in some cases interferences lead to a false positive results is probable (Metcalf et al., 2000). CYN (DLs: 0.05- 2 μg/L) (Aranda-Rodriguez et al., 2015; Gurbuz et al., 2012) and STX (DLs <0.02 μg/L) are also detectable by ELISA (de la Cruz et al., 2013; Kleinteich et al., 2013). Recently, several commercial ELISA-based kits have been proposed by various manufacturers, however concerns over their specificity, accuracy, the frequency of false positives, and potential underestimation still exist (Aranda-Rodriguez et al., 2015; Watson et al., 2017).

MCs and NODs as protein phosphate inhibitors can be indirectly measured by the Phosphatase Inhibition Assay (PPIA) technique. This is a rapid and simple test, but it cannot differentiate between MCs and NODs or detect MCs' variants separately. The minimum DL of this technique

can be around 0.01 μg/L (Gaget et al., 2017; Kaloudis et al., 2013; Merel et al., 2013; Rapala et al., 2002; Sanseverino et al., 2017).

Separation techniques using liquid and gas chromatography (LC and GC, respectively) coupled with mass, fluorescence, and ultraviolet spectroscopy, are widely common in measurement of cyanotoxins (Picardo et al., 2019). High Performance Liquid Chromatography (HPLC) coupled with a Photodiode-Array (PDA) has been used to detect MCs and NODs with the DL between 20-50 ng/L. However, this technique cannot identify the structure of cyanotoxins (Agence Française de Sécurité Sanitaire des Aliments (AFSSA) and Agence Française de Sécurité Sanitaire de l'Environnement et du Travail (AFSSET), 2006b). Liquid Chromatography/ Mass Spectrometry (LC/MS) is a sensitive and selective standard method for cyanotoxin detection, identification, and measurement. This technique is appropriate for MCs, NODs, CYN and ATX-a detection. DLs vary between 10 and 100 ng/L (Agence Française de Sécurité Sanitaire des Aliments (AFSSA) and Agence Française de Sécurité Sanitaire de l'Environnement et du Travail (AFSSET), 2006b; Sanseverino et al., 2017). Triple Quadrupole Mass Spectrometry (LC-MS/ MS) has higher selectivity and sensitivity to identify and measure cyanotoxins. In addition, unknown structures can be detected by mass spectral data. DLs reported for this technique vary: for MCs 2 ng/L, ATX-a 13 ng/L and CYN 30 ng/L (Agence Française de Sécurité Sanitaire des Aliments (AFSSA) and Agence Française de Sécurité Sanitaire de l'Environnement et du Travail (AFSSET), 2006b; Bogialli et al., 2006; Kaushik and Balasubramanian, 2013; Messineo et al., 2009; Roy-Lachapelle et al., 2014; Sanseverino et al., 2017; Zhang et al., 2004). On-line solid phase extraction coupled with liquid chromatography tandem mass spectrometer (SPE-LC-MS/MS) is one of the recently developed techniques to simultaneously detect MC (and its analogues), ATX-a, NODs and CYN with very low detection limits of (~ 0.5- 100 ng/L) (Fayad et al., 2015; Munoz et al., 2017; Zervou et al., 2017).

Table 2-4 Cyanotoxin measurement methods adapted from (Loftin et al., 2010; United States Environmental Protection Agency (USEPA), 2019)

Method	ATX-a	CYN	MCs	NODs	STXs
Biological methods					
Animal bioassay tests	Yes	Yes	Yes	Yes	Yes
Biochemical methods					
ELISA	Yes	Yes	Yes	Yes	Yes
Protein Phosphatase Inhibition Assays (PPIA)	No	No	Yes	No	No
Neurochemical assays (eg. acetylcholinesterase-based)	Yes	No	No	No	Yes
Chromatographic Methods (gas)					
Gas Chromatography with Flame Ionization Detection (GC/FID)	Yes	No	No	No	No
Mass Spectrometry (GC/MS)	Yes	No	No	No	No
Liquid Chromatography/Ultraviolet-Visible Detection (HPLC or LC/UV)	Yes	Yes	Yes	Yes	Yes
Chromatographic methods (liquid)					
Liquid Chromatography / Fluorescence (LC/FL)	Yes	No	No	No	Yes
Liquid Chromatography/Ultraviolet-Visible Detection (LC/UV or LC/PDA)	Yes	Yes	Yes	-	Yes
Chromatography combined with mass spectrometry (liquid)					
Liquid Chromatography Ion Trap Mass Spectrometry (LC/IT MS)	Yes	Yes	Yes	Yes	Yes
Liquid Chromatography Time-of-Flight Mass Spectrometry (LC/TOF MS)	Yes	Yes	Yes	Yes	Yes
Liquid Chromatography Single Quadrupole Mass Spectrometry (LC/MS)	Yes	Yes	Yes	Yes	Yes
Liquid Chromatography Triple Quadrupole Mass Spectrometry (LC/MS/MS)	Yes	Yes	Yes	Yes	Yes
SPE-LC-MC/MC	Yes	Yes	Yes	Yes	-

Hydrophilic interaction liquid chromatographic (HILIC)–MS/MS is an appropriate method for polar toxins such as STXs and BMAA in which the minimum detectable value can be 0.4 pg (Beach et al., 2015). High-resolution mass spectrometry (HRMS) can also detect BMAA, ATX-a, and CYN with below DL of 20 ng/L (Roy-Lachapelle et al., 2015a; Roy-Lachapelle et al., 2015b). It must be mentioned that although new generations of chromatography-based techniques of cyanotoxin measurement are accurate, precise, and enable to identify structures of cyanotoxins, they are sophisticated and needs experienced technicians. Also, they are quite expensive. It is expected that estimation of the techniques varies; reflecting the required sample preparations and

analytical limitations. Therefore, the selection of a measurement technique must be done based on the condition, type of cyanotoxins in the sample, cost, and sensitivity of the tests (Gaget et al., 2017).

2.3.4 Prediction of Cyanotoxins

Genes are responsible to produce cyanotoxins and other metabolites such as geosmin and MIB. PCR can be applied for the detection of the responsible genes. The idea behind the identification and measurement of these genes is to estimate the potentiality of metabolite production. Although toxic and non-toxic cyanobacterial cells are morphologically similar (Lawton et al., 1999), toxicogenic cells contain cyanotoxin synthesis genes. For instance, toxic *mcyA–mcyJ* genes are detected in toxic *Microcystis* strains (Davis et al., 2009). Similarly, *nda*, *ana*, *pks,* and *sxt* are responsible to produce NODs, ATX-a, CYN, and STX, respectively (Ballot et al., 2018; Chiu et al., 2017; Kellmann et al., 2008; Müller et al., 2017). Identification and measurement of toxic genes can be an appropriate approach for monitoring of toxic cyanobacterial blooms in water resources (Fortin et al., 2010; Müller et al., 2017).

Several studies reported a correlation between MC-producer toxic genes (*mcyA- mcyJ*) and MC concentrations in water resources (Chiu et al., 2017; Davis et al., 2009; Fortin et al., 2010; Ha et al., 2009; Joung et al., 2011; Martins et al., 2011; Otten et al., 2015; Singh et al., 2015; Te and Gin, 2011). For instance, a good correlation (r= 0.87- 0.93) was reported between *mcyD* gene copy numbers and MC concentrations in Missisquoi Bay (Canada) using qPCR during two cyanobacterial blooms (Fortin et al., 2010). Similarly, another study on water reservoirs in Taiwan showed a correlation (r= 0.63- 0.83) between MCs and *mcyB* as well as CYN and *pks* (Chiu et al., 2017). In contrast, several investigations reported no correlation between *mcy* gene copies and MC concentrations (Guedes et al., 2014; Oh et al., 2013; Pobel et al., 2012; Rinta-Kanto et al., 2009; Sabart et al., 2010; Ye et al., 2009). A positive correlation between *Microcystis* and *mcyD* gene copy numbers measured by 16S and qPCR techniques has been reported in several investigations (Davis et al., 2009; Fortin et al., 2015; Ha et al., 2009; Joung et al., 2011; Rinta-Kanto et al., 2009). However, no (or weak) correlation was also reported in some studies (Baxa et al., 2010; Crawford et al., 2017; Hotto et al., 2008; Vaitomaa et al., 2003). This shows the limitation of molecular techniques in the risk assessment of toxic blooms. This limitation can be related to the complexity

of cyanotoxin expression/ not expression of toxic genes (Pacheco et al., 2016) as well as gene extraction challenges (Kim et al., 2013).

Finally, most of the molecular-based investigations have been conducted on *Microcystis*, MCs, and *mcy* genes and, there is not enough information about other cyanobacteria species and genes.

2.4 Regulations, Guidelines and Management

The previous WHO guideline (1999) suggested three levels for the management of cyanobacterial cells (Chorus and Bartram, 1999a): The vigilance level is set when detecting one cyanobacterial colony or five filaments in 1 mL of the sample. This level can also be defined by taste and odor (T&O) or muddy smell related to geosmin presence. In this step, a visual inspection of water and weekly cell counts are recommended, Alert Level 1: When cyanobacterial cell counts exceed either 2,000 cells/mL, 0.2 mm^3/L biovolumes, or 1 μg chla /L. In this condition, changing the intake point of water and adding algaecides are recommended. The toxicity of the bloom is also verified in this step, and Alert Level 2: When cell counts rise over 100,000 cells/mL (or 10 mm^3/L biovolumes) or 50 μg chla/L. This is considered as a high risk for human health.

The recent revision of WHO (2021) was prepared based on the combination of cyanobacterial biovolume and cyanotoxin concentrations (MCs, ATXs, CYNs and STXs). When cyanobacterial biovolume is ≥ 0.3 mm^3/L, or chla is ≥ 1 μg/L, the concentration of cyanotoxins should be measured. If CYNs > 0.7 μg/L, or MCs > 1 μg/L, or ATXs > 3 μg/L, or STXs > 0.3 μg/L, then Alert Level 1 will be applied. A source with mentioned biovolumes and chla concentration (≥ 0.3 mm^3/L, chla ≥ 1 μg/L) but with lower cyanotoxin concentrations remains at the Vigilance Level and needs weekly monitoring. Combination of biovolume higher than 4 mm^3/L (or chla ≥ 12 μg/L) with either CYNs > 3 μg/L, or MCs > 12 μg/L, or ATXs > 12 μg/L, or STXs > 3 μg/L will lead to Alert Level 3 (Chorus and Welker, 2021).

In Quebec, intake water and water body in which test results show cell counts over 10,000 and 20,000 cells/mL, respectively, are considered sources with historical cyanobacteria presence. In that case, water quality before treatment must be monitored and sufficient treatment barriers must be applied accordingly (Ellis, 2009; Zamyadi et al., 2013a).

Regulations and guidance levels for cyanotoxins and cyanobacterial levels in source water are summarized in Table 2-5.

So far, there are no recommendations or guidelines for cyanobacteria or cyanotoxins in the sludge or recycled supernatant to the head of the DWTP.

Table 2-5 Cyanotoxin standards and guidelines

Cyanotoxins	Recommended concentration	References
MCs	Ohio: 0.3 µg/L (age under 6), 1.6 µg/L (children 6 and older and adults), 20 µg/L (do not use)- MAC	(Ohio Environmental Protection Agency, 2016)
	Canada: 1.5 µg/L (Drinking water)- MAC[1],	(Health Canada and Federal-Provincial-Territorial Commitee on Drinking Water, 2016)
	20 µg/L, 100,000 cells/mL (Recreational water)- Recommended guideline	(Health Canada, 2012)
	New Zealand: 1.0 µg/L- MAC	(Ministry of Health, 2008)
	Brazil: 1.0 µg/L- MAC	(Codd et al., 2005)
MC-LR	WHO: 1.0 µg/L (lifetime), 12 µg/L (short term[2]), 24 µg/L (recreational)	((WHO), 2020c; World Health Organization (WHO), 1998)
	Minnesota: 0.04 µg/L- Health based value	(Minnesota Department of Health (MDH), 2015)
	Oregon: 1.0 µg/L- Guideline, acute and short term exposures	(Farrer et al., 2015)
	Australia: 1.3 µg/L- Guideline	(Australian Government et al., 2011; Svrcek and Smith, 2004)
	South Africa: 0.8 µg/L- Maximum target water quality range	(Department of Water Affairs and Forestry, 1996)

[1] MAC: Maximum allowable concentration
[2] Short term: Exposure for two weeks

Table 2-5 Cyanotoxin standards and guidelines (continue)

Cyanotoxins	Recommended concentration	References
ATX-a	WHO: 30 µg/L (acute), 60 µg/L (recreational)	((WHO), 2020a)
	Minnesota: 0.1 µg/L- Health based value	(Minnesota Department of Health (MDH))
	Ohio: 20 µg/L- MAC	(Ohio Environmental Protection Agency, 2016)
	Oregon: 3.0 µg/L- Guideline, acute and short term exposures	(Farrer et al., 2015)
	Quebec: 3.7 µg/L MAC	(Barbeau et al., 2008; Ministry of Health, 2008)
	New Zealand: 6 µg/L- MAC	(Ministry of Health, 2008)
ATX-a (s)	New Zealand: 1 µg/L- MAC	(Ministry of Health, 2008)
STX	WHO: 3 µg/L (acute), 30 µg/L (recreational)	((WHO), 2020d)
	Ohio: 0.3 µg/L (age under 6), 1.6 µg/L (children 6 and older and adults), 3 µg/L (do not use)- MAC	(Ohio Environmental Protection Agency, 2016)
	Oregon: 1.0 µg/L (as STX-eq)- Guideline, acute and short term exposures	(Farrer et al., 2015)
	Australia: 3.0 µg/L (as STX-eq)- Alert Level	(Australian Government et al., 2011; Svrcek and Smith, 2004)
	Brazil: 3.0 µg/L- MAC	(Codd et al., 2005)
CYN	WHO: 0.7 µg/L (lifetime), 3 µg/L (short term), 6 µg/L (recreational),	((WHO), 2020b)
	Ohio: 0.7 µg/L (Age under 6), 3.0 µg/L (children 6 and older and adults), 20 µg/L (do not use)- MAC	(Ohio Environmental Protection Agency, 2016)
	Oregon: 1.0 µg/L- Guideline, acute and short term exposures	(Farrer et al., 2015)
	Brazil: 15.0 µg/L- MAC	(Codd et al., 2005)
	New Zealand: 1 µg/L- MAC	(Burch, 2008; Ministry of Health, 2008)

2.5 Cyanobacteria and Cyanotoxins in DWTPs and Sludge

2.5.1 Critical Points in DWTPs

Cyanobacteria will accumulate in solid separation processes that are in place to capture and accumulate particles. This accumulation can occur even in sources with low incoming cyanobacteria levels. A longitudinal study of two low-risk DWTPs (no cyanobacteria history) in Quebec showed high cell accumulation in the sludge, even in low cell incoming concentrations (below 400 cells/mL) in the source (Zamyadi et al., 2013b). The two studied DWTPs were equipped with similar chemically enhanced conventional treatment processes, but only one of the two DWTPs was used pre-ozonation. In the plant with pre-ozonation, low levels of cyanobacteria in the raw water (150 cells/mL) accumulated on the surface of the clarifier in small and rare agglomerations of scum (37,000 cells/mL). Subsequently, cell counts in the sludge surface, middle of sludge, and concentrator of the clarifier were very low (350- 720 cells/mL). Cyanobacteria were quasi absent from the down flow filter feed, filtered water and treated water (below 50 cells/mL). In the 2nd plant, receiving similar low cyanobacteria but without pre-ozonation, cyanobacteria accumulated in scum as 353,000 cells/mL on the surface of clarifier and 1000, 5300, and 1400 Cells/mL in the sludge surface, middle of sludge and concentrator of the clarifier, respectively. Downflow cyanobacteria levels were higher, as cell number on the surface of the filters reached 73,000 Cells/mL and below DL in both filtered water and treated water. A summary of these findings is presented in Figure 2-1, showing the considerable concentration of cyanobacterial cells accumulated in the clarifier and then spilling over to the filter feed even in the absence of a bloom. Pre-oxidation enhances cell removal of the coagulation/sedimentation process (Chu et al., 2017; Lapsongpon et al., 2017; Lin et al., 2018; Naceradska et al., 2017; Petrusevski et al., 1996; Wang et al., 2015b; Xie et al., 2016; Xie et al., 2013), however, it may lead to cell lysis and algal organic matter (AOM) release into water (Chen et al., 2009; Henderson et al., 2010). Similarly, an investigation on 4 DWTP in Great Lakes with low cyanobacterial cell influx (<1000 cells/mL) showed that cyanobacterial cells were accumulated in the sludge 10-100 times more than cell counts in the intake water. MCs were measured at 7.2 µg/L in the sludge showing 12 times increase compared to those of the intake water.

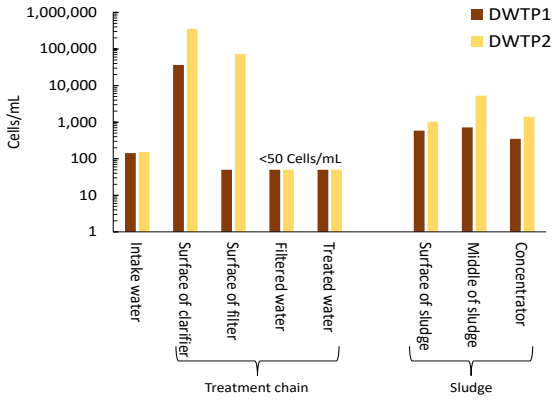

Figure 2-1 Cyanobacterial cells in two low risk DWTPs. DWTP1 has pre-ozonation. Average values from August to October 2011, adapted from (Zamyadi et al., 2013b).

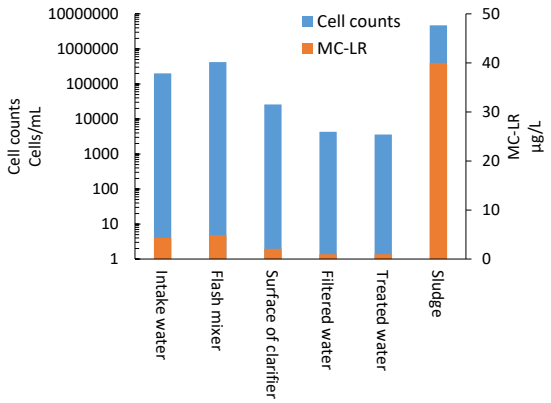

Figure 2-2 Cyanobacterial cells in different points of a high risk DWTP in Missisquoi Bay on August 2008 (average values), adapted from (Zamyadi et al., 2013a; Zamyadi et al., 2012c).

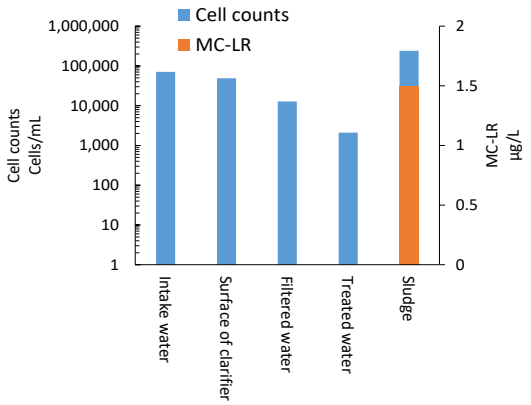

Figure 2-3 Cyanobacterial cells in different points of a high risk DWTP in Missisquoi Bay on July 2009 (average values), adapted from (Zamyadi et al. 2013a, Zamyadi et al. 2012b).

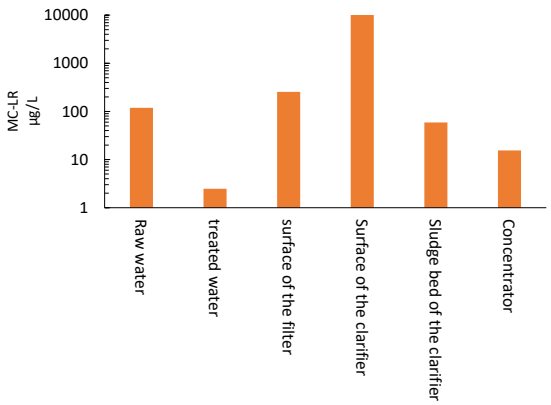

Figure 2-4 MC-LR concentrations in a high risk DWTP during a bloom in Missisquoi Bay on August 2010 (average values), adapted from (Zamyadi et al. 2013a, Zamyadi et al. 2012b).

Cell accumulation over the surface of the clarifier was up to 10 times higher than the intake water influx and both MCs and ATX-a were detected in concentrations up to 1.5 µg/L. While cell counts on the surface of filters and the treated water was below 1000 cells/mL, accumulation of ATX-a

through the DWTPs caused the concentration of these cyanotoxins increased up to 0.8 µg/L on the surface of filters and the filtered water (Almuhtaram et al., 2018).

Similar but with more extreme accumulation pattern trends are reported in the case of a high-risk plant, receiving frequent and elevated levels of cyanobacterial cells (Zamyadi et al., 2013a; Zamyadi et al., 2012c). When cyanobacterial cells in the intake water reached around 200,000 cells/mL, the cell counts in the flash mixer, the surface of the clarifier, and the sludge increased to 420,000, 26,000, and 4,700,000 cells/mL, respectively. Cyanobacterial cells in the filtered water and treated water remained relatively high (4300 and 3600 cells/mL, respectively), however, the cell reduction efficiency in treated water was also high, around 98%. Taxonomic cell counts in the sludge remained more than 23 times higher than the raw water. Similarly, the presence of 4.3 µg/L MC-LR in the raw water caused that the MC-LR concentration in the sludge blanked of the clarifier raised to 40 µg/L. However, cyanotoxin concentrations in the filtered and chlorinated water were reported below DL (Figure 2-2). Another cyanobacterial bloom (below 100,000 cells/mL) in July 2009, caused a marked accumulation in the water over the clarifier and in the sludge blanket (49,000 and 240,000 cells/mL), and cyanobacterial cells remained higher than Alert Level 2 (2,100 cells/mL) in the treated water. In this situation, although the cyanotoxin concentration in the raw water was below DL, a concentration of 1.5 µg/L MC-LR was measured in the sludge (Figure 2-3).

An intense bloom situation was monitored at this DWTP in Missisquoi Bay in 2010. The cyanotoxin concentration reached a peak of 109 µg/L in MC-LR the intake water. This influx of toxic cells caused toxin levels to increase to 10,300 and 59 µg/L MCLR at the surface and in the sludge of the clarifier, and 255 µg/L MC-LR in the water over the filters. As a result, toxin breakthrough in the treated water was documented at a level of 2.47 µg/L MCLR, which is around 2.5 times higher than the WHO guideline (Figure 2-4) (Zamyadi et al., 2013a; Zamyadi et al., 2012c).

Another study in a full-scale DWTP shows that although the dissolved air flotation (DAF) process could remove 89% of cyanobacterial cells, these cells were concentrated at 406 and 2600% in the thickened and centrifuged sludge, respectively (Zamyadi et al., 2016b). Even in DWTPs without clarifiers (eg: direct filtration), agglomerated particles by means of coagulation will be trapped by

a rapid filter, and the backwash water of the filter becomes a reservoir of cyanobacteria (Ho et al., 2012).

In many DWTPs, the supernatant of the storage sludge is recycled to the head of the plant as spent water (Zamyadi et al., 2019). The presence of cyanobacteria and cyanotoxins in this recycled water can be associated with health challenges. A study on a low-risk DWTP in Australia (3400 cells/mL in the intake water) showed a 43% increase in the cell counts after recycling the supernatant sludge. Interestingly, 80% of the transferred cells from the spent water were viable (Zamyadi et al., 2019). Similarly, a recent laboratory investigation on an influent containing 1 x 10^6 cells/mL of *M. aeruginosa*, documented that although conventional treatment could keep the effluent below WHO and US EPA guidelines, recycling of the spent water caused 7 x 10^4 cells/mL, 0.26 µg/L and 0.171 NTU increase in cell counts, MC-LR concentration and turbidity, respectively (Pinkanjananavee et al., 2021).

The impact of coagulation on cell integrity is not still well-understood. One of the first studies on *M. aeruginosa*, and *Planktothrix rubescens* at laboratory and pilot scales hypothesized that shear stress combined with pre-oxidation impact may affect cell lysis and cyanotoxin release (Pietsch et al., 2002). Several studies documented that coagulation at optimum dose does not significantly impact the cell integrity of *M. aeruginosa*, *D. circinale, C. raciborskii, and Oscillatoria sp.* (Li et al., 2018; Ma et al., 2016b; Sun et al., 2013; Sun et al., 2012; Sun et al., 2018). In fact, flocs generated during coagulation and direct filtration can protect cyanobacteria from cell damage (Ho et al., 2012; Pestana et al., 2019). This might be due to: i) Charge neutralization phenomenon after coagulation (Li et al., 2015; Sun et al., 2012), and ii) The presence of coagulation as a stressful factor causes that *M. aeruginosa* cells produce extracellular polymeric substances playing a role as a shield to protect cells against lysis and MC release (Sun et al., 2012).

Filamentous cyanobacteria are susceptible to trichome truncation in the presence of hydraulic stress during conventional treatment and direct filtration. The higher trichomes (>30 cells, eg. *Dolichospermum, Planktothrix* and *Geitlerinema*), the higher susceptibility to trichome truncation and loss of cell integrity. In contrast, the shorter filamentous (<12 cells) such as *Pseudanabaena* and *Planktolyngbya* showed a lower risk of breakage. Accordingly, trichome breakage is more observed in direct filtration than sedimentation due to higher shear stress in filtration than

sedimentation (Clemente et al., 2020; Pestana et al., 2019). This can increase the risk of cell breakthrough in DWTPs. However, it should be noted that most of these investigations were conducted on a laboratory scale and on cultured cyanobacteria. The knowledge about the impact of the coagulation process on natural blooms is limited.

2.5.2 Fate of Cyanobacteria and Cyanotoxins in the Stored Sludge

A combination of coagulation, flocculation, and sedimentation is an appropriate approach to removing cyanobacterial cells from water with the least cell damage. The idea of intact cell removal can be a promising approach to protecting DWTPs from cell lysis and consequent cyanotoxin release (Westrick et al., 2010). However, as discussed in section 2.5.1, this process can cause a huge accumulation of cyanobacteria in the sludge.

Several studies demonstrated that accumulated cyanobacterial cells can survive in the stored sludge for a certain period. The importance of these investigations on stored sludge is summarized in (Table 2-6).

The first study on the sludge of coagulated water containing cultured *M. aeruginosa* (initial cell counts in water/ sludge: 10^6 cells/mL/ 8 x 10^6 cells/mL, coagulant/dose: alum/ 70 mg/L), revealed that while cells were viable in the sludge before storage, they lost 90% of viability after one day. Accordingly, cell counts dropped to half after 2 days. While almost all total MC-LR was cell-bound before storage, dissolved MC-LR composed the majority of total MC-LR after 2 days confirming complete cell lysis and MC-LR release on the 2^{nd} day of the storage. From the 2^{nd} to 4^{th} storage day, total and dissolved MC-LR remained almost constant showing periodic MC-LR release from the lysed cells. After the 4^{th} day, MC-LR progressively decreased probably due to biodegradation. on the 8^{th} and 12^{th} days of storage, cell counts and MC-LR decreased to around zero, respectively (Drikas et al., 2001). Next investigations performed on cyanobacteria-laden sludge prepared by coagulation of water and spiked cultured *M. aeruginosa* confirmed that *M. aeruginosa* cells can stay viable up to 10 days in the stored sludge. In this period, cells can be damaged and cell-bound MCs can be released leading to increasing the dissolved MC concentration (Li et al., 2015; Ma et al., 2016b; Sun et al., 2015; Sun et al., 2013; Sun et al., 2012; Xu et al., 2017; Xu et al., 2016). Another investigation justified that coagulated *D. circinale* and *C. raciborskii* cells (initial cells: 2

x 10^5 cells/mL- culture) released cell-bound STX and CYN into the supernatant led to increasing STX concentration from 0.4 µg/L to 1.4 µg/L and CYN concentration from 1.8 µg/L to 9 µg/L within 7 days. The cells remained viable for up to 10 days (Ho et al. 2012a). Another investigation on *C. raciborskii* spiked in raw water and coagulated by 10 mg/L PAFC demonstrated that *C. raciborskii* cells remained intact for up to 6 days. After 6 days, 67% increase in dissolved CYN was observed confirming cell damage and cell-bound CYN release. The concentration of dissolved CYN decreased after 10 days due to biodegradation (Li et al., 2018). A study on coagulated (5-10 mg/L PAFC) water spiked by cultured *Oscillatoria sp*. reported a 2-30% increase in dissolved CYN within 2-8 days. A 4-40% increase in released cell-bound protein was observed in this period confirming cell damage during sludge storage (Sun et al., 2018).

Recent investigations showed that cyanobacterial cells can not only survive in the stored sludge but also may re-grow (Dreyfus et al., 2016; Pestana et al., 2016; Water Research Foundation (WRF) and Water Research Australia, 2015). Measured cyanotoxin concentrations in the sludge of coagulated water spiked with *M. aeruginosa* and *C. raciborskii* (two separated experiments- alum: 80 mg/L) exceeded the expected concentration (combined cell-bound and dissolved MCs and CYN), suggesting growth and the expression of toxicity due to regrowth of the cyanobacterial cells in the stored sludge. Data from these experiments are illustrated in (Figure 2-5). The experiments were performed in a lab condition and on two different water sources in Australia (Myponga and River Murray). In certain scenarios (*M. aeruginosa*: Myponga 2 and *C. raciborskii*: Myponga 3 and River Murray), dissolved cyanotoxins and organic carbon concentration raised during storage higher than the expected values based on the cell quota. One experiments on the cultured *M. aeruginosa* (3.1 x 10^5 cells/mL) showed that DOC remained 3 times higher than the expected values (based on cell quota) within 16 days of storage. Meanwhile, dissolved MC-LR and MC-LA concentrations were measured more than 2.5 and 1.2 times higher than the expected cyanotoxin release, respectively within 7 days and based on the cell quota. Next experiments on two different water sources and with spiked cultured *C. raciborskii* (3.1 x 10^5 cells/mL) showed that DOC concentrations remained 10-14 times higher than the expected values based on the cell quota and within 23 days. Accordingly, CYN concentrations were measured 3.3-4 times higher than the expected values. The authors hypothesized that either i) Cell re-growth, ii) Underestimation during

cell quota values, or, iii) Cell settlement from supernatant to the sludge caused these unexpected results (Water Research Foundation (WRF) and Water Research Australia 2015).

Long-term survival of spiked natural bloom containing *M. aeruginosa* and *D. circinale* (initial cell number: 8.6 x 10^4 cells/mL) in the lagoon sludge (lab-scale) was also reported by (Pestana et al. 2016). Cells stayed alive for around 35 days in the sludge. Coagulated spiked cultured *M.aeruginosa* cells (5.6 x 10^5 cells/mL) into that lagoon sludge not only remained viable for 10 days but also, toxin concentrations (MC-LR and MC-LA) increased up to 80% (Max. initial concentration: 0.25 µg/L). This experiment was repeated in a sterile condition in which the cells remained viable for around 30 days and released MC-LR and MC-LA 500 times more than initial concentrations justifying the impact of biodegradation on the reduction of extracellular toxin concentration. Surpassing the dissolved MCs from the expected values (based on the cell quota) and increasing cell counts hypothesized either cell growth or further cell settling during sludge storage (Pestana et al., 2016). Another study in the laboratory showed that DOC was detected in the supernatant of coagulated water containing cultured *M. aeruginosa* and *C. raciborskii* 5 times more than predicted values (predicted values: assuming all cell-bound metabolites are released). DOC increase continued for 22 days. Accordingly, MC-LR and MC-LA were detected 2.2 times more than the predicted values. The authors hypothesized either cell growth or metabolite increase per cell during sludge storage (Dreyfus et al., 2016).

Cell damage and cyanotoxin release can also be observed during non-coagulated sludge. Although similar MC release was reported in both coagulated (alum) and non-coagulated sludge containing *M. aeruginosa* within 6 days, extended storage time to 8-12 days caused 4 times higher MC release in the non-coagulated sludge compared to coagulated sludge (Sun et al., 2012). Another study reported dissolved MCs increase in both non-coagulated and coagulated sludge (15 mg/L alum) within 2 days, while 2-3 times higher MC release was observed in the non-coagulated sludge within 6 days as compared to the coagulated sludge (Xu et al., 2016). Similarly, MC release in the non-coagulated sludge was 4-8.5 times less than in coagulated sludge by 70 mg/L $FeCl_3$ (Li et al., 2015). Cultured *M. aeruginosa* spiked in the non-coagulated sludge, and had a 25 and 75% increase in dissolved MCs within 1-6 and 8-12, days of storage, respectively. In contrast, MCs release in the non-coagulated sludge was 1.5-4 times less than sludge coagulated by 5 mg/L PACl (Sun et al., 2013).

While none of the aluminum and ferric-based coagulant do have a significant impact on the loss of cell integrity of *M. aeruginosa* during coagulation (Li et al., 2015; Sun et al., 2013; Sun et al., 2012), they have different behavior during storage of cyanobacteria-laden sludge. One investigation on the stored sludge generated by coagulation of cultured *M. aeruginosa* spiked into water demonstrated that common coagulants such as $AlCl_3$, $FeCl_3$ and PAFC did not affect chla concentrations as an indicator of the cell viability within 2 days. While around a 10% decrease in chla concentration was observed after 4 days in the coagulated sludge as well as the control (non-coagulated). Accordingly, 23-52% of chla concentrations in sludge containing $AlCl_3$ decreased after 6-10 days. A similar decrease in chla concentration was observed in $FeCl_3$ after 6 days while cell viability in the sample coagulated by $FeCl_3$ was slightly higher than $AlCl_3$ after 8-10 days. In contrast, chla concentration in the sludge containing PAFC remained constant after 6 days of storage and had a 20-25% decrease after 8-10 days, respectively. Since higher cell lysis was observed in the sludge containing $AlCl_3$, higher dissolved MCs were measured after 6 days in this sample (60- 70 µg/L) as compared to $FeCl_3$ and PAFC (30-40 µg/L). Loss of cell viability is more critical in the sludge without coagulant where chla concentrations decreased 38-75% after 6-10 days, (Xu et al., 2016). The protective impact of flocs in *M. aeruginosa*-laden sludge was explained in the previous section. Another study evaluated the impact of different coagulants on the stored sludge using Illumina 16S rRNA. The authors documented that PAFC provided richer bacterial communities due to lower toxicity. They also suggested that PAFC and $FeCl_3$ are better coagulants because $AlCl_3$ caused an increase in the release of dissolved MCs and organic matter in the sludge (Pei et al., 2017). Some studies documented the impact of PACl on cultured *M. aeruginosa* lysis and cell-bound MC release within 2 days of sludge storage (Sun et al., 2015; Sun et al., 2013). The positive impact of ferric on cyanobacterial growth have been already documented (Chow et al., 1998). In fact, ferric is a contributor to certain enzymes that enhance the growth of algae (Rueter and Petersen, 1987). However, ferric in higher concentrations (> 24 µmol Fe/L) can inhibit *M. aeruginosa* growth (Wang et al., 2010). Aluminum can inhibit phosphatase enzymes and interrupt phosphorus absorption (Gensemer and Playle, 1999). One study reported that stored sludge containing *M. aeruginosa* coagulated by $FeCl_3$ had the lowest cell breakage as compared to cells coagulated by $AlCl_3$. The authors related this low cell breakage to the higher diameter of $FeCl_3$

flocs and suggested that $FeCl_3$ can be an appropriate coagulant that not only decreases cell viability but also inhibit the cell lysis process (Xu et al., 2016).

The impact of flash mixing of DWTP on cell damage during sludge storage is not well-understood. While there is no data in full-scale plants, there are some lab-scale results using the jar tests. Using SEM, one study documented that stirring (without coagulation) can cause slight cell lysis in *M. aeruginosa* during sludge storage after 4 days. However, the authors confirmed that the impact of coagulation (up to 110%) is significantly higher than stirring (Sun et al., 2015). Xu et al. (2016) reported that stirring does not have a significant impact on cell damage. In contrast, another study on *C. raciborskii* documented that stirring increases CYN release up to 94% as compared to non-stirred samples (without coagulation), however up to 32% lower CYN release was observed as compared to the combination of coagulation (PAFC) and stirring scenario (Li et al., 2018).

While cell lysis was not observed during vacuum pressure until 0.9 bar, a 3 bar hydraulic pressure increased cell lysis and dissolved MCs release up to 23% (Sun et al., 2015). However, even applying 0.9 bar vacuum pressure for dewatering of the stored sludge coagulated by $AlCl_3$ and PACl; increases MC concentration up to 160% in the dewatered sludge within 6 days (Sun et al., 2015).

Table 2-6 Summary of studies on fate of cyanobacteria and cyanotoxins in the stored sludge

Initial characteristics of Cyanobacteria/ Coagulation/sedimentation process	Initial condition of cyanobacteria and cyanotoxins in the sludge	Fate of cyanobacteria after sludge storage	Achievements	Reference
• Cultured *M. aeruginosa*– 1 x 10^6 cells/mL (Jar test, 70 mg/L alum)	• 8 x 10^6 cells/mL, • 2,500 µg MC-LR /L	• 90% loss of integrity within 1 day of storage. • 50 and ~100% cell count decrease within 2 and 8 days, respectively. • Almost all cell-bound MC-LR were released within 2 days showing cell damage. • 80 and ~100% MC-LR decrease within 8 and 10 days, respectively.	• Cyanobacteria is subjected to be lysed and cyanotoxin release in the stored sludge and within 2 days, • Biodegradation can decrease cyanotoxin concentration in the stored sludge.	(Drikas et al., 2001)
• Cultured *D. circinale*, and *C. raciborskii* (1.0 x 10^5 cells/mL), (Jar test, 40 mg/L alum)	Sludge supernatant: • *D. circinale* : (1300 cells/mL) • STX: 0.4 µg/L • Geosmin: 9 µg/L	• 83 and < 99% decrease in *D. circinale* cells after 3 and 5 storage days, respectively. • More than 82% of *D. circinale* remained intact within 7 days. • Geosmin concentration remained stable within 1 day then, decreased by up to 87% within 7 days. • STX had 50- 250% increase within 1-7 days.	• Cyanobacteria cells (*D. circinale*) released metabolites in the sludge supernatant after 3 days and remained viable up to 7 days • Since the majority of *D. circinale* cells remained intact within 7 days, authors hypothesized that Geosmin and STX release were due to metabolite produce and release by cells and not cell lysis.	(Ho et al., 2012)

Table 2-6 Summary of studies on fate of cyanobacteria and cyanotoxins in the stored sludge (continue)

Initial characteristics of Cyanobacteria/ Coagulation/sedimentation process	Initial condition of cyanobacteria and cyanotoxins in the sludge	Fate of cyanobacteria after sludge storage	Achievements	Reference
• Cultured *M. aeruginosa* – 2 x 10^6 cells/mL (Jar test, 15 mg/L $AlCl_3$)	• 18 µg/L dissolved MCs	• 0- 6th storage day: 10% increase in dissolved MCs in both non-coagulated and coagulated sludge. • 8-12th storage day: - Non-coagulated sludge: 65-67% increase in dissolved MCs - Coagulated sludge: 15-16% increase in dissolved MCs.	• No cell damage and MCs release during coagulation. • Cell lysis and MC-LR release were observed after 6 days of sludge storage. • Flocs protects cells and prevent cyanotoxin release during sludge storage.	(Sun et al., 2012)
• Cultured *M. aeruginosa* – 1 x 10^6 cells/mL (Jar test, 4 mg/L PACl-optimum dose)	• 20 µg/L dissolved MCs	• 0- 6th storage date: - 110% increase in dissolved MCs in the coagulated sludge by PACl. - 25% increase in dissolved MCs in the non-coagulated sludge. • 8- 12th storage date: - 115% increase in dissolved MCs in the coagulated sludge as compared to before storage date - 75% increase in dissolved MCs in the non-coagulated sludge as compared to before storage.	• No cell damage and MC release during coagulation with PACl, • Negligible cell damage during stirring. • Significant cell damage and MC release during storage within 6-12 days. • Coagulated sludge by PACl should be managed within 2 days.	(Sun et al., 2013)

Table 2-6 Summary of studies on fate of cyanobacteria and cyanotoxins in the stored sludge (continue)

Initial characteristics of Cyanobacteria/ Coagulation/sedimentation process	Initial condition of cyanobacteria and cyanotoxins in the sludge	Fate of cyanobacteria after sludge storage	Achievements	Reference
• Angaston: Cultured *Microcystis flos aquae*– 5.2 x 10^5 cells/mL (Jar test, 100 mg/L alum) • Bolivar: *M. aeruginosa*– cell counts not measured. (Without coagulant)	Sludge supernatant: • Angaston MC-RR, MC-YR: < 2 µg/L • Bolivar MC-YR: < DL	• Angaston: - MC-RR: 5-8 times increase within 5-10 days, then, gradually decreased to < DL at the 15th day of the storage. - MC-YR: remained low within 12 day of storage, then 20-90 times increase within 12-20 days of storage. • Bolivar MC-YR: remained < DL within 8 days of storage then, increased to 40 and 70 µg/L at 12 and 14th days, respectively then, gradually decreased to < DL at 21st day of storage.	• MCs release may occur from immediate release to 8-13 lag days during sludge storage.	(Ho et al., 2013)
• Cultured *M. aeruginosa*– 1 x 10^6 cells/mL (Jar test,15 mg/L $ALCl_3$-optimum dose)	• -0.9 bar vacuum pressure for dewatering the sludge. • 23 µg/L total MCs	• Dewatering after 0- 4th storage day: < 10% increase in MCs. • Dewatering after 6- 10th storage day: 52- 148% increase in MCs.	• Sludge storage and dewatering caused cell lysis and MC release. • Optimum sludge storage time for sludge coagulated by $AlCl_3$ and PACl is 4 and 2 days, respectively.	(Sun et al., 2015)

Table 2-6 Summary of studies on fate of cyanobacteria and cyanotoxins in the stored sludge (continue)

Initial characteristics of Cyanobacteria/ Coagulation/sedimentation process	Initial condition of cyanobacteria and cyanotoxins in the sludge	Fate of cyanobacteria after sludge storage	Achievements	Reference
• Cultured *M. aeruginosa*– 1 x 10^6 cells/mL (Jar test, 4 mg/L PACl- optimum dose)	• -0.9 bar vacuum pressure for dewatering the sludge • 23 µg/L total MCs	• Dewatering after 0-2nd storage day: < 20% increase in MCs. • Dewatering after 4-12th storage day: 100-160% increase in MCs.	*(See above)*	(Sun et al., 2015)
• Cultured *M. aeruginosa*– 1 x 10^6 cells/mL (Jar test, 0-70 mg/L $FeCl_3$)	• ~1 µg/L dissolved MCs	• Non-coagulated sludge: - 400, 800, 2,700 and 3400% increase in dissolved MCs after 2, 4, 6 and 8 days respectively. - MCs dropped to ~0 after 10 days. • Coagulated sludge: - ~100, 200, 300 and 400% increase in dissolved MCs after 2, 4, 6 and 8 days respectively. - MCs dropped to ~0 after 10 days.	• Cells remained viable up to 10 days. • Cells were damaged during sludge storage hence MC release was due to cell lysis. • Biodegradation caused MCs decrease after 8 days.	(Li et al., 2015)

Table 2-6 Summary of studies on fate of cyanobacteria and cyanotoxins in the stored sludge (continue)

Initial characteristics of Cyanobacteria/ Coagulation/sedimentation process	Initial condition of cyanobacteria and cyanotoxins in the stored sludge	Fate of cyanobacteria after sludge storage	Achievements	Reference
• Myponga reservoir: - Cultured *M. aeruginosa*– 2.3 x 10^5 cells/mL - DOC: 11.5 mg/L - Cell-bound MC-LR: 4.7 µg/L - Dissolved MC-LR: 2.0 µg/L (Jar test 80 mg/L alum)	Sludge supernatant after 1 day of storage: • DOC: 6.5 mg/L, • Cells: 4300 cells/mL • Cell-bound MC-LR: 0.5 µg/L • Dissolved MC-LR: 2.5 µg/L	• A 25-38% increase in dissolved MC-LR within 1 day of sludge storage. • Total MC-LR reached to 6 µg/L within 4-7 days of storage (total expected MC-LR: 10 µg/L)	• Cell lysis and MC-LR release can occur even after 1 day sludge storage. • Cell lysis, MC-LR release and MC degradation occurred simultaneously. • During 1-3 days of storage, the rate of MC-LR release exceeded the rate of MC-LR degradation, while within 4-16 storage days, the rate of degradation exceeded the rate of MC-LR release.	(Water Research Foundation (WRF) and Water Research Australia, 2015)
• Myponga reservoir: - Cultured *M. aeruginosa*– 3.1 x 10^5 cells/mL - DOC: 10.1 mg/L - Cell-Bound MC-LR: 5.0 µg/L - Dissolved MC-LR: 2.9 µg/L (Jar test 80 mg/L alum)	Sludge supernatant after 1 day storage: • DOC: 5.2 mg/L, • Cells: 2760 cells/mL • Cell-bound MC-LR: < DL • Dissolved MC-LR: 4.0 µg/L	• The measured DOC after 16 days was 3 times higher than the expected DOC values based on the cell quota. • Total MC-LR reached to 27 µg/L within 7 days of storage (total expected MC-LR: ~10 µg/L) • Total MC-LA reached to 4.2 µg/L within 7 days of storage (total expected MC-LA: ~5 µg/L)	• Cell reproduction might occur, or underestimation of DOC, MC-LR and -LA per cell might interfere with the results.	(Water Research Foundation (WRF) and Water Research Australia, 2015)

Table 2-6 Summary of studies on fate of cyanobacteria and cyanotoxins in the stored sludge (continue)

Initial characteristics of Cyanobacteria/ Coagulation/sedimentation process	Initial condition of cyanobacteria and cyanotoxins in the stored sludge	Fate of cyanobacteria after sludge storage	Achievements	Reference
• Myponga reservoir: - Cultured *C. raciborskii*– 3.1 x 10^5 cells/mL - DOC: 10 mg/L - Cell-bound CYN: 2.5 µg/L - Dissolved CYN: 0.7 µg/L (Jar test 80 mg/L alum)	Sludge supernatant after 1 day storage: • DOC: 6.0 mg/L • Cells: 7080 cells/mL • Cell-bound CYN: 1.0 µg/L • Dissolved CYN: 0.8 µg/L	• The measured DOC after 23 days was 10 times higher than the expected DOC values based on the cell quota • Total CYN reached to 18.5 µg/L within 7-16 days of storage (total expected CYN: ~5 µg/L).	• A tenfold cell reproduction occurred or underestimation of DOC and CYN cell quota might interfere with the results.	(Water Research Foundation (WRF) and Water Research Australia, 2015)
• River Murary: - Cultured *C. raciborskii*– - 3.1 x 10^5 cells/mL - DOC: 8.63 mg/L - Cell-bound CYN: 2.7 µg/L - Dissolved CYN: 0.3 µg/L (Jar test 80 mg/L alum)	Sludge supernatant after 1 day storage: • DOC: 4.9 mg/L • Cells: 4140 cells/mL • Cell-bound CYN: 0.3 µg/L • Dissolved CYN: 0.9 µg/L	• The measured DOC after 23 days was 14 times higher than the expected DOC values based on the cell quota. • Total CYN reached to ~18.0 µg/L within 15 days of storage (total expected CYN: ~5.4 µg/L).	• The increase of metabolites to higher than the cell quota can be related to cell grow or cell quota underestimation.	(Water Research Foundation (WRF) and Water Research Australia, 2015)

Table 2-6 Summary of studies on fate of cyanobacteria and cyanotoxins in the stored sludge (continue)

Initial characteristics of Cyanobacteria/ Coagulation/sedimentation process	Initial condition of cyanobacteria and cyanotoxins in the stored sludge	Fate of cyanobacteria after sludge storage	Achievements	Reference
• Cultured *M. aeruginosa*– 1×10^6 cells/mL No coagulation	• 20 µg/L dissolved MCs • 4.4 mg/L dissolved polysaccharides • 4 mg/L chla	• 63%, 225%, 413%, -63% and -75% changes in MC concentrations after 2, 4, 6, 8 and 10 storage days, respectively as compared to before storage. • 70%, 255%, 539%, 468% and 312% increase in dissolved polysaccharides after 2, 4, 6, 8 and 10 days storage, respectively as compared to before storage. • %15, %40, %63 and 69% decrease in chla after 4, 6, 8 and 10 storage days, respectively as compared to before storage.	• Coagulated sludge had lower MC release as compared to non-coagulated sludge due to charge neutralization and protective shield protection of *M. aeruginosa* cells. • Order of MC release: non-coagulated sludge > $AlCl_3$ > $FeCl_3$ & PAFC, due to destructive impact of Al and Fe on *M. aeruginosa*, • *M. aeruginosa* cells release MCs due to either natural cyanotoxin release or cell lysis for 6th days. After 6 days, dissolved MCs decreased due to biodegradation of MCs • Although biodegradation may decrease MCs, MC concentration remained pretty high (>20 µg/L) in the coagulated sludge. • Dissolved polysaccharides have the highest changes in non-coagulated and $AlCl_3$ showing the highest cell damage in these two samples as compared to FeCl3 and PAFC. *Continued in the next page.*	(Xu et al., 2016)

Table 2-6 Summary of studies on fate of cyanobacteria and cyanotoxins in the stored sludge (continue)

Initial characteristics of Cyanobacteria/ Coagulation/sedimentation process	Initial condition of cyanobacteria and cyanotoxins in the stored sludge	Fate of cyanobacteria after sludge storage	Achievements	Reference
• Cultured *M. aeruginosa*– 1 x 10^6 cells/mL (Jar test 15 mg/L $AlCl_3$)	• 20 µg/L dissolved MCs • 4.2 mg/L dissolved polysaccharides • 4 mg/L chla	• A 64%, 75%, 200%, 163% and 150% increase in MC concentration after 2, 4, 6, 8 and 10 storage days, respectively as compared to before storage. • A 19%, 79%, 198%, 198% and 183% increase in dissolved polysaccharides after 2, 4, 6, 8 and 10 storage days, respectively as compared to before storage. • An 11%, 19%, 40% and 45% decrease in chla after 4, 6, 8 and 10 storage days, respectively as compared to before storage.	• SEM confirmed cell damage after 6th days of storage in the non-coagulated and $AlCl_3$ sample. Cells in the coagulated sludge by $FeCl_3$ and PAFC remained intact after 8 days. • Order of cell viability: PAFC > $AlCl_3$, $FeCl_3$ > non-coagulated sludge. • Cell lysis order: $AlCl_3$ > $FeCl_3$, PAFC > non-coagulated sludge.	(Xu et al., 2016)

Table 2-6 Summary of studies on fate of cyanobacteria and cyanotoxins in the stored sludge (continue)

Initial characteristics of Cyanobacteria/ Coagulation/sedimentation process	Initial condition of cyanobacteria and cyanotoxins in the stored sludge	Fate of cyanobacteria after sludge storage	Achievements	Reference
• Cultured *M. aeruginosa*– 1 x 10^6 cells/mL (Jar test 50 mg/L $FeCl_3$)	• 20 µg/L dissolved MCs • 1.5 mg/L dissolved polysaccharides • 4 mg/L chla	• A 25%, 63%, -25%, 0 and -25% change in MCs concentration after 2, 4, 6, 8 and 10 storage days, respectively as compared to before storage. • A 25%, 233%, 150%, 140% and 65% change in dissolved polysaccharides after 2, 4, 6, 8 and 10 storage days, respectively as compared to before storage. • An 11%, 17%, 38% and 43% decrease in chla after 4, 6, 8 and 10 storage days, respectively as compared to before storage.	*(See above)*	(Xu et al., 2016)
• Cultured *M. aeruginosa*– 1 x 10^6 cells/mL (Jar test, 15 mg/L PAFC)	• 20 µg/L dissolved MCs • 1 mg/L dissolved polysaccharides • 4 mg/L chla	• A 38%, 50%, 52% and 50% changes in MCs concentration after 2, 4, 6, 8 and 10 days storage as compared to before storage. • A 10%, 212%, 338%, 300% and 330% change in dissolved polysaccharides after 2, 4, 6, 8 and 10 days storage, respectively compared to before storage. • A 8%, 8&, 21% and 27% decrease in chla after 4, 6, 8 and 10 days storage as compared to before storage.	*(See above)*	(Xu et al., 2016)

Table 2-6 Summary of studies on fate of cyanobacteria and cyanotoxins in the stored sludge (continue)

Initial characteristics of Cyanobacteria/ Coagulation/sedimentation process	Initial condition of cyanobacteria and cyanotoxins in the stored sludge	Fate of cyanobacteria after sludge storage	Achievements	Reference
• Cultured *M. aeruginosa*– 2 x 10^6 cells/mL (Jar test, 2.6 mg/L chitosan-7.5 mg/L $AlCl_3$ (CTSAC))	• Coagulated sample: 9 µg/L dissolved MCs • Non- coagulation: 18 µg/L dissolved MCs (The difference is due to adsorption on CTSAC)	• 0- 4th days of storage: - Non-coagulated sludge: 56-95% increase in dissolved MCs. - Coagulated sludge: 100-200% increase in dissolved MCs. • 6-10^{th} days of storage: - Non-coagulated sludge: 37-94% decrease in dissolved MCs. - Coagulated sludge: 10-32% decrease in dissolved MCs.	• Adsorption of MCs on the flocs were observed for 6 days. • Less MCs release was observed in coagulated sludge as compared to non-coagulated sludge due to the protective impact of flocs. • EOM remained low during 4 days of storage due to adsorption on CTSAC.	(Ma et al., 2016b)
• Cultured *M. aeruginosa, D. circinale, C. raciborskii*– 3.0 x 10^5 cells/mL (Jar test, 80 mg/L Alum)	Sludge supernatant after 1 day storage: • DOC: 5.2- 6.5 mg/L • Cyanobacterial cells: 2162-7080 cells/mL • Cell-bound MC-LR: < 0.5 µg/L • Dissolved MC-LR: 2.5-4.0 µg/L • Cell-bound CYN: 1.0 µg/L • Dissolved CYN: 0.8 µg/L	• DOC values increased 1.5-5.0 times within 6-22 days of storage. • MC-LR release increased up to 1.6-2.2 times within 6-9 days of storage. • MC-LA release increased up to 1.2 times within 2-10 days of storage. • CYN release increased up to 1.6-2.5 times within 8-18 days of storage.	• In some scenarios, metabolite concentrations surpassed the expected value based on the cell quota. This might be due to either cell growth or increase in metabolite production during sludge storage.	(Dreyfus et al., 2016)

Table 2-6 Summary of studies on fate of cyanobacteria and cyanotoxins in the stored sludge (continue)

Initial characteristics of Cyanobacteria/ Coagulation/sedimentation process	Initial condition of cyanobacteria and cyanotoxins in the stored sludge	Fate of cyanobacteria after sludge storage	Achievements	Reference
• Cultured *M. aeruginosa and D. circinale*– 8.6 x 10^4 cells/mL. (Jar test, 80 mg/L Alum)	• Non-coagulated sludge: 5.0 x 10^6 cells/mL • Coagulated sludge: 5.4 x 10^5 cells/mL	• Without coagulation: Cyanobacterial cells were counted up to ~ 1 x 10^6 cells/mL within 35 days. • With coagulation: Cyanobacteria was detected up to 2.5x106 cells/mL within 11 days. • Metabolites remained 5 times higher than the initial condition during sludge storage.	• Cell survival in the stored sludge was observed in both non-coagulated and coagulated scenario up to 35 days • Either cell re-growth or additional settling during storage led to cell increase in the sludge to the cunts higher than the initial value.	(Pestana et al., 2016)
• Cultured *M. aeruginosa*– 2 x 10^5 cells/mL. (Jar test, 15 mg/L $AlCl_3$, 50 mg/L $FeCl_3$, 15 mg/L PAFC)	• 1 µg/L dissolved MCs	MCs: • 0- 4th storage days: < 10% increase. • 4- 6th storage days: $AlCl_3$: 600%, $FeCl_3$ and PAFC: 400%. • 6- 10th storage day: $AlCl_3$: 300% decrease, $FeCl_3$ and PAFC: 200% decrease as compared to 4- 6th storage.	• Sludge should be treated or disposed within 4 days of storage. • $FeCl_3$ and PAFC are recommended for coagulation due to lower cell damage and MC release.	(Xu et al., 2017)

Table 2-6 Summary of studies on fate of cyanobacteria and cyanotoxins in the stored sludge (continue)

Initial characteristics of Cyanobacteria/ Coagulation/sedimentation process	Initial condition of cyanobacteria and cyanotoxins in the stored sludge	Fate of cyanobacteria after sludge storage	Achievements	Reference
• Cultured *Oscillatoria sp.* – 1.0 x 10^4 cells/mL (No coagulation)	• 1.0 mg/L chla • 2.3 µg/L cell-bound protein • 8.6 µg/L dissolved CYN • Stored in a 12/12 (light/ dark) cycle.	• A 110%, 200%, 410% and 850% increase in chla after 2, 4, 6 and 8 storage days, respectively as compared to before storage. • A -8%, -10%, 52% and 74% changes in cell-bound protein after 2, 4, 6, 8 storage days, respectively, as compared to before storage. • A 22%, 35%, 49% and 47% increase in dissolved CYN after 2, 4, 6, 8 storage days, respectively as compared to before storage. • A 80%, 120%, 200% and 300% increase in chla after 2, 4, 6 and 8 days, respectively, as compared to before storage. • A 4%, 20%, 32% and 40% increase in cell-bound protein after 2, 4, 6, 8 days, respectively, respectively, as compared to before storage. • A 2%, 8%, 12% and 30% increase in dissolved CYN after 2, 4, 6, 8 days, respectively, as compared to before storage.	• Flocs protect cells and limit cell growth in the coagulated stored sludge. • Increase in chla and cell-bound protein concentrations after 6 days showed either an increase in cell counts, cell viability or increase in metabolite production during storage. • Cell viability gradually decreased after 2 days, but cells stayed viable up to 8 days. • According to SOD and MDA data, stress had the highest impact on the cells within 4 days, after this period. Cells can adapt themselves with sludge environment. • Dissolved CYN concentration increased in all samples after 4 days. The order of increase was non-coagulated > overdose of coagulation > optimum dose of coagulation, • An optimum dose of PAFC may control CYN level by adsorption.	(Sun et al., 2018)
• Cultured *Oscillatoria sp.* – 1.0 x 10^4 cells/mL (Jar test, PAFC 5 mg/L- optimum dose)	• 1.0 mg/L chla • 2.5 µg/L cell-bound protein • 10 µg/L dissolved CYN			

Table 2-6 Summary of studies on fate of cyanobacteria and cyanotoxins in the stored sludge (continue)

Initial characteristics of Cyanobacteria/ Coagulation/sedimentation process	Initial condition of cyanobacteria and cyanotoxins in the stored sludge	Fate of cyanobacteria after sludge storage	Achievements	Reference
• Cultured *Oscillatoria sp.* – 1 x 10^4 cells/mL (Jar test, PAFC 10 mg/L-overdose)	• 1.0 mg/L chla • 2.2 µg/L cell-bound protein • 11.4 µg/L dissolved CYN	• An 80%, 130%, 350% and 600% increase in chla after 2, 4, 6 and 8 storage days, respectively as compared to before storage. • A 10%, 55%, 86% and 100% increase in the cell-bound protein after 2, 4, 6, 8 days, respectively as compared to before storage. • A 14%, 0, 12% and 25% change in dissolved CYN after 2, 4, 6, 8 storage days, respectively as compared to before storage.	*(See above)*	(Sun et al., 2018)
• Cultured *C. raciborskii* – 1 x 10^6 cells/mL at the late exponential phase (No coagulation, no stirring)	• 0.9 µg/L dissolved CYN • 2 mg/L cell-bound protein	• A 67% increase in dissolved CYN after 6 days • A 10^ and 25% decrease in cell-bound protein after 8 and 10 days, respectively as compared to before storage.	• Dissolved CYN can be adsorbed by sediments and flocs in the stored sludge. • C. raciborskii started to be damaged or lysed after 6 days of storage. • Biodegradation occurred after 10 days can reduce CYN concentration	(Li et al., 2018)

Table 2-6 Summary of studies on fate of cyanobacteria and cyanotoxins in the stored sludge (continue)

Initial characteristics of Cyanobacteria/ Coagulation/sedimentation process	Initial condition of cyanobacteria and cyanotoxins in the stored sludge	Fate of cyanobacteria after sludge storage	Achievements	Reference
• Cultured *C. raciborskii*– 1 x 10^6 cells/mL at the late exponential phase (No coagulation, stirring)	• 0.8 µg/L dissolved CYN • 2 mg/L cell-bound protein	• A 50%, 100%, 188% and 213% increase in dissolved CYN after 4, 6, 8 and 10 days, respectively as compared to before storage. • A 10%, 30%, 50% and 60% decrease in cell-bound protein after 4, 6, 8 and 10 days, respectively as compared to before storage.	• EOM level reached the highest level at the 6^{th} day of storage showing cell damage. • Stirring and coagulation by PAFC have negative impact on cell lysis of *C. raciborskii* and CYN release during sludge storage.	(Li et al., 2018)
• Cultured *C. raciborskii* – 1 x 10^6 cells/mL at the late exponential phase (Jar test, 10 mg/L PAFC+ stirring)	• 1.1 µg/L dissolved CYN • 2 mg/L cell-bound protein	• A 110%, 200% and 160% increase in dissolved CYN after 6, 8 and 10 days, respectively as compared to before storage. • A 15%, 34%, 56% and 62% decrease in cell-bound protein after 4, 6, 8 and 10 days, respectively as compared to before storage.		

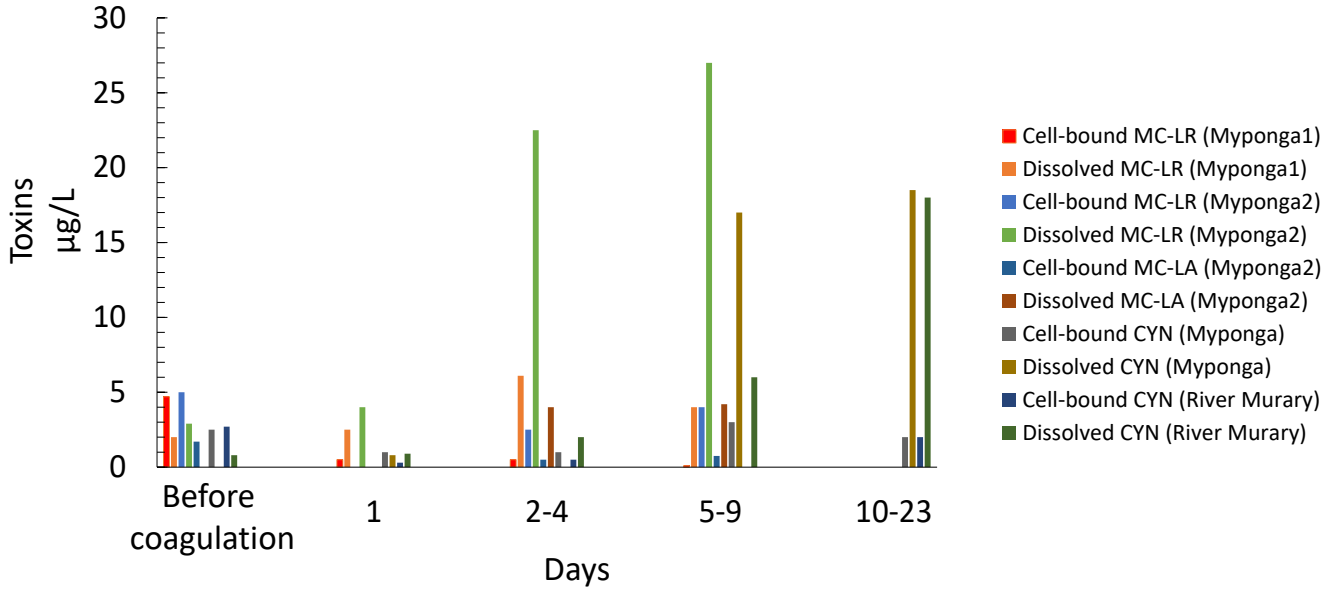

Figure 2-5 Fate of cyanotoxins during sludge storage (adapted from (Water Research Foundation (WRF) and Water Research Australia, 2015)

2.5.3 NGS Studies on Sources, DWTPs and Sludge

Several studies focused on the fate of microbial and bacterial compositions in DWTPs using NGS techniques (Chao et al., 2013; Hou et al., 2018; Lautenschlager et al., 2014; Li et al., 2017; Lin et al., 2014; Ma et al., 2020; Pinto et al., 2012; Poitelon et al., 2010; Zhang et al., 2017b). Although the bacterial community of the intake water is an important parameter affecting the bacterial composition of the treated water, microbial and bacterial communities are also influenced by water treatment processes (Chao et al., 2013; Li et al., 2017; Lin et al., 2014; Pinto et al., 2012). Additionally, nutrient parameters such as DOC and NH_4 affect bacterial communities through water treatment processes (Hou et al., 2018).

The impact of the treatment chain on bacterial communities is different. Bacterial communities are not only different in water and biofilms but also may vary from one source to another source and from one biofilm to another biofilm (Lautenschlager et al., 2014; Lautenschlager et al., 2013; Li et al., 2017). One study documented that in one DWTP, *Bradyrhizobiaceae* was predominant in GAC filters, while *Nitrospira* was predominant in rapid and slow sand filters. It was also reported that aromatic degradation functions such as *Rhizobiales* were largely detected in the GAC filters attributed to the removal of aromatic-based NOMs (Oh et al., 2018).

The impact of conventional treatment processes on the bacterial community is not still well-understood. One investigation demonstrated that conventional treatment does not change bacterial composition, although decreased cell counts (Ma et al., 2020). In contrast, another study documented that coagulation can shift the community from *Sawyeria spp.* to *Plectus spp.* (Zhang et al., 2017b). Similarly, two studies showed that biofilm in the sand filter affects bacterial communities in the filtration effluent (Lin et al., 2014; Pinto et al., 2012). The effluent of biofilters can be impacted by the bacterial compositions of the filters' biofilms (Lautenschlager et al., 2014). In fact, bacterial communities in the biofilm are resistant enough that communities after chlorination are still shaped by those in biofilters (Hou et al., 2018; Pinto et al., 2012). Similarly, a combination of ozone with BAC can influence the bacterial community by decreasing the relative abundance of heavy bacteria such as *Sphingomonas* (Ma et al., 2020). The chlorination process using chlorine, ClO_2, and NaOCl significantly affect bacterial communities (Hou et al., 2018; Li et al., 2017; Lin et al., 2014; Ma et al., 2020; Poitelon et al., 2010). It is reported that chlorination shifted the bacterial community towards the dominancy of *Proteobacteria* (Poitelon et al., 2010). Accordingly, one study documented that bacterial communities in the chlorinated water were quite stable and composed of *Pseudomonas*, *Citrobacter*, and *Acinetobacter* (genus of Proteobacteria) (Hou et al., 2018).

NGS is an efficient tool to study the occurrence of cyanobacterial blooms in water resources (Berry et al., 2017; Casero et al., 2019; Tromas et al., 2017). Eight-year monitoring of Lake Champlain (Canada) from 2006 to 2013 using 16S revealed that cyanobacterial blooms changed bacterial communities without decreasing diversity. The authors reported that microbial communities altered periodically within a year; hence cyanobacterial blooms can be predicted based on community changes (Tromas et al., 2017). Similarly, monitoring of Lake Erie (Canada) in 2014 demonstrated that cyanobacterial blooms affect the diversity of bacterial communities (other than Cyanobacteria). The authors confirmed the hypothesis that cyanobacterial communities dynamically changed even during blooms, however, *Microcystis* and *Synechococcus* remained predominant (Berry et al., 2017). 16S rRNA combined with cyanotoxin-biosynthesis genes has been applied to monitor a cyanobacterial bloom in a Rosarito reservoir (Spain) in 2013. The authors identified *Planktothrix agardhii,* and *Microcystis spp.* as producers of MCs, *Cuspidothrix issatschenkoi,* and *Phormidium/Tychonema spp.* as the producers of ATX and *Aphanizomenon*

gracile as the producer of STX. The results also confirmed the simultaneous presence of *mcyE*, *anaF* and *sxtI*, the toxic genes of MCs, ATX, and STX, respectively (Casero et al., 2019).

One study on two DWTPs in Australia showed that the relative abundance of Synechococcales (order of potentially cyanotoxin producer genera such as *Aphanocapsa*) slightly increased (< 11%) after mixing of recycled sludge supernatant and the intake water followed by PAC injection as compared to the DAF effluent. In another DWTP in Australia equipped with pre-ozonation followed by conventional treatment, the relative abundance of Chroococcales (order of *Microcystis*) increased by up to 30% in the effluent of the clarifier as compared to the head of the plant (i.e. mix of recycled sludge supernatant and raw water slightly). In contrast, a clarifier could reduce the relative abundance of Nostocales (order of *Dolichospermum* and *Cylindrospermopsis*) and Synechococcales to below DL (Zamyadi et al., 2019).

To date, only two investigations conducted on bacterial and cyanobacterial communities of sludge generated by coagulation (Pei et al., 2017; Xu et al., 2018). Xu et al. (2018) investigated the sludge of six DWTPs in China equipped with conventional treatment followed by chlorination. While Proteobacteria, Cyanobacteria, Bacteroidetes, Firmicutes, Verrucomicrobia, Planctomycetes, and Actinobacteria were predominant phyla in sludge samples, the relative abundance of these phyla was different. This difference might be due to differences of i) Bacterial communities, and ii) Variation of TP, DO, Al, Fe, and chloride parameters in the intake water of the studied DWTPs. However, bacterial communities in the intake water were not analyzed. Various potentially toxic cyanobacteria (*Planktothrix, Microcystis,* and *Cylindrospermopsis*) and pathogens (*Escherichia coli*, *Bacteroides ovatus, Prevotella copri,* and *Rickettsia*) were also detected in the sludge samples. The authors reported that the cyanobacterial genera (*Microcystis, Planktothrix,* and *Cylindrospermopsis*) were influenced by nutrient parameters (TOC, TN, and TP) as well as temperature (Xu et al., 2018).

Bacterial communities in the sludge generated by different coagulants such as $AlCl_3$, $FeCl_3$, and PAFC in the laboratory scale have been studied using Illumina 16S rRNA within 10 days of sludge storage (Pei et al., 2017). Cyanobacteria, Proteobacteria, Firmicutes, Bacteroidetes, Verrucomicrobia, and Planctomycetes were predominant in the sludge samples. While richness (Chao index) was almost similar in all the sludge samples before storage (<16%), richness in the

sludge generated by PAFC remained higher than the richness in the coagulated sludge by $AlCl_3$, $FeCl_3$. The authors observed that the relative abundance of the predominant genera as *Microcystis*, *Rhodobacter*, *Phenylobacterium*, and *Hydrogenophaga* decreased after 4 days. MC and DOC concentrations increased up to 500% (day 6) and 60% (day10), respectively from 4-10th days of storage due to cell damage, metabolite release, and degradation. They suggested that sludge should be disposed of 4 days to avoid cell-bound metabolite release. (Pei et al., 2017).

2.6 Conventional Treatment

Around 50- 99% of cyanotoxins are intracellular, bound within cyanobacteria cells. Therefore, the removal of intact cells from sources reduces the risk of cyanotoxin release/accumulation through DWTPs (Chorus and Bartram, 1999a; Drikas et al., 2001; Newcombe and Nicholson, 2004; Zamyadi et al., 2013a; Zamyadi et al., 2012c). Both anionic and cationic coagulants have been recommended in different studies for cyanobacteria removal (Agence Française de Sécurité Sanitaire des Aliments (AFSSA) and Agence Française de Sécurité Sanitaire de l'Environnement et du Travail (AFSSET), 2006b; Zamyadi et al., 2012c). Similarly, both trivalent iron (Fe^{+3}) and aluminum (Al^{+3}) based coagulants have been investigated for their ability to ensure the removal of cells by a clarification in previous studies (Drikas et al., 2001; Ho et al., 2012; Liu et al., 2014b; Ma et al., 2016a; Teixeira and Rosa, 2007; Widrig et al., 1996; Xie et al., 2013; Zamyadi et al., 2012c). However, during natural blooms, the use of a coagulant aid is recommended since the flocs are light and more agglomeration is required (Bernhardt and Clasen, 1994). According to the previous studies, cyanobacterial cell counts do not influence significantly the coagulant demand. In this case, the optimum coagulant dose can be selected based on the NOM removal (Ho et al., 2012; Water Research Foundation (WRF) and Water Research Australia, 2015). In contrast, cell morphology (size and shape) may influence coagulant dosage. In fact, the smaller cells, the more coagulant dosage. Finally, elevated turbidity may contribute to cyanobacterial cell removal since, particles may play a role as coagulant aid (Water Research Foundation (WRF) and Water Research Australia, 2015).

More than 95% of cultured *M. aeruginosa* (initial counts up to 2 x 10^6 cells/mL) can be removed during coagulation by common coagulants such as alum (15-80 mg/L), $FeCl_3$ (~50) and PACl (~4 mg/L) (Drikas et al., 2001; Li et al., 2015; Sun et al., 2013; Sun et al., 2012; Water Research

Foundation (WRF) and Water Research Australia, 2015). Additionally, 60- 80 mg/L of alum is sufficient to remove more than 90% of cultured *D. circinale*, *C. raciborskii* and *Oscillatoria sp.* With an initial cell concentration of less than 3 x 10^5 cells/mL (Ho et al., 2012; Water Research Foundation (WRF) and Water Research Australia, 2015).

Several studies reported that common coagulants such as alum, $FeCl_3$ and PACl do not cause cell damage and cyanotoxin release during the coagulation process in cultured *Microcystis*, *Dolichospermum,* and *Cylindrospermopsis* and in the laboratory scale (Chow et al., 1998; Drikas et al., 2001; Li et al., 2015; Peterson et al., 1995; Sun et al., 2013; Sun et al., 2012; Water Research Foundation (WRF) and Water Research Australia, 2015). In contrast, one study in the laboratory and pilot scales documented that both alum (as 4 mg/L Al) and $FeCl_3$ (as 6 mg/L Fe) could cause cell damage in *M. aeruginosa* and *Planktothrix rubescence* in the stationary phase resulting in MC-LR release up to 13% (Pietsch et al., 2002). Finally, the impact of coagulants during sludge storage is an important topic (*See 2.5.2*). However, the probability of cyanotoxin release due to some unknown phenomena during coagulation should be considered (Ho et al., 2012).

The removal efficiency of cyanobacterial cells may vary depending on the species and the coagulation conditions (Zamyadi et al., 2013a; Zamyadi et al., 2019). In one full-scale study, *Microcystis*, *Dolichospermum*, and *Pseudanabaena* were well removed by clarification using alum (70-224 mg/L), while *Aphanizomenon* was not. The same preferential removal order was also observed in the down flow filtration step (Zamyadi et al., 2013a). One investigation suggests that coagulation is not species-dependent for *M. aeruginosa* and *D. Circinale*. However, *C. raciborskii* is less efficiently removed by coagulation/sedimentation (Water Research Foundation (WRF) and Water Research Australia, 2015).

Overall, the efficacy of conventional treatment varies from 62% to 99% according to the operational conditions, cyanobacterial species, and water quality (NOM, turbidity, and pH) (Chorus and Bartram, 1999a; Dreyfus et al., 2016; Drikas et al., 2001; Henderson et al., 2010; Teixeira and Rosa, 2007; Zamyadi et al., 2012c). In fact, NOM and turbidity are important parameters during coagulation that can be used for the optimization of cyanobacterial cell removal (Water Research Foundation (WRF) and Water Research Australia, 2015).

Since the cell removal in coagulation never reaches 100% in a full-scale plant and coagulation is ineffective on dissolved (extracellular) metabolites, therefore, other cyanobacteria and cyanotoxins treatment processes after conventional treatment are required.

Filtration is only effective on cyanobacteria and cell-bound removal and not on dissolved toxins and other metabolites (Chorus and Bartram, 1999a; De Julio et al., 2010; Scharf et al., 2010). The size, shape, and surface structure of algae influence the removal efficiency of cells in filters (Ma et al., 2007).

Overall, conventional treatment as a combination of coagulation/sedimentation with filtration can decrease *Microcystis*, *Dolichospermum*, and *Pseudanabaena* to below the detection limit. While, same as coagulation/sedimentation, filtration is species-dependent with a lower impact on *Aphanizomenon*. (Zamyadi et al., 2013a; Zamyadi et al., 2012c). As mentioned before, conventional processed lead to the accumulation of cyanobacteria and cyanotoxins in the sludge *(See 2.5.2).*

2.7 Sludge Treatment and Management

2.7.1 Oxidation

Oxidation is a common approach to control cyanobacterial cells as well as dissolved metabolites (cyanotoxin and T&O agents). Several studies investigated the impact of chlorination (chlorine, chloramine and chlorine dioxide) (Acero et al., 2005; Cheng et al., 2009; Chu et al., 2017; Daly et al., 2007; Fan et al., 2014b; Ho et al., 2006; Lin et al., 2009; Ma et al., 2012a; Merel et al., 2010a; Merel et al., 2010b; Nicholson et al., 2003; Rodriguez et al., 2007b; Rodriguez et al., 2007c; Xagoraraki et al., 2006; Zamyadi et al., 2013a; Zamyadi et al., 2013b; Zamyadi et al., 2013c; Zamyadi et al., 2012b; Zamyadi et al., 2010; Zhang et al., 2017a; Zhang et al., 2016), ozonation (Al Momani et al., 2008; Al Momani and Jarrah, 2010; Coral et al., 2013a; Jasim and Saththasivam, 2017; Merel et al., 2013; Newcombe and Nicholson, 2004; Onstad et al., 2007; Orr et al., 2004; Rodriguez et al., 2007b; Rositano et al., 2001; Rositano et al., 1998; Shawwa and Smith, 2010; Yan et al., 2016; Zamyadi et al., 2015a), $KMnO_4$ (Chen et al., 2005; Fan et al., 2014b; Ou et al., 2012; Rodriguez et al., 2007a; Rodriguez et al., 2007b; Rodriguez et al., 2007c; Xie et al., 2013), and H_2O_2 (Bauza et al., 2014; Drábková et al., 2007; Matthijs et al., 2012; Papadimitriou et al.,

2016; Piezer et al., 2020b; Zamyadi et al., 2020) on cyanobacteria and cyanotoxins in water in laboratory and full scales. Recently, the impact of oxidation by chlorine, ozone, $KMnO_4$ and H_2O_2 on bacterial and cyanobacterial communities was studied in water sources during natural blooms (Lusty and Gobler, 2020; Moradinejad et al., 2020).

Oxidation was also studied to control cyanobacteria and cyanotoxins in waste stabilization ponds using chlorine and H_2O_2 (Barrington et al., 2011; Barrington et al., 2013a; Barrington et al., 2013b; Praptiwi et al., 2017) and WWTP's effluent using chlorine, ozone, $KMnO_4$ and H_2O_2 (Fan et al., 2014a).

Sludge oxidation in the sludge thickener of an Australian DWTP using 10 mg/L $KMnO_4$ followed by 20 mg/L PAC was studied in a full-scale plant. The objective was to decrease *Pseudanabaena* cells (Max. initial condition: 3.5 x 10^5 cells/mL) and MIB concentration (Max. 1.5 μg/L) in the sludge thickener and its supernatant. In situ treatment of the sludge decreased MIB producer (*Pseudanabaena*) cell counts as well as MIB concentration by up to 90% and 22%, respectively. However, MIB was still detected (~ 20 ng/L) in the treated water attributed to the MIB presence in the supernatant recycled from the sludge thickener to the head of the plant (Zamyadi et al., 2016b). This study provided not only a new approach towards cyanobacteria-laden sludge treatment but also justified the potential health risk related to the recycling of the supernatant of cyanobacteria-laden sludge to the head of DWTPs.

2.7.2 Adsorption, Biodegradation

Several studies document the efficacy of PAC to remove dissolved MCs, CYN, and STXs from water (Cook and Newcombe, 2002; Ho et al., 2011; Merel et al., 2013; Newcombe et al., 2010). Combination of PAC with conventional treatment is a common approach to control organic matters in DWTPs including cyanotoxins and T&O agents (Zamyadi et al., 2013a; Zamyadi et al., 2013b). However, there are little data on the sludge. A 10-50 mg/L PAC can remove 74-100% of MIB (initial concentration) in the sludge and in a laboratory scale experiment. Accordingly, 22% MIB degradation was reported using 20 mg/L PAC in the sludge thickener of full DWTP (Zamyadi et al., 2016b).

While cyanobacteria can survive (or even grow) and produce cyanotoxins in the stored sludge. The dissolved cyanotoxins can be degraded in different ways due to degradation by adsorption on PAC (Ho et al., 2013; Zamyadi et al., 2016b), and biodegradation by cyanotoxin degrader microorganisms (Xu et al., 2016).

Degradation of dissolved MC analogs, CYN, and STXs was reported in the stored sludge. A 2-8 lag day was reported before degradation (Drikas et al., 2001; Li et al., 2015; Ma et al., 2016b; Water Research Foundation (WRF) and Water Research Australia, 2015; Xu et al., 2017). In contrast, some studies did not report degradation of MCs, CYN, and STXs during sludge storage (Ho et al., 2012; Sun et al., 2013; Sun et al., 2012). Interestingly, one study reported that in one sludge sample (Angaston- Australia), MC-RR was degraded, while MC-YR was not. The authors attributed this difference to hydrophilic and net charge characteristics of MC-YR resulting in adsorption on PAC (Ho et al., 2013). Similarly, although remarkable MC degradation (>99%, initial: 100 μg/L) was reported in a non-coagulated sludge after 6 days, reported degradation in coagulated stored sludge ($FeCl_3$ and PAFC) was lower (<48%). Also, degradation in the coagulated sludge by alum was not observed (Xu et al., 2016).

Different species of Proteobacteria (*Sphingomonas sp., Burkholderia sp., Sphingopyxis sp., Sphingomonas sp., Novosphingobium sp., Rhizobium gallicum, Burkholderia sp., Methylobacillus sp., Paucibacter toxinivorans, Ralstonia solanacearum, Morganella morganii, Pseudomonas aeruginosa,* and *Stenotrophomonas sp.*), Actinobacteria (*Arthrobacter sp., Brevibacterium sp., Rhodococcus sp., Microbacterium sp., Bifidobacterium lactis,* and *Bifidobacterium longum 46*) and Firmicutes (*Bacillus sp.,* and *Lactobacillus rhamnosus*) participate in biodegradation of MCs, NODs and CYN (Bourne et al., 1996; Chen et al., 2010; Ho et al., 2012; Hu et al., 2012; Ishii et al., 2004; Kormas and Lymperopoulou, 2013; Lemes et al., 2008; Manage et al., 2009; Nybom et al., 2007, 2008; Saito et al., 2003; Stepanauskas, 2012).

While *mlr*A, *mlr*B, *mlr*C and *mlr*D are considered the responsible genes for MC degradation, the degradation pathway of all MC degraders has not still been identified (Ho et al., 2012; Kormas and Lymperopoulou, 2013; Lawton et al., 2011; Maghsoudi et al., 2016). More than 99% of MC analogs (MC-LR, -YR, -LY, -LW, LF, initial concentration: 10 μg/L) could be degraded in the

clarifier sludge within 19 days. Accordingly, degradation of CYN was also observed within 6 days. The authors highlighted the importance of particulate attached bacteria (PAB) during degradation. In the absence of PAB, complete degradation of MC-LR and -YR was prolonged by up to 21 days, while negligible degradation was observed in other MC analogs even after 22 days. Moreover, more than 85% of CYN (initial concentration: 3 µg/L) was degraded in the clarifier sludge within 17 days, while in the absence of PAB, degradation was not observed. The authors suggested that cyanotoxin degrader bacteria are mostly accumulated in PAB (Maghsoudi et al., 2015).

CHAPTER 3 RESEARCH OBJECTIVES, HYPOTHESES AND METHODOLOGY

3.1 Critical Review and Problem Statement

Coagulation, flocculation, and sedimentation processes efficiently can remove intact cyanobacterial cells (and cell-bound cyanotoxins) throughout DWTPs. However, this treatment leads to remarkable cell/ cyanotoxin accumulation in the sludge. Current evidence suggests that cyanobacterial cells in the stored sludge may survive for more than 10 days and then be lysed leading to cyanotoxin release. Recent studies hypothesized that cyanobacteria could re-growth in the stored sludge, which increases health and technical risks related to sludge storage and management (however this has not been justified yet).

Despite these important findings, there are major data gaps on the fate of cyanobacteria and cyanotoxins in the stored sludge.

The data gaps on the fate of cyanobacteria in DWTP's sludge are the result of:

1) Using cultured cyanobacteria as opposed to cells from natural blooms in a DWTP sludge matrix.

   Previous studies were mostly performed on spiked unicellular cultured *M. aeruginosa* into the raw water and performing jar test. Similarly, there are only a few data about jar test-made sludge using spiked cultured *Dolichospermum circinale*, *Oscillatoria sp*. and *Cylindrospermopsis raciborskii* into raw water. Furthermore, the previous studies were conducted using cells in the stationary or exponential phases. Thus, the cell ages and integrity were almost the same in those studies. There are no investigations about the fate of natural cyanobacteria (blooms) containing multicellular cells with different ages and cell integrities during sludge storage. Moreover, the presence of other microorganisms, which can affect cyanobacterial communities, has not been investigated.

2) Conducting studies at the laboratory scale as opposed to investigating full-scale dynamic sludge facilities.

   Operational parameters such as sludge age, solids retention time and nutrient concentrations can play a role in the accumulation of cyanobacteria and cyanotoxins in the sludge and supernatant.

Some studies suggest that cell re-growth during sludge storage containing cultured cyanobacteria (eg. *M. aeruginosa*) can occur. However, the authors hypothesized either cell growth or i) Underestimation of cell-quota, ii) Metabolite increase per cells, iii) Additional cell settling from the supernatant to the sludge during storage. These may show an increase in cell counts and metabolites during sludge storage but are not related to cell growth (Dreyfus et al., 2016; Pestana et al., 2016; Water Research Foundation (WRF) and Water Research Australia, 2015). Also, the fate of cyanobacteria-laden sludge in the dark condition has not been studied.

Previous studies highlighted the importance of cyanobacterial-laden sludge handling. Some investigations suggested that sludge should be disposed prior to 4 days avoiding cell lysis and cyanotoxin release (Pei et al., 2017; Sun et al., 2015). However, these studies focused on cell lysis and metabolite release, while the importance of cell survival (or cell growth), and prolonged metabolite release have not been studied. In certain DWTPs, sludge supernatant is recycled to the head of the plant or is discharged to the source. Therefore, a solution for control of cyanobacteria and cyanotoxins before disposal is necessary. There is only one study suggested the combination of $KMnO_4$ and PAC to control cyanobacteria in the stored sludge (Zamyadi et al., 2016b). Thus, more studies about cyanobacteria-laden sludge management are required.

Finally, if treatment is applied, oxidized sludge will be stored for certain period of times in the holding tank before its disposal. During this period, the supernatant of the sludge can be recycled to the head of the DWTP as spent water. For the health risk assessment of cyanobacterial presence in this supernatant, the impact of oxidation on cyanobacterial cells during sludge storage should be studied.

This thesis research was conducted to fill some of these data gaps using microscopy taxonomic cell counts, shotgun metagenomic sequencing (taxonomy and functions), ddPCR, MC, and physico-chemical analyses to investigate cyanobacteria-laden sludge in a DWTP.

3.2 Objectives and Hypotheses

3.2.1 General Objective

The general objective of this research study is to investigate the fate of cyanobacteria and cyanotoxins in the sludge of DWTPs during storage and handling.

3.2.2 Specific Objectives

The specific objectives are to:

1) Diagnose critical points of DWTPs where cyanobacteria cells and their associated cyanotoxins accumulate;
2) Determine the relationship between cyanobacterial communities in the intake water, sludge, and sludge supernatant;
3) Determine the impact of nutrients on cyanobacterial community shifts in the intake water, sludge, and sludge supernatant;
4) Compare taxonomic cell counts with shotgun metagenomic sequencing results.
5) Validate cyanobacterial cell growth during sludge storage,
6) *S*tudy the dynamics of the cyanobacterial compositions in the stored sludge under controlled conditions,
7) Investigate the most resistant and susceptible cyanobacterial genus during sludge storage,
8) Study the potential health impact (i.e. cyanotoxin release) of the genera surviving sludge storage,
9) Investigate the impact of oxidation on cyanobacteria and cyanotoxins in both lab- and full-scales,
10) Determine the efficiency of oxidation during sludge storage,

11) Evaluate the impact of oxidation and stagnation on microbial and cyanobacterial communities.

12) Conduct a critical review on the fate of cyanobacteria and cyanotoxins in conventional treatment plants' sludge and during sludge storage,

13) Develop an operational decision framework to determine the best practice to minimize risks associated with cyanobacteria and cyanotoxin presence in DWTPs.

The objectives were prepared according to the following hypotheses:

1) The capture of cyanobacteria in sludge depends on the species and water quality for a given treatment condition.

Originality: This is the first comparative study to predict the fate of cyanobacteria and cyanotoxins in the stored sludge using microscopy taxonomic cell counts, MC measurement, shotgun metagenomic sequencing, and nutrient parameters.

2) Cyanobacterial communities in the stored sludge reflect intake water compositions during storage time, while the supernatant community is affected by intake water and sludge communities. Different phenomena such as cell survival, growth, and lysis influence this trend.

Originality: This is the first study to evaluate the impact of microbial/ cyanobacterial communities of intake water on sludge and supernatant using microscopy taxonomic cell counts, shotgun metagenomic sequencing, and MC measurement.

3) Shotgun metagenomic results support microscopy taxonomic cell counts.

Originality: Comparison of the microscopy taxonomic cell counts and shotgun metagenomic results is performed for the first time in the sludge samples at the order, genus, and species levels.

4) Cyanobacteria can survive, be lysed or even grow during sludge storage. These depend on cyanobacterial composition and sludge characteristics. The survived/grown cyanotoxins can produce cyanotoxins.

Originality: This is the first investigation to evaluate the fate of cyanobacteria, MC concentrations, microbial /cyanobacterial communities using microscopy taxonomic cell counts, SPE-UHPLC-

MS/MS and taxonomic shotgun metagenomic sequencing, respectively in the sludge containing natural cyanobacterial cells in various sludge ages and bloom conditions (pre-blooms, blooms, post-blooms).

5) Oxidation of sludge can degrade cyanobacterial cells and released cyanotoxins.

Originality: This is the first study evaluating the impact of oxidation on ensemble cyanobacterial cells (microscopy taxonomic cell counts), MC concentrations, and microbial/ cyanobacterial communities (shotgun metagenomic sequencing).

6) Oxidation followed by stagnation, as stressors, can induce toxicity expression in surviving toxigenic cells.

Originality: This is the first study to monitor the impact of oxidation followed by stagnation on cyanobacterial cell counts, MC concentrations, microbial/ cyanobacterial communities and toxic *mcyD* gene copy numbers using microscopy taxonomic cell counts, on-line SPE-UHPLC-MS/MS, taxonomic shotgun metagenomic sequencing, and ddPCR analysis, respectively.

7) (7-1) Cyanobacteria and cyanotoxin monitoring in the intake water, inside the treatment chain, sludge and supernatant is an important preventive action to mitigate cyanobacteria/cyanotoxin concerns during cyanobacterial events, (7-2) Adjustment of coagulation/sedimentation process, (pre-/post-) oxidation and activated carbon can mitigate cyanobacteria and cyanotoxins in DWTPs, and (7-3) The impact of sludge and sludge supernatant on health and environment should be assessed before decision about cyanobacteria-laden sludge handling.

Originality: This hypothesis is studied based on our achievements combined with other available data in the format of a critical review. This is the first suggestive decision framework for monitoring and controlling of cyanobacteria and cyanotoxin in DWTP and associated sludge.

The experimental approaches are presented as follows and are summarized in Table 3-1:

- *Article 1 (Chapter 4):*

The fate of cyanobacteria and cyanotoxins throughout a DWTP chain and in the sludge holding tank and sludge holding tank supernatant were studied. Microscopy taxonomic cell counts were

applied in the intake water, clarified water, filtered water and finished (chlorinated) water, sludge and sludge supernatant. Meanwhile, microbial and cyanobacterial communities (shotgun metagenomic sequencing), MC concentrations and nutrient parameters were investigated in the intake water, sludge, and sludge supernatant. The important parameters that shaped cyanobacterial community in the sludge and sludge supernatant were investigated.

The results were published in the journal of *Toxins* (January 2021).

- *Article 2 Chapter 5):*

The fate of cyanobacterial cell counts, MC concentrations, microbial/ cyanobacterial communities and cyanobacterial functions during sludge storage (stagnation) were investigated.

The results were submitted in the journal of *Toxins* (August 2022).

- *Article 3 (Chapter 6):*

In this article oxidation as a solution to control cyanobacteria-laden sludge was studied in laboratory and full scales (on-site). Then, oxidized sludge was monitored during storage (stagnation). Samples for microscopy taxonomic cell counts, microbial/ cyanobacterial communities, MCs and *mcyD* gene copy numbers (ddPCR) were taken and analyzed.

The results were published in the Journal of *Water* (February 2022).

- *Article 4 (Chapter 7):*

This chapter presents a critical review including our achievements in this research study and previous available data. The objective of this article is to develop a decision framework for management and handling of cyanobacteria and cyanotoxins in DWTPs and associated sludge.

The results were published in the Journal of *Toxins* (June 2022).

Table 3-1 Experimental approach for validation of hypotheses (Article 1- Chapter 4)

Objectives	Hypothesis	Experimental approach
Diagnose critical points of DWTPs where cyanobacteria cells and their associated cyanotoxins accumulate	The capture of cyanobacteria in sludge depends on the species and water quality for a given treatment condition.	• Study on natural cyanobacteria in a full scale DWTP located South East of Montreal (Missisquoi Bay- Lake Champlain). • The fate of cyanobacteria (taxonomic cell counts) in the treatment chain, sludge holding tank and sludge holding tank supernatant were studied. • Shotgun metagenomics and on-line SPE-UHPLC-MS/MS were applied to investigate microbial /cyanobacterial communities and MC concentrations, respectively in the intake water, sludge and supernatant. • The impact of nutrient parameters (TOC, TN, TP, DN, DP, OP, NH4, NO_2-NO_3) on cyanobacterial diversity shifts from the intake water to the sludge and supernatant were studied. • Microscopy taxonomic cell counts and shotgun metagenomic results were compared.
Determine the impact of nutrients on cyanobacterial community shifts in the intake water, sludge, and sludge supernatant		
Determine the relationship between cyanobacterial communities in the intake water, sludge, and sludge supernatant	Cyanobacterial communities in the stored sludge reflect intake water compositions during storage time, while supernatant communities is affected by intake water and sludge communities. However, different phenomena such as cell survival, growth, and lysis influence this trend.	
Compare taxonomic cell counts with shotgun metagenomic sequencing results	Shotgun metagenomic results support microscopy taxonomic cell counts.	

Table 3-2 Experimental approach for validation of hypotheses (Articles 2 and 3- Chapter 5 and 6)

Specific objectives	Hypothesis	Experimental approach
To validate cyanobacterial cell growth during sludge storage To study the dynamics of the cyanobacterial compositions in the stored sludge under controlled conditions To investigate the most resistant and susceptible cyanobacterial genus during sludge storage To study the potential health impact (i.e. cyanotoxin release) of the genera surviving sludge storage	Cyanobacteria can survive, be lysed or even grow during sludge storage. These depend on cyanobacterial composition and sludge characteristics. The survived/ grown cyanotoxins can produce cyanotoxins.	Sludge samples taken from the sludge holding tank were stagnated for 8-38 days in the dark. Analysis: microscopy taxonomic cell counts, MCs and taxonomic/function shotgun metagenomics. Site location: South East of Montreal (Missisquoi Bay).
To investigate the impact of oxidation on cyanobacteria and cyanotoxins in both lab- and full- scales	Oxidation of sludge can degrade cyanobacterial cells and released cyanotoxins.	Lab-scale: Performed by $KMnO_4$ (5 & 10 mg/L) and H_2O_2 (10 & 20 mg/L). On-site oxidation: using 10 mg/L of $KMnO_4$. Analysis: microscopy taxonomic cell counts, MCs, taxonomic shotgun metagenomics. Site location: South East of Montreal (Missisquoi Bay).
To determine the efficiency of oxidation during sludge storage To evaluate the impact of oxidation and stagnation on microbial and cyanobacterial communities	Oxidation followed by stagnation, as stressors, can induce toxicity expression in surviving toxigenic cells.	Oxidized sludge was stagnated for 7-38 days in the dark. Analysis: microscopy taxonomic cell counts, MCs, taxonomic shotgun metagenomics and ddPCR (*mcyD*) Site location: South East of Montreal (Missisquoi Bay).

Table 3-3 Experimental approach for validation of hypotheses (Chapter 7)

Specific objectives	Hypothesis	Experimental approach
To conduct a critical review on the fate of cyanobacteria and cyanotoxins in conventional treatment plants' sludge and during sludge storage,	-	A critical review on our achievements and previous available data about cyanobacteria/ cyanotoxin management/handling in DWTP and associated sludge/ supernatant
To develop an operational decision framework to determine the best practice to minimize risks associated with cyanobacteria and cyanotoxin presence in DWTPs.	i) Cyanobacteria and cyanotoxin monitoring in the intake water, inside the treatment chain, sludge and supernatant is an important preventive action during cyanobacterial events. ii) Adjustment of coagulation/ sedimentation process, (pre-/ post-) oxidation and activated carbon can mitigate cyanobacteria and cyanotoxins in DWTPs, iii) The impact of sludge and sludge supernatant on health and environment should be assessed before decision about cyanobacteria-laden sludge handling.	

3.2.3 Research Strategy and Methodology

This research focused on natural cyanobacteria during pre-blooms, blooms, and post-blooms. The studied DWTP was in South East of Montreal. The intake water of the plant was taken from Missisquoi Bay- Lake Champlain (Figure 3-1). The treatment chain comprised of coagulation flocculation followed by PAC, sedimentation, filtration and disinfection (Figure 4-1).

Samples were taken throughout the DWTP and after each treatment process as intake water, clarified water, filtered water, finished (chlorinated) water. Sludge and supernatant samples were taken from the sludge holding tank. This sampling campaign were conducted in 2017 and 2018 in summer and fall.

Sludge stagnation as well as laboratory and on-site oxidation were performed in 2018.

Sludge stagnation were performed by taking sludge samples and stored in autoclaved polypropylene bottles stored in the dark in the room temperature (20 ± 2 °C).

Laboratory oxidation were performed using 5 and 10 mg/L of $KMnO_4$ and 10 and 20 mg/L of H_2O_2 at CREDEAU laboratory (Polytechnique Montréal).

On-site oxidation was performed using 10 mg/L of $KMnO_4$ in the sludge holding tank of the DWTP (Figure 3-2).

Microscopy taxonomic cell count samples were analyzed at Université du Québec à Montréal (department of biological sciences). MC samples (total, cell-bound and dissolved) were analyzed at University of Montreal (department of chemistry). Shotgun metagenomic samples were extracted at CREDEAU (Polytechnique Montréal) and then, the extracted DNA were sent to McGill University and Genome Quebec Innovation Centre for sequencing. Nutrient samples (TOC, TN, TP, DN, DP, OP, NH_4, and NO_2-NO_3) were analyzed at GRIL lab (University of Montreal). Solid parameters (TSS and TVS) were measured at Wastewater lab (Polytechnique Montréal). The ddPCR analysis on *mcyD* gene copies was conducted at National Research Council of Canada (NRCC).

Detailed analytical methods are explained in the following chapters:

A) Parameter measurements:

- Microscopy taxonomic cell counts is explained in section 4.2.3.1.

- MC measurement is explained in 4.2.3.2.
- Nutrient parameters is explained in section 4.2.3.3
- Shotgun metagenomic including DNA extraction, sequencing, bioinformatics and statistical analysis is explained in sections 4.2.4, 4.2.5 and 4.2.6.
- *mcyD* gene copy quantification by ddPCR is explained completely by Fortin et al. (2010) and (Moradinejad et al., 2021a).

In brief, genomic DNA samples were normalized to 1.0 ng/µL. Initial concentrations below 1.0 ng/µL were concentrated to the ideal 1.0 ng/µL using a speed vacuum. Primers for the detection of the *mcyD* gene involved in microcystin synthesis were as follows: *mcyD*(KS)F1: 5'-TGGGGATGGACTCTCTCACTTC-3' and *mcyD*(KS)R1: 5' GGCTTCAACATTCGGAAAACG-3' (Fortin et al., 2010).

The PCR mixture consisted of 12.5 µL Bio-Rad 2x QX200 ddPCR EvaGreenR Supermix (SM) (BioRad Laboratories Ltd, Mississauga, ON), *mcyD*(KS) primers at a final concentration of 0.1 µM, 0.2 mg/mL BSA and genomic DNA input concentrations ranging from 1 to 5 ng DNA per 25 µL ddPCR reaction. Sample mixtures were vortexed gently avoiding the formation of bubbles, centrifuged for 20 sec, then kept on ice until droplet generation. Samples were packaged into droplets by adding 20 µL of the PCR mixture in each sample well of the single-use DG8 cartridge followed by addition of 70 µL of droplet generation oil for EvaGreenR to each of the corresponding oil wells. The cartridge was then placed into the QX200 droplet generator for droplet production. Forty microliters of generated droplets were transferred from the cartridge to a semi-skirted ddPCR 96-well plate (Bio-Rad). Samples were successively prepared in cartridges by groups of eight, transferred to the PCR plate and subsequently heat sealed with a pierceable foil seal. The plate was transferred to a thermal cycler and reactions were run under the following standard cycling conditions: 95°C for 10 min followed by 40 cycles of 94°C for 30 sec; 55°C for 60 sec, 98°C for 10 min, and 4°C hold; ramp rate = 50% (2°C/sec). Upon completion of the PCR phase, plates were loaded onto the QX200 Droplet Digital reader, which automatically reads the droplets from each well of the plate (17 000 droplets/well). Data analysis was performed using QuantasoftTM software (Bio-Rad). Negative droplets, lacking target and/or reference gene DNA, and positive droplets,

containing either or both DNAs, were counted to give the fraction of positive droplets. Using Poisson statistics, the concentrations of both DNA species were determined, and copies/ng calculated.

B) Treatment/ handling (stagnation and oxidation) procedure description:

- Stagnation without/ without oxidation is explained in Section 5.2.2.
- Laboratory and on-site oxidation is explained in section 6.2.2.

Figure 3-1 Missisquoi Bay during a cyanobacterial bloom (August 2018)

Figure 3-2 On-site oxidation using $KMnO_4$ in the sludge holding tank

Figure 3-3 Sample preparation in the DWTP

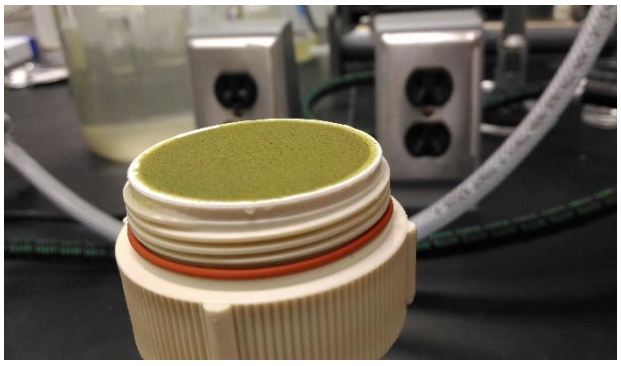

Figure 3-4 A filtered sample

CHAPTER 4 ARTICLE 1: CAN CYANOBACTERIAL DIVERSITY IN THE SOURCE PREDICT THE DIVERSITY IN SLUDGE AND THE RISK OF TOXIN RELEASE IN A DRINKING WATER TREATMENT PLANT?

In this chapter, we discussed about the impact of treatment chain on accumulation of cyanobacterial cells and MCs through a DWTP. Also, bacterial and cyanobacterial communities in the raw water, sludge holding tank and supernatant were studied. The results will help for better understanding the fate of cyanobacteria and MCs accumulated in the sludge during storage, and the impact of raw water and sludge on risks related to sludge supernatant recycling to the head of the DWTP. This chapter was published as a research article in the Toxins journal on January, 2021. Supplementary data is presented in Appendix A.

CAN CYANOBACTERIAL DIVERSITY IN THE SOURCE PREDICT THE DIVERSITY IN SLUDGE AND THE RISK OF TOXIN RELEASE IN A DRINKING WATER TREATMENT PLANT?

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Abstract

Conventional processes (coagulation, flocculation, sedimentation, and filtration) are widely used in drinking water treatment plants and are considered a good treatment strategy to eliminate cyanobacterial cells and cell-bound cyanotoxins. The diversity of cyanobacteria was investigated using taxonomic cell counts and shotgun metagenomic sequencing over two seasons in a drinking water treatment plant before, during, and after the bloom. Changes in the community structure over time at the phylum, genus, and species levels were monitored in samples retrieved from raw water (RW), sludge in the holding tank (ST), and sludge supernatant (SST). *Aphanothece clathrata brevis*, *Microcystis aeruginosa*, *Dolichospermum spiroides*, and *Chroococcus minimus* were predominant species detected in RW by taxonomic cell counts. Shotgun metagenomic sequencing revealed that Proteobacteria was the predominant phylum in RW before and after the cyanobacterial bloom. Taxonomic cell counts and shotgun metagenomic showed that the *Dolichospermum* bloom occurred inside the plant. Cyanobacteria and Bacteroidetes were the major bacterial phyla during the bloom. Shotgun metagenomic sequencing also showed that *Synechococcus*, *Microcystis*, and *Dolichospermum* were the predominant detected cyanobacterial genera in the samples. Conventional treatment removed more than 92% of cyanobacterial cells but led to cell accumulation in the sludge up to 31 times more than in the RW influx. Coagulation/sedimentation selectively removed more than 96% of *Microcystis* and *Dolichospermum*. Cyanobacterial community in the sludge varied from raw water to sludge during sludge storage (1–13 days). This variation was due to the selective removal of coagulation/sedimentation as well as the accumulation of captured cells over the period of storage time. However, the prediction of the cyanobacterial community composition in the SST remained a challenge. Among nutrient parameters, orthophosphate availability was related to community profile in RW samples, whereas communities in ST were influenced by total nitrogen, Kjeldahl nitrogen (N- Kjeldahl), total and particulate phosphorus, and total organic carbon (TOC). No trend was observed on the impact of nutrients on SST communities. This study profiled new health-related, environmental, and technical challenges for the production of drinking water due to the complex fate of cyanobacteria in cyanobacteria-laden sludge and supernatant.

Keywords

Cyanobacteria; microcystins (MCs); water treatment; sludge; shotgun metagenomic sequencing; cyanobacterial community; high-throughput sequencing

Key Contribution

High-throughput sequencing was applied to investigate cyanobacterial community in raw water, stored sludge, and supernatant of sludge holding tanks in a drinking water treatment plant.

4.1 Introduction

Cyanobacterial cells and their associated cyanotoxins are considered to represent an important challenge due to i) Their health threat to humans and animals; ii) Their negative aesthetic impacts with respect to taste, odor, and color; iii) The implications of extra water treatment requirements for ozonation and membrane filtration; and iv) The increased consumption of coagulants, flocculants, and activated carbon (Shang et al., 2018; Westrick et al., 2010; Zamyadi et al., 2013a).

Conventional treatment using coagulation, flocculation, sedimentation, and filtration is a common approach for cyanobacterial removal from intake water (Drikas et al., 2001; Oehrle et al., 2010; Zamyadi et al., 2013a). However, conventional treatment is not efficient at removing dissolved cyanotoxins (Newcombe and Nicholson, 2004). In addition, hydraulic and chemical stresses during treatment may cause damage to cells and trichomes, leading to the release of cyanotoxins (Pestana et al., 2019; Pietsch et al., 2002). Another challenge of conventional treatment is the increase in cyanobacteria and cyanotoxin concentrations in the clarifiers and filters of water treatment plants (DWTPs) and their accumulation in the sludge of clarifiers (Drikas et al., 2001; Ho et al., 2012; Zamyadi et al., 2013a; Zamyadi et al., 2012c). Cyanobacterial cells can be present at concentrations 10–100 times higher in the sludge than in intake water, even in plants with low cyanobacterial flux (<1000 cells/mL) (Almuhtaram et al., 2018; Zamyadi et al., 2013b). Moreover, some investigations have shown that coagulated cells can stay viable in the sludge for 2 to 10 days (Drikas et al., 2001; Ho et al., 2012; Li et al., 2015; Sun et al., 2013; Sun et al., 2012). More recently, cell viability in the sludge was observed for more than 20 days (Water Research Foundation (WRF) and Water Research Australia, 2015). During this period, microcystin-LR (MC-LR) and cylindrospermopsin

concentrations increased to 3–7 times their initial levels. The authors of (Pestana et al., 2016) reported that metabolite concentrations in the sludge supernatant after storage were up to five times greater than those within the sludge before storage. This shows a new challenge in sludge management during storage and when the sludge supernatant is recycled to the head of the plant (Pestana et al., 2016; Zamyadi et al., 2019). The fate of cyanobacteria and cyanotoxins during and after coagulation and in the sludge is not fully understood. The impacts of coagulation on cyanobacterial cells are still controversial. Although some studies have demonstrated that coagulation depends on cyanobacterial species (Water Research Foundation (WRF) et al., 2009; Zamyadi et al., 2013a; Zamyadi et al., 2019), another study showed that cells are not selectively captured by coagulation (Water Research Foundation (WRF) and Water Research Australia, 2015). It has been shown that cell damage and metabolites released in sludge are associated with various environmental conditions; however, due to the complex interactions of cyanobacteria with treatment processes, the primary factors behind this complex behavior are still not determined (Dreyfus et al., 2016; Pestana et al., 2016). Additionally, although the positive impact of powdered activated carbon (PAC) on cyanotoxin degradation in raw water (RW) has been widely studied (Cook and Newcombe, 2002; Ho et al., 2011; Merel et al., 2013; Newcombe et al., 2003; Newcombe and Nicholson, 2004), there are no data about the role of injected PAC in RW in the degradation of accumulated cyanotoxins within sludge.

Recently, high-throughput sequencing and metagenomics techniques have been successfully applied to describe microbial communities in the water resources to predict the occurrence of cyanobacterial blooms (Berry et al., 2017; Tromas et al., 2017). During the last decade, several studies have investigated bacterial communities in DWTPs and have demonstrated that while microbial communities in the water treatment chain are represented by water intake, treatment processes have an impact on the microbial community structure through DWTPs (Chao et al., 2013; Lautenschlager et al., 2014; Li et al., 2017; Lin et al., 2014; Ma et al., 2020; Pinto et al., 2012; Zamyadi et al., 2019; Zhang et al., 2017b). Few studies have investigated bacterial communities within sludge in WTPs (Pei et al., 2017; Xu et al., 2018). The authors of (Xu et al., 2018) reported similar bacterial communities in sludge samples collected from six different Chinese WTPs with the same treatment processes. They reported similar bacterial communities in sludge samples, suggesting that bacterial communities in sludge might be shaped by RW communities; however,

they did not compare the bacterial composition in sludge with that in RW. The authors of (Pei et al., 2017) studied the impact of different coagulants on bacterial communities and metabolite release in sludge. They found that the relative abundance of the dominant genera *Microcystis*, *Rhodobacter*, *Phenylobacterium*, and *Hydrogenophaga* decreased, reflecting their damage and the subsequent release of extracellular microcystin and organic matter. They suggest that the sludge should be treated or disposed of within 4 days to avoid the proliferation of the pathogens.

There are basically no studies exploring the impact of RW cyanobacterial communities on cyanobacteria-laden sludge and its supernatant in DWTPs. Moreover, previous studies considered sludge as a batch samples, while in DWTPs cyanobacteria-laden sludge might be dynamically affected by different parameters such as RW characteristics, treatment process functionality, and sludge storage time. The impact of these parameters on the cyanobacterial community structure of sludge has not been investigated. No comparative analysis has been carried out on bacterial/cyanobacterial community composition within RW, sludge, and sludge supernatant. Due to the knowledge gaps related to the fate of cyanobacteria and cyanotoxins in sludge, high-throughput sequencing techniques could be useful to better understand the community dynamics of cyanobacteria-laden sludge through a DWTP. This study is the first to use both shotgun metagenomic sequencing and taxonomic cell count approaches to provide an overview of cyanobacterial composition in RW, a sludge holding tank (ST), and the corresponding sludge supernatant (SST) in a DWTP.

The general objective of this research was to study the fate of cyanobacteria and its associated cyanotoxins in a DWTP. The specific objectives were to: (1) diagnose critical points of DWTPs where cyanobacteria cells and their associated cyanotoxins accumulate; (2) determine the relationship between cyanobacterial communities in RW, sludge, and its supernatant; (3) determine the impact of nutrients on cyanobacterial community shifts in RW, the sludge holding tank, and its supernatant; and (4) compare taxonomic cell counts with shotgun metagenomic sequencing results.

4.2 Materials and Methods

4.2.1 Description of the Studied Water Body and Plant, Including Treatment Schematics

A plant located on the Canadian side of Missisquoi Bay, Lake Champlain, located South East of Montreal was monitored from July 27th to October 27th 2017. The plant intake water is situated 180 m within the bay. The treatment chain is presented in Figure 4-1. Briefly, powdered activated carbon (PAC) injection followed by conventional treatment (coagulation, flocculation, sedimentation) and a post-chlorination step are applied as treatment. The clarifier sludge is stored in a sludge holding tank. The supernatant of this sludge is discharged into the lake and the sludge is transferred to the local WWTP.

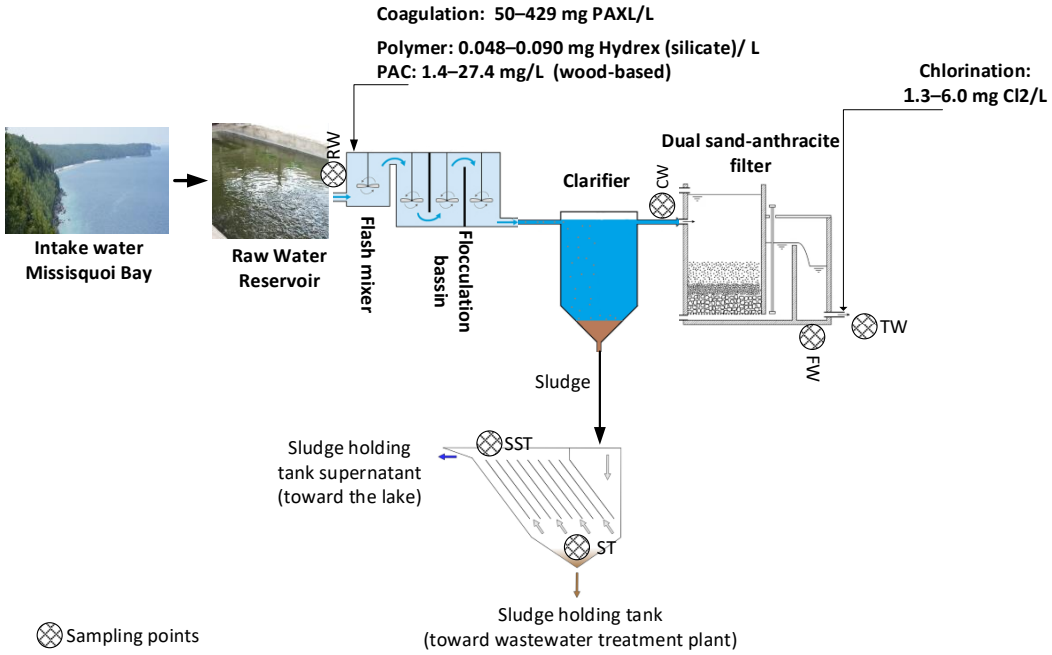

Figure 4-1 The treatment chain of the DWTP and sampling points. The water intake is from Missisquoi Bay. Sampling points are indicated by ⊗. RW: raw water; CW: clarified water; FW: filtered water; TW: treated water; ST: sludge holding tank; SST: supernatant of the sludge holding tank.

Overall, seven sampling campaigns were performed on the following dates: July 27th, August 15th, 25th, and 30th, September 1st and 5th, and October 27th 2017. Specifications of the plant and treatment are summarized in Table A- 1.

4.2.2 Description of the Sampling Points and Sample Preparation

Samples were taken following each treatment step from raw water (RW), clarified water (CW), filtered water (FW), treated water (TW), the sludge holding tank (ST), and sludge holding tank supernatant (SST).

Autoclaved 1-L polypropylene bottles, 5-L polypropylene containers, and 40-mL glass vials were used for each sampling point. Before sampling, all containers and vials were rinsed with the water from the sampling point. The 40-mL vials were used for taxonomic cell counts. The taxonomic samples were preserved with Lugol's iodine. Subsamples were taken for metagenomics from the 1-L bottles; the 5-L containers were used for cell-bound and dissolved microcystins (MCs) and nutrient samples.

Genomic subsamples were prepared by sample filtration via 0.2-μm polyether sulfone hydrophilic membranes (Millipore Sigma, Oakville, ON). Membranes were stored in the sterile Falcon tube at −80 °C. Cell-bound and dissolved microcystin sub-samples were prepared by sample filtration using pre-weighted 0.45-μm GHP mem-branes (Pall, Mississauga, ON). The filters were kept in the petri dish and the filtrate was kept in 125-mL graduated polyethylene terephthalate glycol (PETG) amber bottles (Thermo Fisher, Mississauga, ON). Subsamples for total nitrogen (TN), total phosphorus (TP), and total organic carbon (TOC) were aliquoted directly. Dissolved nitrogen (DN), Kjeldahl nitrogen (N- Kjeldahl), ammonium nitrogen (NH4), nitrite/nitrate (NO2/NO3) and dissolved phosphorus (DP) subsamples were filtered on 0.45-μm membranes (Millipore Sigma, Oakville, ON).

Genomic subsamples were taken in triplicate, while MC and nutrient samples were taken in duplicate. MC, N- Kjeldahl, and NO_2/NO_3 subsamples were frozen at −25 °C. TN, TP, and TOC samples were stored at 4 °C. Taxonomic cell count samples were stored in a dark place at ambient temperature.

4.2.3 Description of Analytical Methods

4.2.3.1 Taxonomic Cell Counts

Taxonomic cell counts were performed by an inverted microscope in a Sedgwick-Rafter chamber at magnifications of 10 and 40× according to (Lund, 1959; Lund et al., 1958; Planas et al., 2000).

4.2.3.2 4.3.2. Microcystin Analysis

Total microcystins (MCs) were analyzed by on-line solid-phase extraction ultra-high-performance liquid chromatography coupled to tandem mass spectrometry (on-line SPE-UHPLC-MS/MS). Firstly, samples were oxidized by potassium permanganate and sodium (meta) periodate (Sigma Aldrich, Oakville, ON, Canada). Secondly, samples were quenched by a 4M sodium bisulfite solution (Sigma Aldrich, Oakville, ON, Canada). Thirdly, the standard solutions of 4-phenylbutyric acid (50 ng/L) (Sigma Aldrich, Oakville, ON, Canada) and erythro-2-Methyl-3-methoxy-4-phenylbutyric acid (D3-MMPB, 10 ng/L) (Wako Pure Chemicals Industries, Ltd., Osaka, Japan) were added to the samples. Fourthly, 10 mL of solution were filtered using 0.22-μm nylon filters (Sterlitech Corporation, Kent, WA, USA). Aliquots were taken for analysis using the Thermo EQUAN™ interface (Thermo Fischer Scientific, Waltham, MA, USA). The “in-loop” injection was controlled by an HTC Thermopal autosampler (CTC analytics, Zwingen, Switzerland). Then, samples were loaded into a Thermo Hypersil Gold aQ C18 (on-line SPE) column (20 mm × 2.1 mm, 12 μm). Separation of toxins was performed on a Thermo Hypersil Gold C18 column (100 mm × 2.1 mm, 1.9 μm). MS/MS detection was performed by thermo TSQ QUANTIVA triple quadrupole mass spectrometer (Thermo Fischer Scientific) following UHPLC. Water, methanol, and acetonitrile for HPLC were purchased from Fisher Scientific (Whitby, ON, Canada) and formic acid (>95%), potassium carbonate, ammonium hydroxide (28–30% NH3), and ammonium acetate (≥99.0%) were obtained from Sigma Aldrich (Oakville, ON, Canada). More details are provided by Munoz et al. (2017) and Roy-Lachapelle et al. (2019).

4.2.3.3 Nutrient Analysis

Nitrogen, nitrite, nitrate, and ammonium nitrogen were analyzed by the colorimetric technique based on EPA 350.1 and 353.2 standard methods methods (United States Environmental Protection

Agency (USEPA), 1993a, b). Phosphorus and phosphate were measured by the colorimetric technique based on EPA 365.1 and 365.3 methods (United States Environmental Protection Agency (USEPA), 1978). TOC was analyzed by Sievers InnovOX Laboratory TOC analyzer (USA) based on USEPA 415.1 method (United States Environmental Protection Agency (USEPA), 1974).

4.2.4 DNA Extraction and Metagenomics Preparation

The extraction of total nucleic acid from frozen filters was performed with the RNeasy PowerWater Kit (Qiagen, Toronto, ON, USA) with slight modifications. To avoid formation of disulfide bonds protein residuals, dithiothreitol (DTT) was spiked into the pre-warmed (55 °C) PM1 buffer. Since substantial biomass remained on the surface of the membrane after the bead-beating step, the filters were transferred alongside the supernatant and were incubated with the IRS solution. Total nucleic acids were eluted with 60 µL of nuclease-free water. Half of the sample was treated with RNase If (New England Biolabs, Whitby, ON) to remove the RNA. The resulting DNA was purified using the Genomic DNA Clean & Concentrator TM-10 (Zyimo Research Corporation, Irvine, CA, USA), following the instructions of the manufacturer.

For the sludge samples, the RNeasy PowerSoil Total RNA Kit (Qiagen, Toronto, ON, Canada) was used with two modifications to improve the recovery of DNA. The modifications consisted of (1) a centrifugation step for 5 min at 13,000 rpm at 4 °C to separate the water from the sludge; and (2) incubation at −20 °C for 60 min after the addition of solution SR4 to precipitate nucleic acids. DNA was eluted using the PowerSoil DNA Elution Kit (Qiagen, Toronto, ON, Canada), following the instructions of the manufacturer. DNA quantification was done by Qubit V2.0 fluorometer (Life Technologies, Burlington, ON, Canada). The metagenomic libraries (Roche 454 FLX instrumentation with Titanium chemistry) were sent to McGill University and Genome Quebec Innovation Centre for sequencing. Ninety six libraries were then pooled together and sequenced on a NovaSeq 6000 S4 with paired end of 150 bp and an insert of 360 bp.

Metagenomic analysis were performed on all RW, ST, and SST samples collected on August 30th and September 5th. Community dynamics was assessed using shotgun metagenomic sequencing at

the phylum, order, genus, and species levels. The number of reads for taxonomic data was normalized by relative abundance.

4.2.5 Bioinformatics Analysis

DNA libraries were sequenced on the Illumina NovaSeq 6000 platform using S4 flow cells. Paired-end raw reads of 150 base pairs (bp) were further analyzed using a home-made bioinformatics pipeline. Firstly, quality trimming of raw reads was performed by the SolexaQA v3.1.7.1 program with default settings (Cox et al., 2010). Trimmed reads shorter than 75 nt were removed for further analysis. Artificial duplicate removal was performed using an in-house script based on the screening of identical leading 20 bp. From the trimmed high-quality reads, gene fragments were predicted using FragGeneScan-Plus v3.0 (Kim et al., 2015). Cd-hit v4.8.1 was applied to cluster predicted protein fragments at a 90% similarity (Fu et al., 2012). One representative of each cluster was used for a similarity search on the M5nr database (https://github.com/MG-RAST/myM5NR) using the Diamond engine (Buchfink et al., 2015). For assessment of taxonomic affiliation of gene fragments encoding proteins, we took into account best hits (minimal e-value of 1×10^{-5}) combined with a last common ancestor approach.

4.2.6 Statistical Analysis

Statistical analysis was performed by R (3.6.2). Bacterial communities at the phylum, order, and genus level were analyzed by phyloseq (1.28.0) (McMurdie and Holmes, 2013). Taxonomic data was normalized by centered log-ratio transformation using easyCODA (0.31.1) (Greenacre, 2018). Then, the cyanobacteria species community was analyzed based on the first 25 most frequent species by pheatmap (1.0.12) (https://CRAN.R-project.org/package=pheatmap). The richness index was analyzed by phyloseq's estimate_richness function. For visualization of the species community and diversity variation, heat trees were illustrated using the metacoder (0.3.3) (Foster et al., 2017). Beta diversity was performed by vegan package (2.5–6) (https://CRAN.R-project.org/package=vegan). Similarity matrices were calculated according to Euclidean distance. A redundancy analysis (RDA) was performed to evaluate the impact of constrained variables on sampling points 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 nutrient parameters on the distribution of cyanobacterial communities in the RW, ST, and SST at the order level. 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.

4.3 Results and Discussion

4.3.1 Impact of Conventional Treatment on Cyanobacteria and Cyanotoxins

During the period prior to the bloom (July 1st to August 30th 2017), taxonomic cell counts in RW were below 5.0×10^4 cells/mL and *Aphanothece clathrata brevis* was the most dominant species, representing 65%–100% of total cell counts. *Dolichospermum spiroides* (0%–26%), *Chroococcus minimus* (0%–34%), *Microcystis aeruginosa* (0%–2%), and *Dolichospermum circinale* (<1%) were detected frequently (Figure 4-1). During this period, low concentrations of MCs were detected, with dissolved microcystin (MC) levels below 90 ng/L and cell-bound MC levels below 10 ng/L (Figure 4-3, Table A-2).

A cyanobacterial bloom appeared in Missisquoi Bay in late August, and total taxonomic cell counts increased in RW to 3.1×10^5 cells/mL on September 1st. The dominant species was *D. spiroides*, representing 52% of total cell counts with a concentration of 1.6×10^5 cells/mL. Other identified species were *A. clathrata brevis* (1.0×10^5 cells/mL, 33%), *M. aeruginosa* (4.0×10^4 cells/mL, 13%), and *Coelosphaerium kuetzingianum* (1.7 × 103 cells/mL, 1%) (Figure 4-2). The low total MC level increased to 260.1 ng/L, of which 191.9 ng/L were dissolved (Figure 4-3). On the sampling dates following the bloom (September 5th and October 27th), cell counts decreased to around 3.9×10^4 cells/mL and remained constant. The species *A. clathrata brevis* was dominant during those two dates (68%–85%). *C. minimus* (31%) was also found on September 5th and *M. aeruginosa* (31%) and *Aphanizomenon gracile* (1%) were found on October 27th (Figure 4-2). MCs remained below the detection limit (DL) on September 5th. On October 27th, the total MC concentration increased to 142.6 ng/L, of which 121.9 ng/L were dissolved (Figure 4-3).

Through the treatment process, 86%–99% of total cyanobacterial cells were removed by the clarifier. In particular, 85%–100% of *M. aeruginosa*, *A. clathrata brevis*, *C. minimus*, *A. gracile*, and *D. spiroides* were eliminated. These results are in agreement with previous studies

documenting coagulation efficiency of between 62% and 99% (Chorus and Bartram, 1999a; Drikas et al., 2001; Sun et al., 2012; Teixeira and Rosa, 2007; Zamyadi et al., 2012c). Meanwhile, 14%–71% of the escaped cells from the clarifier were removed by filtration (Figure 4-2). Overall, 92%–99% of cells were eliminated by conventional treatment. A previous study on this plant found a similar reduction (Zamyadi et al., 2013a). Furthermore, taxonomic cell counts increased from 25% to 120% in treated water (TW) on all sampling dates. *A. clathrata brevis* (72%–99%) remained dominant in TW, except on the bloom date (September 1st). On September 1st, the cyanobacterial composition in TW consisted of *D. spiroides* (62%), A. clathrata brevis (29%), and *Pseudanabaena mucicola* (9%) (Figure 4-2). Cell counts in TW (after chlorination) were 1.3–2 times greater than cell counts in filtered water (FW) (before chlorination). This accumulation can be problematic if accumulated cells produce cyanotoxins.

Total taxonomic cell counts in ST remained around 3–31 times greater than in RW (Figure 4-2). The cell percentage of *A. clathrata brevis* in ST decreased from ~100% on July 27th to 32% on September 1st (bloom date). In contrast, during this period, the percentages of *Aphanocapsa delicatissima* and *D. spiroides* increased from 0% to 29% and 27%, respectively. On September 1st, *M. aeruginosa* counts in ST were four times greater than in RW. Furthermore, *A. delicatissima, P. mucicola, Aphanizomenon flos-aquae, D. circinale,* and *C. kuetzingianum* were detected in small amounts (0–1.7 × 10^3 cells/mL) in RW, whereas their cell counts increased to between 2.5 × 10^2 to 1.2 × 10^6 cells/mL in ST on the corresponding dates (Figure 4-2).

Cell counts in SST remained around 94%–98% lower than those in ST and 69%–97% lower than those in RW. *A. clathrata brevis* was dominant (83%–99%) in SST, with small percentages of *D. spiroides* (1%–6%) and *P. mucicola* (5%–11%).

Total MCs in ST and SST remained below 281 and 128 ng/L, respectively, during the sampling campaign (Figure 4-3). These MC trends are inconsistent with previous investigations which reported MC-LR concentrations in the clarifier sludge to be around 10 times greater than in RW (Zamyadi et al., 2013a; Zamyadi et al., 2012c). This low concentration of MCs measured in the ST also contradicts the results of (Pestana et al., 2016), where it was shown that cyanobacterial metabolites in lagoon supernatant were 2 to 5 times greater than the initial concentrations. One reason behind the low MC concentrations in the ST as well as in the SST in our study may be the

impact of injected PAC in RW on accumulated MCs in stored sludge. Indeed, levels of dissolved MCs were reduced from 121.7 ng/L (September 1st) to below DL on September 5th when the PAC dose increased from 9.2 to 27.3 mg/L. In contrast, the concentration of dissolved MCs increased to 116.7 ng/L on October 27th when the PAC dose decreased to 7.0 mg/L (Figure 4-3 and Figure 4-4). A second reason may be the biodegradation of MCs during sludge storage. However, the authors of (Maghsoudi et al., 2015) documented biodegradation of MCs as being very low compared to individual microcystin analogues.

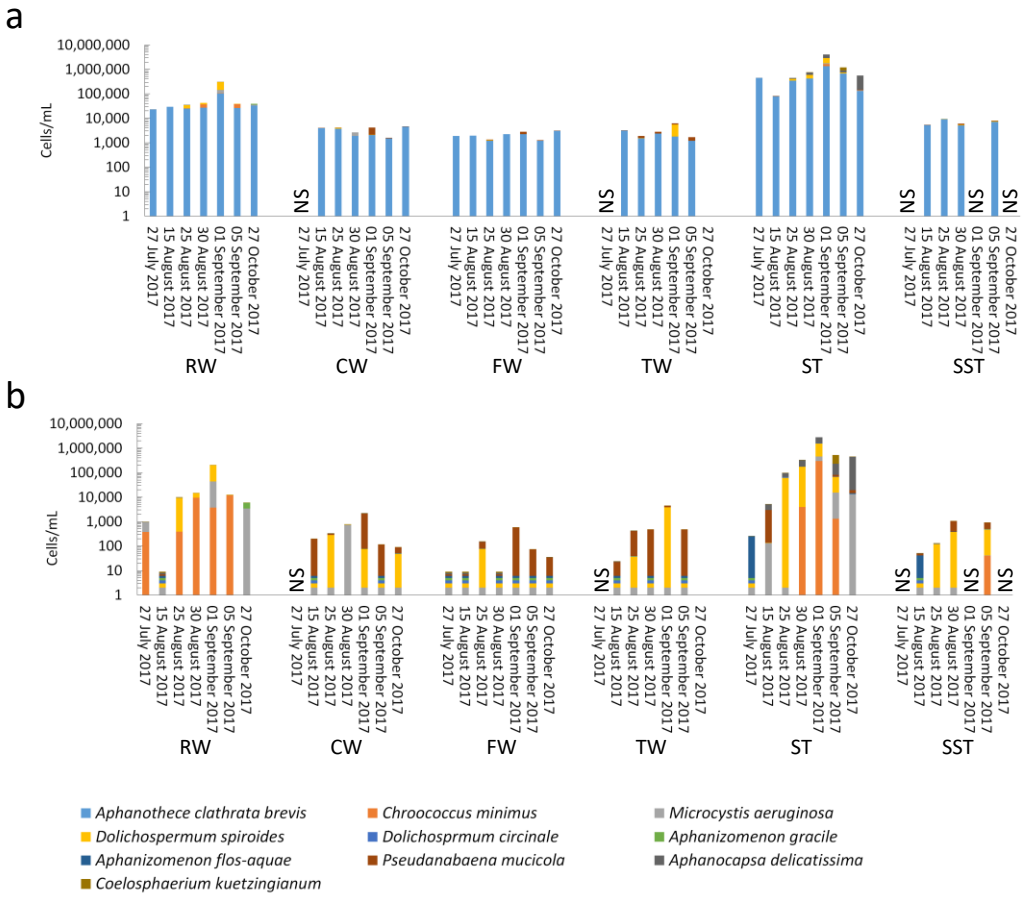

Figure 4-2 Distribution of cyanobacterial species in the water treatment plant (WTP) by taxonomic cell counts: (a) all species, (b) speciation of species other than *Aphanothece clathrata brevis*. RW: raw water, CW: clarified water, FW: filtered water, TW: treated water, ST: sludge holding tank, SST: sludge holding tank supernatant. Sludge storage times: July 27th 2017: 8 days; August 15th 2017: 3 days; August 25th 2017: 8 days; August 30th 2017: 13 days; September 1st and 5th 2017: 1 day; October 27th 2017: 2 days. NS: sample not taken.

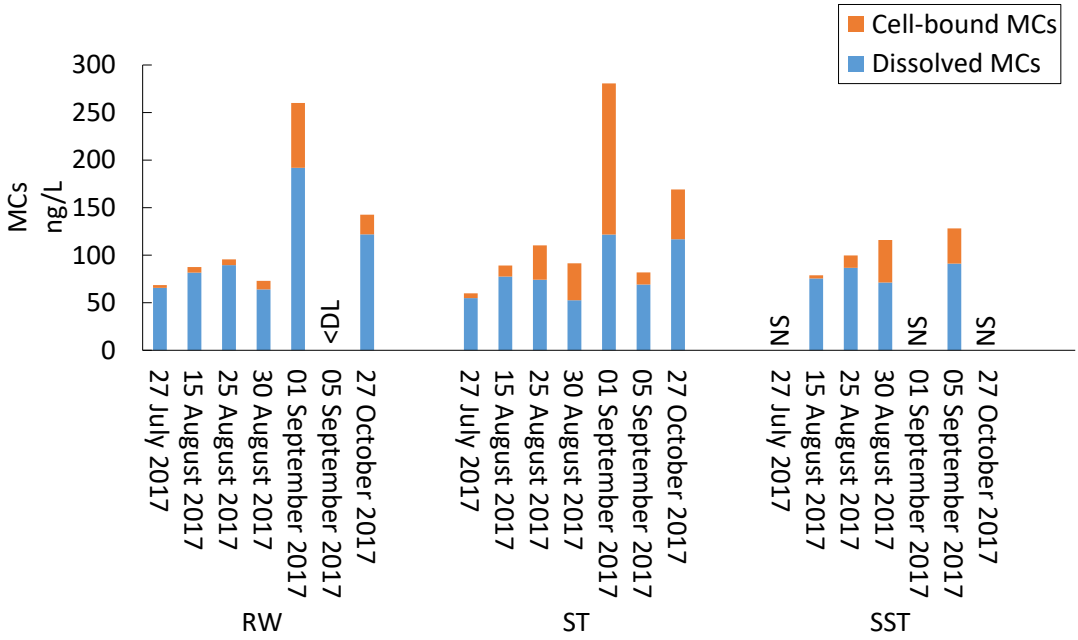

Figure 4-3 Concentration of dissolved and cell-bound microcystins (MCs) in raw water (RW), in the sludge holding tank (ST), and in sludge holding tank supernatant (SST). NS: sample not taken, DL: below detection limit.

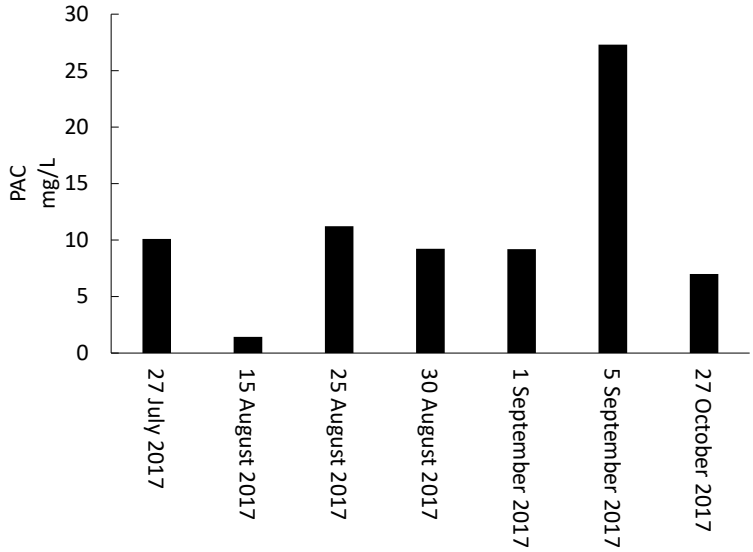

Figure 4-4 Powder activated carbon (wood-based PAC) doses injected into raw water (RW) during the 2017 sampling campaigns.

4.3.2 Cyanobacterial Diversity in Sludge and Supernatant Assessed by Shotgun Metagenomic Sequencing

From July 27th to August 25th 2017, Proteobacteria remained dominant in RW and their relative abundance increased from 26% to 56%. Actinobacteria (12%–26%) and Bacteroidetes (14%) were the following dominant phyla in RW. During this period, Cyanobacteria, Verrucomicrobia, and Firmicutes had small relative abundances below 6%, 5%, and 3%, respectively. On August 30th, the abundance of Proteobacteria and Actinobacteria decreased to 35% and 14.6%, respectively, while that of Cyanobacteria and Bacteroidetes increased to 19%. On September 1st, the community profile was associated with high cyanobacterial levels (38%) and was distinct from those of other sampling dates where there were lower cyanobacterial levels. This is coherent with trends observed in taxonomic cell count results on September 1st. Similarly, the relative abundance of Bacteroidetes reached its highest level (32%) on that date (Figure 4-5), as supported by previous reports linking cyanobacterial blooms with Bacteroidetes (Guedes et al., 2018; Kim et al., 2020). Indeed, Bacteroidetes is associated with nutrient loadings which promote the growth of Cyanobacteria (Cai et al., 2014). In this work, we observed that total (TN) and dissolved nitrogen (DN) were significantly associated with the Bacteroidetes community (Table A-3, Figure A- 1). On the next sampling dates (September 5th and October 27th), the abundance of Cyanobacteria and Bacteroidetes decreased to 12.4% and 4.5% on September 5th, and 19% and 14% on October 27th, respectively. The abundance of Proteobacteria increased from 37% to 48% (Figure 4-5). On August 30th (before the bloom) and September 5th (after the bloom), Proteobacteria and Actinobacteria were the two dominant phyla in the ST (56%–57% and 14%–17%, respectively) and SST (56%–68% and 18%–20%, respectively) (Figure 4-5). During this period, the relative cyanobacterial abundance in the ST and SST was about 7% and 4%, respectively. Interestingly, Bacteroidetes was also found at low levels, fluctuating from 5% to 12% and from 7% to 18% in the ST and SST, respectively.

At the genus level within Cyanobacteria in RW, *Synechococcus* and *Microcystis* were predominant on July 27th and August 15th (Figure 4-6). In late August, the relative abundance of *Synechococcus* and *Microcystis*, declined, while that of *Dolichospermum* and *Nostoc*, increased. The relative abundance of *Dolichospermum* reached its maximum level on August 30th and September 1st (bloom date). After the bloom date (September 5th and October 27th), the relative abundance of

Dolichospermum decreased, while that of *Microcystis* and *Synechococcus* increased, and the diversity of cyanobacterial communities almost returned to pre-bloom conditions (Figure 4-6). A previous investigation in Missisquoi Bay documented that the relative abundance of *Dolichospermum* and *Microcystis* repeatedly alternated in bloom and non-bloom events (Tromas et al., 2017), while our study showed that Synechococcus also shifted from being a highly abundant taxa before and after the bloom to being present with very low abundance during the bloom.

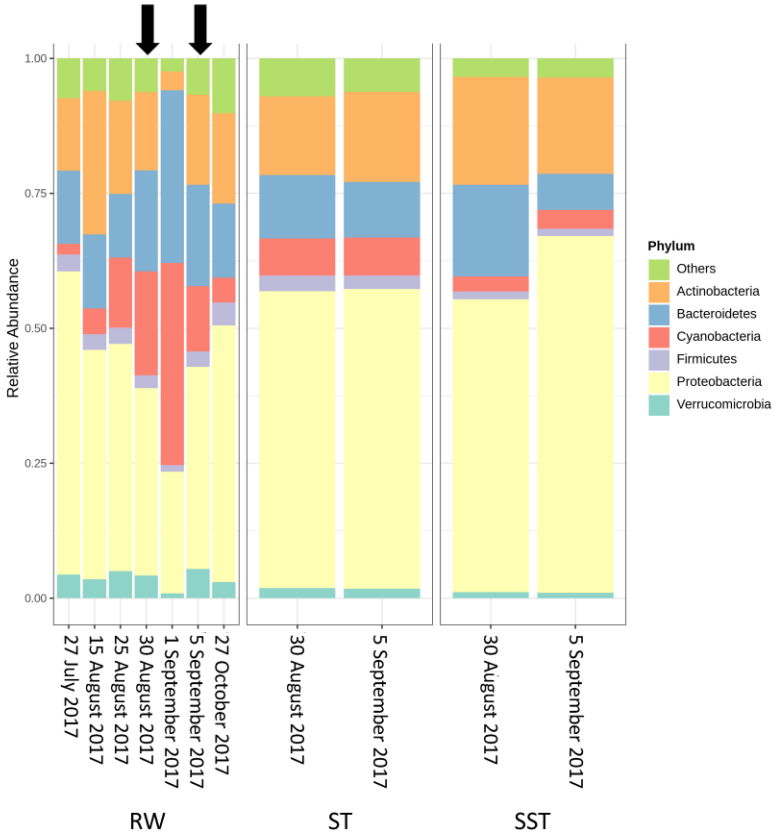

Figure 4-5 Bacterial community at the phylum level in raw water (RW) (July 27th, August 15th, 25th, and 30th, September 1st and 5th, and October 27th 2017), in the sludge holding tank (ST), and in the sludge holding tank supernatant (SST) (August 30th and September 5th 2017). The black arrows show the corresponding dates with the ST and SST samples.

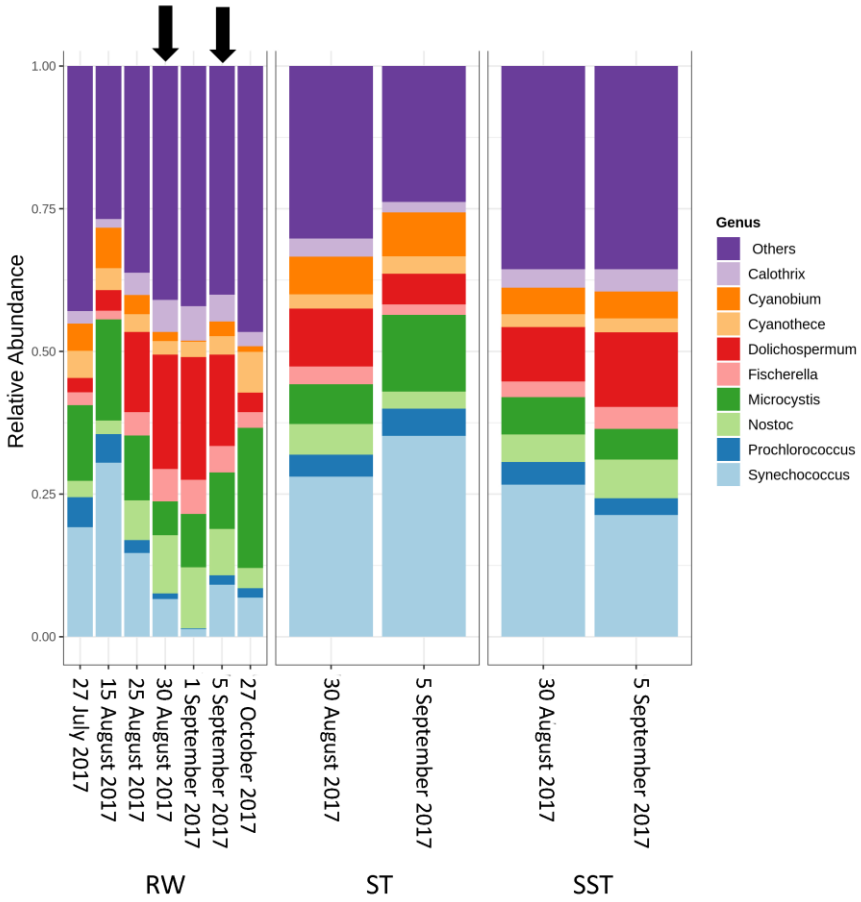

Figure 4-6 Cyanobacterial community at the genus level in raw water (RW) (on July 27th, August 15th, 25th and 30th, September 1st and 5th, and October 27th 2017), in the sludge holding tank (ST), and in sludge holding tank supernatant (SST) (on August 30th and September 5th 2017). The black arrows show the corresponding dates for the ST and SST samples.

The relative abundance of the genera and species changed between RW and ST/SST stages (Figure 4-6). It is important to note that the structural composition of the sludge communities was not expected to match because sludge is the result of several days of cyanobacterial cell accumulation in the holding tank. For example, when considering samples from August 25th 2017, the sludge holding time was estimated as 13 days, while that of September 5th was 1 day. A comparison between RW (August 25th and 30th) and ST (August 30th) showed that there was a higher abundance of *Synechococcus* and a lower abundance of *Dolichospermum* in the ST as compared to RW. This trend was also observed on September 5th. On August 25th, the relative abundance of *Microcystis* was lower in the ST in comparison to RW, while the opposite trend was observed on August 30th.

On September 5th, the abundance of *Microcystis* was higher in the ST than in the RW. The abundance of *Synechococcus* in SST (August 30th) was higher than in RW (August 25th and 30th).

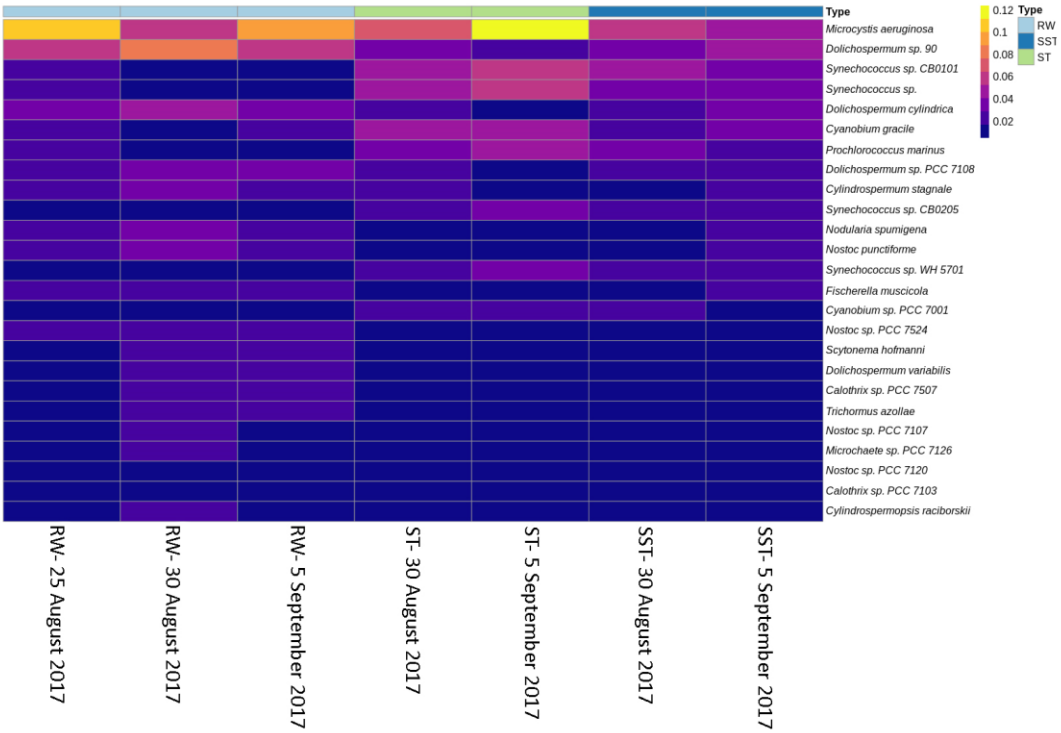

Figure 4-7 Relative abundance of the top 25 major abundant species in raw water (RW) (August 25th and 30th, and September 5th 2017), in the sludge holding tank (ST), and in sludge holding tank supernatant (SST) (August 30th and September 5th 2017).

This trend was also observed on September 5th. The opposite trend of *Synechococcus* was observed within that of *Dolichospermum*. The abundance of *Microcystis* in SST (August 30th) was lower than in RW on August 25th but higher than in RW on August 30th. On September 5th, the abundance of *Microcystis* in SST was lower than that in RW. The relative abundance of *Synechococcus* in the ST was higher than that in SST on both sampling dates (August 30th and September 5th). On August 30th, the relative abundances of *Microcystis* and *Dolichospermum* were similar in the ST and SST. Interestingly, on September 5th, the abundance of *Microcystis* decreased in SST compared to the ST, while *Dolichospermum* showed the opposite trend (Figure 4-6). At the species level, similar trends were observed in *M. aeruginosa*, *Dolichospermum sp. 90*, and *Synechococcus sp. CB0101* (Figure 4-7). Additionally, other genera with lower relative abundance (<6%) were detected in the

samples. For example, *Prochlorococcus, Cyanobium, Fischerella, Calothrix,* and *Cyanothece* did not show significant changes between the RW, ST, and SST (Figure 4-7, Figure A- 2 and Figure A- 3). The richness of cyanobacterial species (Chao1 index) remained approximately constant in the RW (578 and 598 on August 30th and September 5th, respectively) and the ST (599 and 620 on August 30th and September 5th, respectively), while it decreased in the SST (275 and 475 on August 30th and September 5th, respectively) (Figure 4-8a). The difference in richness between RW and ST with respect to SST suggests that the cyanobacterial communities in SST and ST were different but that there were similarities between the ST and RW.

Between July 27th and August 30th, the Shannon index increased from 4.18 to 4.51 in RW, while it decreased to 4.06 on bloom on September 1st (Figure A- 4). Our results showed that the diversity decreased during the bloom, in agreement with (Tromas et al., 2017). The Shannon index in the ST on August 30th (4.4) indicated similar diversity profiles to RW on August 25th (4.39) and August 30th (4.28). Due to 13 days of sludge storage time on August 30th, the diversity in the ST was affected by the bacterial populations in RW in samples on both August 25th and August 30th. The Shannon index in the sludge decreased to 4.16 on September 5th, which is a lower value than the Shannon index in the RW (4.38) on the same date (Figure 4-8b).

Overall, when relating structural composition of the communities found in raw water (RW), in the sludge holding tank (ST), and in sludge holding tank supernatant (SST), several factors should be considered:

- As expected, changes in composition of RW communities were observed and affected the microbial populations in the ST. Since sludge accumulates over the period of time (1–13 days in this case), the ST profile is expected to reflect accumulative diversity considering both the relative abundance and biomass. Furthermore, the efficacy of coagulation and settling is species-dependent, as shown by (Zamyadi et al., 2013a; Zamyadi et al., 2019). Previous investigations also demonstrated that 96%–100% of Dolichospermum and Microcystis cells were more likely to be captured by the clarifier (Zamyadi et al., 2013a; Zamyadi et al., 2012c), and that the coagulation efficiency for these genera was twice the value observed for Synechococcus (Aktas et al., 2012).

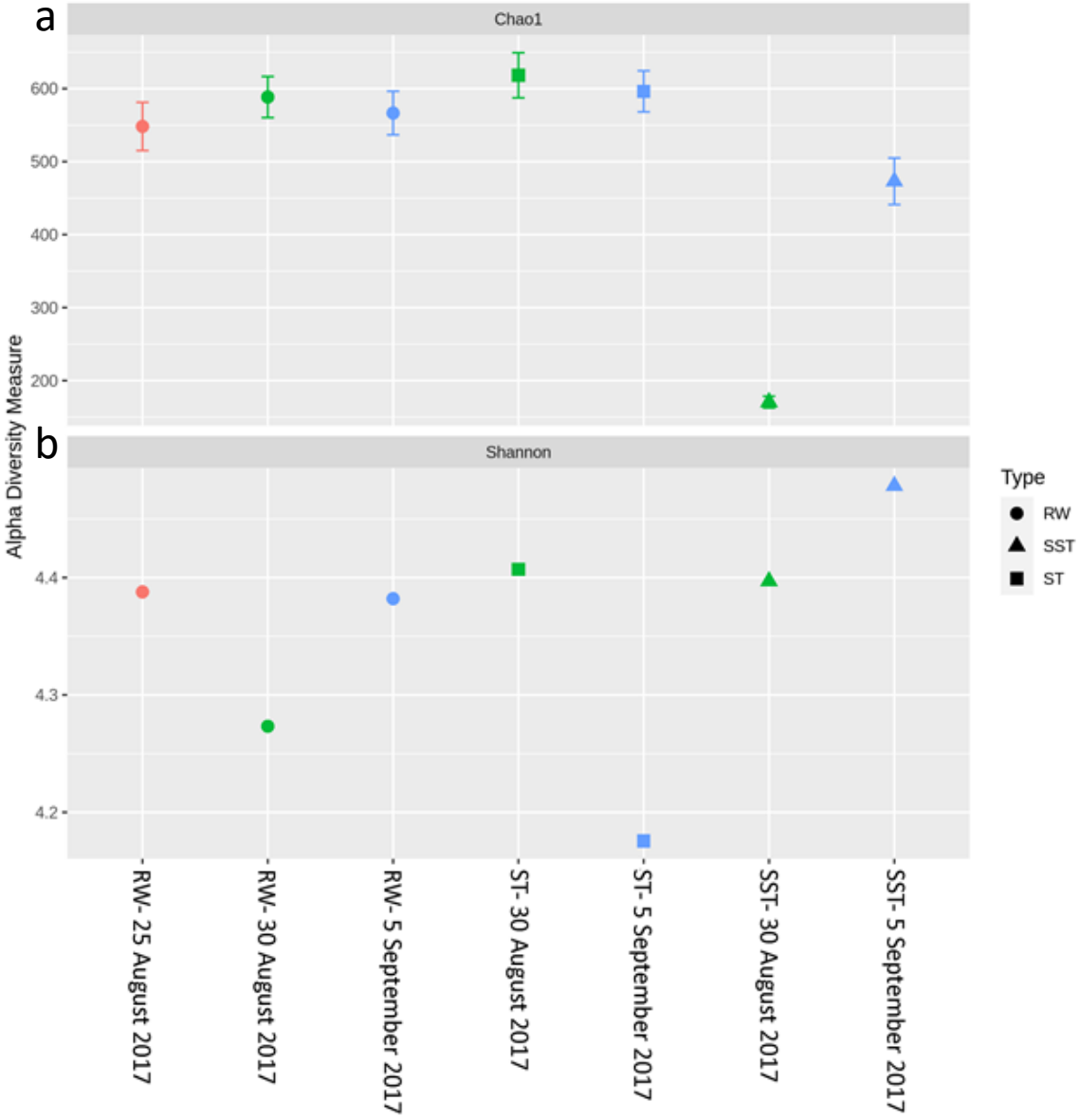

Figure 4-8 Evaluation of cyanobacterial richness and diversity in raw water (RW) (August 25th and 30th, and September 5th 2017), in the sludge holding tank (ST), and in sludge holding tank supernatant (SST) on August 30th and September 5th 2017 using (a) Chao1 and (b) the Shannon index.

- The communities found in the ST and SST showed different trends at the phylum and genus level as shown by the Shannon index (Figure A- 5, Figure 4-5 and Figure 4-6). In fact, at

the phylum level, Cyanobacteria was selectively removed and retained within sludge (Figure 4-5). The cyanobacterial community distribution in the supernatant reflects the incoming sludge and the subsequent buoyancy of the community in the sludge. Storage in the holding tank of the sludge may cause cell breakage, leading to vesicle damage (Arii et al., 2015) and interruption of buoyancy regulation (Reynolds et al., 1987). This would affect the profile of the supernatant in our work. The increase in cyanobacterial richness in SST on September 5th might be due to the longer sludge storage time in the August 30th sample (13 days) compared to that of September 5th (1 day), providing more time for cell damage.

- Cell survival, re-growth, and damage might have occurred in ST during sludge storage. The longer storage of the sludge might have led to cell lysis in the sludge. These phenomena were documented in several studies for various dominant genera including the most dominant genera in this study (*Microcystis* and *Dolichospermum*) after 2 days of sludge storage (Drikas et al., 2001; Pestana et al., 2016; Sun et al., 2015; Water Research Foundation (WRF) and Water Research Australia, 2015). Furthermore, trichome damage of Dolichospermum due to the treatment stress has been already reported (Pestana et al., 2019). However, there are no data on the fate of Synechococcus in stored sludge.

Understanding the community structure and dynamics in the ST and SST is important for quantitative cyanobacterial risk assessment. Water operators need to be able to predict the exchanges between the sludge and the supernatant. Supernatant (SST) can be discharged into water resources or recycled to the head of the DWTP and could constitute a risk for the water intake or an additional burden on the plant treatment processes. Sludge (ST) can be disposed of in wastewater collectors, processed as sludge in lagoons or sludge facilities, or land-applied.

Other studies have shown that environmental conditions can impact sludge communities (Dreyfus et al., 2016; Pestana et al., 2016). Redundancy analysis (RDA) analyses were performed to evaluate the relationship between nutrients (Table A-3) and cyanobacterial communities. Orthophosphate (OP), total nitrogen (TN, sum of Kjeldahl nitrogen (N- Kjeldahl), organic nitrogen, nitrite, and nitrate), N-Kjeldahl, total phosphorus (TP), particulate phosphorus (PP), and total organic carbon (TOC) exerted significant effects (p <0.05) on community profiles in different ways (Figure 4-9). A clear correlation was observed between OP in RW, with Nostocales (reported to have a 4.5 times

higher relative abundance of genes related to phosphorus metabolism than Chroococcales) found at low concentrations of phosphorus (Lu et al., 2019). Other studies have demonstrated that higher concentrations of nitrogen, phosphorus, and carbon resulted in better conditions for bacterial communities and led to an increase in microbial growth (Jankowiak et al., 2019; Pei et al., 2017; Xu et al., 2018). In our study, RDA analyses showed that OP was more available in RW than in the ST and SST and that TN, N- Kjeldahl, TP, PP, and TOC had a strong impact on the cyanobacterial population in the ST, which mostly contained Chroococcales. None of these nutrient parameters seemed to affect the SST. This is in accordance with our previous observations on the different patterns of cell accumulation in SST. However, it must be noted that the mass of nutrients measured in sludge (2.0–32.8 mg/L of TN and 0.48–5.9 mg/L of TP), was not associated with cyanobacterial cell-bound nutrients nor with dissolved nutrients. Using reference values for cell nutrient content, cell-bound nutrients consist of less than 0.8% nitrogen and 0.4% phosphorus (Lopez et al., 2016). The persistence of Chroococcales in the sludge environment could be the result of the high environmental resistance and the ability to thrive in the presence of elevated levels of nutrients (Figure 4-9).

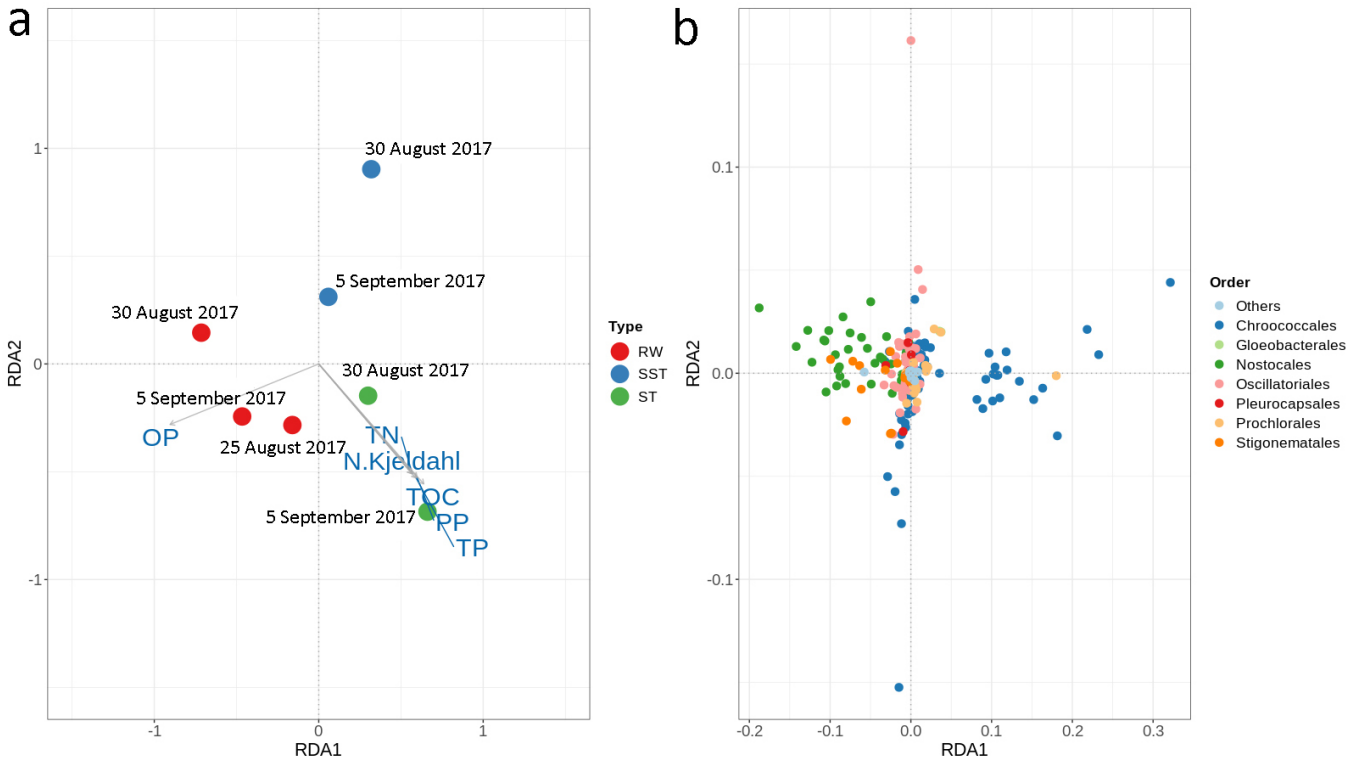

Figure 4-9 Redundancy analysis (RDA) of cyanobacterial communities with respect to nutrient parameters in (a) raw water (RW), in the sludge holding tank (ST), and in sludge holding tank supernatant (SST); (b) cyanobacterial distribution at the order level. RDA1: 65.6%, RDA2: 8.7%. Only significant parameters (p <0.05) are shown.

4.3.3 Comparison Between Shotgun Metagenomic and Microscopy Taxonomic Cell counts

The observed genera from microscopic cell counts do not completely match the high-throughput sequencing results, as *Aphanothece, Chroococcus, Aphanocapsa* and *Coelosphaerium* were not detected by shotgun metagenomic sequencing. In RW, *M. aeruginosa*, *D. circinale*, *D. spiroides*, *A. gracile*, and *P. mucicola* were detected through both taxonomic cell counts and metagenomics (data not shown). In contrast, *A. clathrata brevis, C. minimus, and C. kuetzingianum* were detected only by taxonomic cell counts and not by metagenomics (data not shown). In ST, only *M. aeruginosa*, *D. spiroides*, and *D. circinale* were detected by both approaches, while *A. clathrata brevis*, *C. minimus*, *P. mucicola*, *A. delicatissima*, *A. flos-aquae*, and *C. kuetzingianum* were only detected in taxonomic cell counts (data not shown). Overall, 76% and 88% of the detected species by metagenomics were not detected by taxonomic cell counts in RW and ST, respectively (Figure

4-2Figure 4-6). As recently discussed (Moradinejad et al., 2020), taxonomic cell counts and high-throughput sequencing can yield different community profiles because of the limitations inherent to each of these methods. Physical and chemical stress in DWTPs may cause damaged cells and affect taxonomic cell counts (Pestana et al., 2019), while DNA can be extracted from lysed and dead cells and provide metagenomics shotgun reads (Ellegaard et al., 2020). Despite the advantages of taxonomic cell counts, measurement bias such as misidentification of morphologically similar species, the impact of the conservation agent on biovolume, and the complexity of counting species in aggregates should be considered (America Water Works Association (AWWA), 2010; Hawkins et al., 2005; Park et al., 2018). In sludge samples, the presence of debris, sediments, and a high number of cells might increase the probability of cross interferences. New metagenomic approaches based on direct cloning and shotgun sequencing of environmental DNA represent a powerful tool for species classification and the evaluation of community dynamics through water treatment processes. However, use of metagenomics also represents some challenges such as: (1) an inadequate recovery rate of DNA (Bag et al., 2016); (2) contamination of DNA during extraction (Gevers et al., 2012; Kuczynski et al., 2011); and (3) a lack of a standard identification pipeline that includes all species (Teeling and Glockner, 2012).

4.4 Conclusions

- Bacterial communities shifted before and after the cyanobacterial bloom. Proteobacteria was the predominant phylum in RW before the bloom. Levels of Cyanobacteria and Bacteroidetes progressively increased to reach their greatest abundance on the bloom date. This high abundance of Bacteroidetes was associated with nutrient-rich conditions which occurred during the cyanobacterial bloom. After the bloom, bacterial communities returned to almost the same composition as prior to the bloom.
- Conventional treatment eliminated 92%–97% of the cyanobacterial cells, as revealed by cell counts. Overall, 96% of Microcystis and Dolichospermum were eliminated by this process. At first glance, this is an effective approach to controlling the cyanobacterial flux. However, coagulation leads to accumulation of cyanobacterial cells in the sludge. Even a low cell number in intake water (3.9×10^4 cells/mL) led to 31 times as much cell accumulation in the sludge.

- Selective removal of cyanobacteria at the genus and species levels by coagulation/sedimentation has been highlighted by both metagenomic shotgun sequencing and taxonomic cell counts. Sludge (ST) cyanobacterial composition differs from RW if only samples from the same day are considered. Sludge diversity reflects both selective removal by coagulation/sedimentation and the accumulation of captured cells over a period of time as determined by sludge age.
- Monitoring strategies focusing on sporadic measurement of the diversity in raw water cannot capture the risk associated with the storage and disposal of the sludge. Sludge community profiles also appear to be a better indicator for evaluating the influx of cyanobacterial communities in WTPs. Indeed, the sludge profile reflects a cumulative community in terms of relative abundance and biomass.
- Bacterial and cyanobacterial communities of sludge in the holding tank (ST) markedly differed from those measured in sludge supernatant (SST). The communities found in the ST and SST showed different trends at the phylum and genus level as shown by the Shannon index. The prediction of cyanobacterial communities in the supernatant remains a challenge as it is often recycled, possibly adding cyanobacteria and cyanotoxins to the intake water.
- Considering environmental parameters monitored, nutrients were the most discriminating factors affecting cyanobacterial communities. Cyanobacterial communities in RW were influenced by OP, while the sludge communities were correlated with TN, N- Kjeldahl, TP, PP, and TOC.
- Storage, management, and disposal of the cyanobacteria-laden sludge are technical and health-related challenges. By adjusting the storage time and adding PAC, risk assessment of supernatant recycling can be applied to minimize the impact of cyanobacteria and cyanotoxin accumulation.

Author Contributions

Conceptualization, F.J., H.T., S.D., and M.P.; Methodology, F.J., H.T., J.F.G.M., N.F., B.J.S., Y.T., A.Z., S.D., S.S., and M.P.; Software, F.J., J.F.G.M., and Y.T.; Validation, F.J., H.T., J.F.G.M., N.F., Y.T., S.D., and M.P.; Formal analysis, F.J., H.T., J.F.G.M., and Y.T.; Investigation, F.J., H.T., N.F., J.F.G.M., S.D., A.Z., and M.P.; Resources: F.J., H.T., and N.F.; Data curation, F.J. and J.F.G.M.; Writing—original draft preparation, F.J., H.T., and J.F.G.M.; Writing—review and editing, F.J., H.T., N.F., J.F.G.M., S.S., S.D., and M.P.; Visualization, F.J., H.T., J.F.G.M., N.F., S.D., and M.P.; Supervision, A.Z., S.D., and M.P.; Project administration, A.Z., S.D., B.J.S., S.S., and M.P.; Funding acquisition, S.D., S.S., B.J.S., and M.P. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement

Not applicable.

Informed Consent Statement

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Data Availability Statement

Data is contained within the article and supplementary material.

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Conflicts of Interest

The authors declare no conflict of interest.

CHAPTER 5 ARTICLE 2: IMPACT OF STAGNATION ON THE DIVERSITY OF CYANOBACTERIA IN DRINKING WATER TREATMENT PLANT SLUDGE

In this chapter, we discussed about the impact of storage (stagnation) on cyanobacteria and cyanotoxins during sludge stagnation (storage). In spite of previous investigations on cyanobacteria-laden sludge, the fate of cyanobacteria and cyanotoxins in the DWTP's sludge still needs more studies. Although previous studies demonstrated that cyanobacteria can survive in the stored sludge, probability of cell growth in the sludge stored in the dark is not understood. The results of this chapter will help for better understanding the dynamics of cyanobacteria and cyanotoxins during storage and handling. This chapter was submitted as a research article in the Journal of Toxins on August 2022. Supplementary data is presented in Appendix C.

IMPACT OF STAGNATION ON THE DIVERSITY OF CYANOBACTERIA IN DRINKING WATER TREATMENT PLANT SLUDGE

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Abstract

Health-related concerns about cyanobacteria-laden sludge of drinking water treatment plants (DWTPs) has been raised in the past years. Microscopic taxonomy, shotgun metagenomic sequencing, and microcystin (MC) measurement were applied to study the fate of cyanobacteria and cyanotoxin after controlled sludge storage (stagnation) in a full-scale drinking water treatment plant in the dark within 7 to 38 days. For 4 out of 8 dates, cyanobacterial cell growth was observed by total taxonomic cell counts during sludge stagnation. The highest observed cell growth was 96% after 16 days of stagnation. Growth of cells was dominated by potential MC producers such as Microcystis, Aphanocapsa, Chroococcus, and Dolichospermum. Shotgun metagenomic sequencing unveiled that stagnation stress shifts the cyanobacterial communities from the sensitive Nostocales (Dolichospermum) order towards less compromised orders and potential MC producers such as Chroococcales (Microcystis) and Synechococcales (Synechococcus). The relative increase of toxin producers presents a health challenge when the supernatant of the stored sludge is recycled to the head of the DWTP or is discharged into the source. These findings emphasize the importance of a strategy to manage cyanobacteria and cyanotoxins in the sludge and suggest sufficient approaches to limit the accumulation and mitigation of cyanobacteria in sludge should be considered.

Keywords

Taxonomic cell counts, Shotgun metagenomic sequencing, DWTP, Storage, Waste, Microcystins

Key Contribution

Sludge storage shifts diversity from a sensitive order towards persistent orders. Persistent genera after storage are potential microcystin-producers.

5.1 Introduction

Cyanobacteria and cyanotoxins are a challenge in water resources worldwide that may affect drinking water quality (Ho and Michalak, 2019; Ho et al., 2019; Kimambo et al., 2019; Pick, 2016; Winter et al., 2011).

Conventional treatment (flocculation, coagulation, sedimentation and filtration) is widely applied to manage cyanobacterial cells and cell-bound metabolites in drinking water treatment plants (DWTPs) (Chorus and Welker, 2021; Drikas et al., 2001; Newcombe et al., 2010; Zamyadi et al., 2013a). Although these processes can remove 60- 99% of cyanobacterial cells from the water, they cause accumulation of cyanobacterial cells and cyanotoxins in the sludge (Drikas et al., 2001; Shang et al., 2018; Sun et al., 2012; Teixeira and Rosa, 2007; Zamyadi et al., 2012c). It is reported that even low influent cell counts below 1000 cells/mL may lead to cyanobacterial accumulation in the sludge by up to 100-fold. Additionally, cyanotoxin concentrations detected below the detection limit (DL) in the intake water may increase 12-fold in the sludge (Almuhtaram et al., 2018; Zamyadi et al., 2013b).

Several studies have highlighted that *Microcystis aeruginosa*, *Dolichospermum circinale*, *Oscillatoria sp*., and *Cylindrospermopsis raciborskii* can remain alive in the stored sludge for 2-12 days. During sludge storage, cells can undergo lysis leading to cyanotoxin release (Drikas et al., 2001; Ho et al., 2012; Li et al., 2018; Sun et al., 2013; Sun et al., 2012; Sun et al., 2018; Xu et al., 2016). An investigation showed that concentrations of microcystin-LR, -LA and cylindrospermopsin in the stored sludge containing *Microcystis aeruginosa* and *Cylindrospermopsis raciborskii* cells remained 1.2-4 times higher than the maximum expected

concentration calculated based on the cell toxin quota (if all cells release cyanotoxins) after 7- 16 days (Water Research Foundation (WRF) and Water Research Australia, 2015). This revealed that cells not only were able to survive in the stored sludge, but also could retain the ability to grow. However, the authors mentioned that the underestimation of cell quota might affect the results. Thus, cell growth of cyanobacteria in the stored sludge remains questionable. Recent studies reported that *Microcystis aeruginosa* and *Cylindrospermopsis raciborskii* cells can stay alive and proliferate in the sludge for around 35 days (Dreyfus et al., 2016; Pestana et al., 2016). Additionally, cyanotoxin release can increase by up to 2.5 times during sludge storage.

Accumulation of cyanobacteria in the sludge could lead to technical problems and health issues. Some studies suggest that cyanobacteria-laden sludge should be disposed of within 2- 4 days to minimize risks associated with metabolite release (Pei et al., 2017; Sun et al., 2015). However, the negative impact of the sludge supernatant containing cyanobacteria and cyanotoxins still remains a challenge. One study reported that the recycling of cyanobacteria-laden sludge supernatant to the head of the plant caused a 40% increase in the cyanobacterial cell counts in the intake water (Zamyadi et al., 2019). In our previous study, we demonstrated the dynamics of bacterial and cyanobacterial diversity in the stored sludge and its impact on the sludge supernatant in a full-scale plant. (Jalili et al., 2021). In addition, we showed the selective accumulation of Microcystis and Dolichospermum in the sludge after flocculation, coagulation, and sedimentation processes (Jalili et al., 2021). These results highlighted concerns about MC accumulation in the sludge and its impact on water quality when the sludge supernatant is recycled to the head of the DWTP or is discharged into the source.

However, previous studies fall short of demonstrating the potential for cyanobacteria to regrow during storage in the dark. Additionally, most of the previous sludge investigations were performed using cultured cyanobacteria, not on natural cyanobacterial blooms. Secondly, the dynamic nature of sludge holding tank operation precludes the quantification of the impact of storage on the regrowth of cyanobacterial cells. Furthermore, the survival and growth of cyanobacteria in the sludge is best investigated using shotgun metagenomic sequencing to observe microbial/ cyanobacterial community dynamics during storage.

The general objective of this study was to assess the fate of cyanobacteria during sludge storage in a full scale DWTP. The specific objectives were to *i)* validate cyanobacterial cell growth during sludge storage, *ii)* study the dynamics of the cyanobacterial compositions in the stored sludge under controlled conditions, iii) investigate the most resistant and susceptible cyanobacterial genus during sludge storage, and iv) study the potential health impact (i.e. cyanotoxin release) of the genera surviving sludge storage.

To the best of our knowledge, this is the first descriptive study on the fate of natural cyanobacteria-laden sludge during stagnation (storage) in a full-scale DWTP using taxonomic cell counts, shotgun metagenomic sequencing, MC and physico-chemical parameter quantification.

5.2 Materials and Methods

5.2.1 Description of the Studied plant, Treatment process and Sampling

A DWTP located in Southeast of Montreal was monitored during July to November 2018. The influent (intake water) of the DWTP is taken from Missisquoi Bay (Lake Champlain). The

treatment chain includes powdered activated carbon (PAC) injection followed by conventional treatment (coagulation, flocculation, sedimentation, filtration) and post-chlorination. The characteristics and operational data of the studied DWTP are presented in (Table 5-1). The clarifier sludge is stored in a sludge holding tank (volume: 200 m^3). The solid phase of the holding tank is transferred to a wastewater treatment plant (WWTP) for treatment. The supernatant of the holding tank is discharged into Missisquoi Bay (source). Sludge storage time in the holding tank varies from 7 to 38 days (prior to transferring to the WWTP). More details are explained in Jalili et al. (2021). Samples were taken from the bottom of the sludge holding tank (solid phase) on July 27th and 31st, August 7th, 10th and 17th, September 5th, October 16th and November 1st 2018.

Table 5-1 Water characteristics of the studied DWTP in Missisquoi Bay during the sampling campaign from July to October 2018.

Treatment Step	Parameters	July	August	September	October	Specifications
Raw water (RW)	Turbidity (NTU)	2.1- 153.7	9.4- 153.1	11.6- 152.7	17.5- 152.9	-
	pH	6.1- 8.1	5.8- 8.3	6.2- 9.0	5.9- 8.8	-
Clarifier (CW)	Turbidity (NTU)	0.01-20.1	0.30- 20.0	0.33- 10.2	0.01- 10.3	PAC (wood-based): 1.5- 27.0 mg/L, Coagulant: PAXL, 49- 410 mg/L, Polymer: Hydrex (silicate), 0.05-0.1 mg/L Effective clarifier depth: 4.90 m, Max. sludge bed: 2.95 m, Hydraulic retention time: 1 h, Solid retention time: 48 h
	pH	6.1- 7.2	6.7- 7.2	6.2- 7.1	6.6- 7.2	
Dual sand-antrachite filter (FW)	Turbidity (NTU)	0.14- 0.4	0.16- 0.4	0.11- 0.6	0.17- 0.6	Retention time: 2 h
Treated water (TW)	Turbidity (NTU)	0.21- 0.60	0.25- 0.49	0.21- 0.43	0.23- 0.48	Injected chlorine: NaOCl, 1.3-6.0 mg/L
	pH	6.6- 8.1	6.9- 8.0	7.1- 8.6	7.1- 8.2	

5.2.2 Sludge Stagnation

Sludge stagnation was performed by storage of the sludge samples in the capped autoclaved polypropylene bottles stored in the dark and at room temperature (20 ±2 ^{o}C) for 7 to 38 days. Stagnation times were selected based on the applied sludge storage times in the studied DWTP.

5.2.3 Sample Preparation

Sub-samples were prepared for taxonomic cell counts, shotgun metagenomic sequencing, cell-bound (intracellular) microcystins (MCs), dissolved (extracellular) MCs, dissolved organic carbon (DOC) and solids analysis.

The sub-samples of the time zero points (before stagnation) were prepared on-site at the DWTP using a portable laboratory prepared by our group at Polytechnique Montreal. An autoclaved 1-L propylene bottle ware used to collect sludge samples and bring back to Polytechnique Montreal for stagnation (storage). The bottles containing sludge samples were stored in a dark place during transportation to Polytechnique Montreal.

A 40-mL vial was used for taxonomic cell counts. Lugol's iodine was added to the taxonomic cell count sub-samples for preservation (Jalili et al., 2021). A 10 mL of sludge was collected in the sterile Falcon tube for shotgun metagenomic sequencing analysis. For cell-bound and dissolved MCs, samples were filtered using pre-weighted 0.45-µm GHP membranes (Pall, Mississauga, ON). The filters were kept in the petri dish as cell-bound MCs and the filtrate was kept in 125-mL polyethylene terephthalate glycol (PETG) amber bottles (Thermo Fisher, Mississauga, ON) as dissolved MCs. DOC subsamples were prepared by filtration using pre-rinsed 0.45-µm membranes (PALL, Port Washington, NY, USA). The filtrate was collected and stored in 40-mL vials.

Shotgun metagenomic sub-samples were taken in triplicate. MC and DOC sub-samples were taken in duplicate. Shotgun metagenomic subsamples were kept at −80 °C prior to analysis. MC subsamples were stored at −25 °C prior to analysis. DOC sub-samples were stored at 4 °C. Taxonomic cell count samples were stored in the dark and at room temperature. Shotgun metagenomic samples were taken in triplicate while, MC, DOC, and solid samples were taken in duplicate. All details are explained in our previous work Jalili et al. (2021).

5.2.4 Sample Analysis

5.2.4.1 Taxonomic Cell Counts

Taxonomic cell count analysis was performed using an inverted microscope and a Sedgwick-Rafter chamber at magnifications of 10X and 40X. All details of the cell count analysis are explained in (Lund, 1959; Lund et al., 1958; Planas et al., 2000). Taxonomic cell counts in the sludge samples are described in our previous works (Jalili et al., 2021; Jalili et al., 2022b). Taxonomic cell counts are widely applied for evaluation of cyanobacteria in water and sludge samples (Almuhtaram et al., 2018; Jalili et al., 2021; Pestana et al., 2016; Water Research Foundation (WRF) and Water Research Australia, 2015; Zamyadi et al., 2013a; Zamyadi et al., 2013b; Zamyadi et al., 2012c). Measurement of chlorophyll-a (chla) and phycocyanin (PC) can provide more information about the cell viability in the sludge. However, measurement of these parameters in the sludge is subjected to interferences due to elevated solids/ turbidity using common approaches such as fluorometry techniques (Zamyadi et al., 2016a). Therefore, taxonomic cell counts were selected for the cell enumeration. Variability and reproducibility of taxonomic cell counts in our laboratory setting was investigated and the relative standard deviation

was shown to be 4% (Zamyadi et al., 2013a). Taxonomic cell counts were conducted by an experienced scientist considering cells in unicellular, aggregated, and filamentous forms.

5.2.4.2 DOC

DOC analysis was performed according to USEPA 415.1 method using total organic carbon analyzer (Sievers Analytical Instruments, Boulder, CO, USA) (United States Environmental Protection Agency (USEPA), 1974).

5.2.4.3 MCs

MC analysis was performed using on-line solid-phase extraction ultra-high-performance liquid chromatography coupled to tandem mass spectrometry (on-line SPE-UHPLC-MS/MS). In brief, potassium permanganate and sodium (meta) periodate (Sigma Aldrich, Oakville, ON, Canada) was applied for oxidation of the samples. After quenching the oxidized samples using a 4 M sodium bisulfite solution (Sigma Aldrich, Oakville, ON, Canada), the standard solutions of 4-phenylbutyric acid (50 ng/L) (Sigma Aldrich, Oakville, ON, Canada) and erythro-2-Methyl-3-methoxy-4-phenylbutyric acid (D3-MMPB, 10 ng/L) (Wako Pure Chemicals Industries, Ltd., Osaka, Japan) were added to the samples. 10 mL of the solution filtered on a 0.22-µm nylon filter (Sterlitech Corporation, Kent, WA, USA) was aliquoted for analysis by Thermo EQUAN™ interface (Thermo Fischer Scientific, Waltham, MA, USA). Then, the samples were loaded into the Thermo Hypersil Gold aQ C18 (on-line SPE) column (20 mm × 2.1 mm, 12 µm). An HTC Thermopal autosampler (CTC analytics, Zwingen, Switzerland) was applied to control the "in-loop" injection. Thermo Hypersil Gold C18 column (100 mm × 2.1 mm, 1.9 µm) was applied for toxin separation. Thermo TSQ QUANTIVA triple quadrupole mass spectrometer (Thermo Fischer

Scientific) followed by UHPLC was applied for MS/MS detection. Water, methanol, and acetonitrile for HPLC were provided by Fisher Scientific (Whitby, ON, Canada). Formic acid (> 95%), potassium carbonate, ammonium hydroxide (28– 30% NH_3), and ammonium acetate (≥ 99.0%) were purchased from Sigma Aldrich (Oakville, ON, Canada). More details are explained by Munoz et al. (2017) and Roy-Lachapelle et al. (2019).

5.2.4.4 DNA extraction, metagenomic sequencing, bioinformatics and statistical analysis

Microbial and cyanobacterial dynamics were analyzed by shotgun metagenomic sequencing at the phylum, order, and genus levels. The number of reads of taxonomic data was normalized using the relative abundance.

Total nucleic acid was extracted by RNeasy PowerSoil Total RNA Kit (Qiagen, Toronto, ON, Canada) and DNA was eluted by the PowerSoil DNA Elution Kit (Qiagen, Toronto, ON, Canada). Qubit V2.0 fluorometer (Life Technologies, Burlington, ON, Canada) was applied for quantification of DNA. Sequencing was performed on the metagenomic libraries (Roche 454 FLX instrumentation with Titanium chemistry) by McGill University and Genome Quebec Innovation Centre using NovaSeq 6000 S4 with paired end of 150 bp and an insert of 360 bp. DNA sequencing was performed on the Illumina NovaSeq 6000 platform using S4 flow cells. SolexaQA v3.1.7.1 program (default settings) was applied for the quality trimming of the raw reads (Cox et al., 2010). FragGeneScan-Plus v3.0 and Cd-hit v4.8.1 were applied for prediction of gene and protein fragments (90% similarity), respectively (Fu et al., 2012). A similarity search was performed on a representative of each cluster using M5nr database (https://github.com/MG-RAST/myM5NR) and the Diamond engine (Buchfink et al., 2015). The best hits from the SEED Subsystems, KEGG and

COG databases (Kanehisa et al., 2012; Overbeek et al., 2014; Tatusov et al., 2000) were used to determine the function fragments. Cyanobacterial functions were studied at Level 4 and the results were presented for following biomarkers: “Cyanobacterial circadian clock”, “Heterocyst formation in Cyanobacteria”, “Transcription factors cyanobacterial RpoD-Like sigma factors”, and “Pentose phosphate pathway”. More details are presented in Moradinejad et al. (2021a).

Overall, 96 sludge samples were analyzed in our sludge management project during 2017 and 2018. Due to the time and cost limitations, replication was not applied on whole genome in this investigation. However, reproducibility and variability between replicates was quantified by comparing results from triplicates of source water (Moradinejad, 2021b). Instead of replication, we focused on the importance of the survival/ growth of the cyanobacterial communities during sludge storage/ handling in batch experiments and relevant required engineering approaches.

Statistical analysis was done using R (3.6.2) and phyloseq (1.28.0) (McMurdie and Holmes, 2013). EasyCODA (0.31.1) was applied for normalization of the taxonomic data using centered log-ratio transformation (Graffelman, 2019). Vegan package (2.5–6) (https://CRAN.R-project.org/package=vegan) was applied for beta diversity analysis based on the Euclidean distance. For evaluation of the constrained variables, the redundancy analysis (RDA) was performed. Prior to implementation of the model, the variance homogeneity was validated (Blanchet et al., 2008). The permutation test was performed to select the significant (> 95%) variables.

All details can be found in our previous investigation (Jalili et al., 2021).

5.2.4.5 Solids

Total suspended solids (TSS) and total volatile solids (TVS) were measured according to the standard method 2540- Solids (American Public Health Association (APHA) et al., 2012).

5.3 Results and Discussion

5.3.1 Overview of Microbial/ Cyanobacterial Diversity, Sludge Characteristics and Microcystin Concentrations

Taxonomic cell counts in the sludge varied from 0.7 x 10^6 cells/mL (min. on July 27th 2018) to 5.6 x 10^6 cells/mL (Max. on August 10th 2018) (Table 5-2). A*phanothece clathrata brevis* (17- 77%), *Aphanocapsa delicatissima* (5- 60%), *Dolichospermum spiroides* (0- 59%) and *Microcystis aeruginosa* (0- 21%) were predominant in the sludge samples during sampling dates (Figure 5-1). Overall, taxonomic cell counts in the sludge progressively increased from July 27th (0.7 x 10^6 cells/mL) to August 10th (5.6 x 10^6 cells/mL) then, they progressively decreased to 1.2 x 10^6 cells/mL on November 1st as the end of the sampling campaign (Table 5-2). The highest total cell counts on August 10th corresponded with a high level of *Dolichospermum spiroides* (3.3 x 10^6 cells/mL), and low level of *Microcystis aeruginosa* (7.0 x 10^3 cells/mL) (Figure 1). Interestingly, the highest cell counts of *Microcystis aeruginosa* (5.8 x 10^6 cells/mL) detected on October 16th corresponded with a low level of *Dolichospermum spiroides* as 1.8 x 10^4 cells/mL. This trend was already documented in our previous work in this studied DWTP (Jalili et al., 2021).

Shotgun metagenomic sequencing revealed that Proteobacteria (35- 52%), Cyanobacteria (5- 39%), Actinobacteria (8- 26%) and Bacteroidetes (8- 14%) were the predominant phyla (Figure 5-2a) in the sludge and during the sampling campaign. Cyanobacteria reached its highest relative

abundance level on August 10th (Figure 5-2a). This is in accordance with taxonomic cell counts (Figure 5-1). Nostocales (order of *Dolichospermum*) was the most abundant order on July 31st (47%), August 10th (76%) and 17th (44%), whereas Chroococcales (order of *Microcystis*) was predominant on August 7th (57%), October 16th (61%) and November 1st (64%) (Figure 5-2b). Meanwhile, Oscillatoriales (< 14%), Stigonematales (< 7%), Prochlorales (< 6%), and Pleurocapsales (< 3%) were detected in a low relative abundance. At the genus level, *Synechococcus* (2- 37%), *Microcystis* (3- 36%), and *Dolichospermum* (2- 32%) were predominant in all dates (Figure 5-2c). In addition, *Cyanobium* (< 10%), *Nostoc* (< 10%), *Calothrix* (< 6%), *Cyanothece* (< 4%), *Fischerella* (< 4%) and *Prochlorococcus* (< 4%) were detected in a lower relative abundance. Previously, at the same studied DWTP, we highlighted the selective accumulation of cyanobacteria at the genus and species levels by both shotgun metagenomic sequencing and taxonomic cell counts following conventional processes (flocculation/ coagulation/ sedimentation) (Jalili et al., 2021). However, the impact of stagnation was not systematically assessed to investigate potential growth, lysis and selective survival of cyanobacterial cells.

Although turbidity (171- 920 NTU), TSS (716- 3394 mg/L), TVS (367- 1230 mg/L) and DOC (3.4- 9.8 mg/L) varied widely (Table 5-2), there was a significant association between pH and cyanobacterial community in the sludge ($p < 0.05$) (Figure 5-3). Values of pH varied from 6.74 (on November 1st) to 7.54 (on August 7th) (Table 5-2). In fact, on August 7th, pH of the incoming water, settled water and sludge were in the high range of pH values for the studied period, with 7.05- 7.94 in the raw water and 6.49- 6.99 in the clarified water. On that day, *Synechococcus* was

the most abundant genus (Figure 5-2c). The growth rate increase of *Synechococcus sp. WH7803* at pH values of 7- 8 in artificial sea water was reported by Traving et al. (2014).

Total MC concentrations remained below 239 ng/L from July 27th to August 17th (Table 5-2). Later in the season (September 5th and October 16th), total MCs increased markedly to 1083 and 7413 ng/L, respectively; most being cell-bound (88% on September 5th, and 96% on October 16th). This increase corresponds to elevated *Microcystis aeruginosa* cell counts on these two days (3.3 x 10^5 cells/mL to 5.8 x 10^5 cells/mL) (Figure 5-1). Total MCs decreased to 579 ng/L on November 1st in which 40% and 60% were cell-bound and dissolved, respectively (Table 5-2). Similarly, *Microcystis aeruginosa* cell counts remarkably deceased (3.8 x 10^4 cells/mL) on this date (Figure 5-1).

Table 5-2 Sludge characteristics throughout sampling campaign. -: shotgun metagenomic sample not taken, *: shotgun metagenomic sample taken.

Sampling date	Shotgun metagenomic sequencing	Taxonomic cell counts		MCs (ng/L)		DOC (mg/L)	pH	Turbidity (NTU)	TSS (mg/L)	TVS (mg/L)	Sludge storage time (d)
		Cells/mL x10^6	mm3/L	Cell-bound	Dissolved						
July 27th 2018	-	0.70	7.11	170.8	67.9	4.10	-	-	-	-	3
July 31st 2018	*	2.25	147.4	24.9	37.9	3.60	7.05	201	716	367	7
August 7th 2018	*	2.71	96.20	22.0	138.5	3.19	7.54	171	728	456	5
August 10th 2018	*	5.57	608.9	138.2	55.8	5.20	6.8	258	1022	409	8
August 17th 2018	*	2.35	138.3	41.6	46.6	3.35	7.12	327	1092	434	3
September 5th 2018	-	2.37	52.76	951.8	131.2	9.80	6.81	701	1957	1230	6
October 16th 2018	*	2.70	25.95	7129.0	284.2	3.46	6.87	2300	3394	1084	8
November 1st 2018	*	1.21	4.42	230.8	348.2	2.76	6.74	225	740	546	6

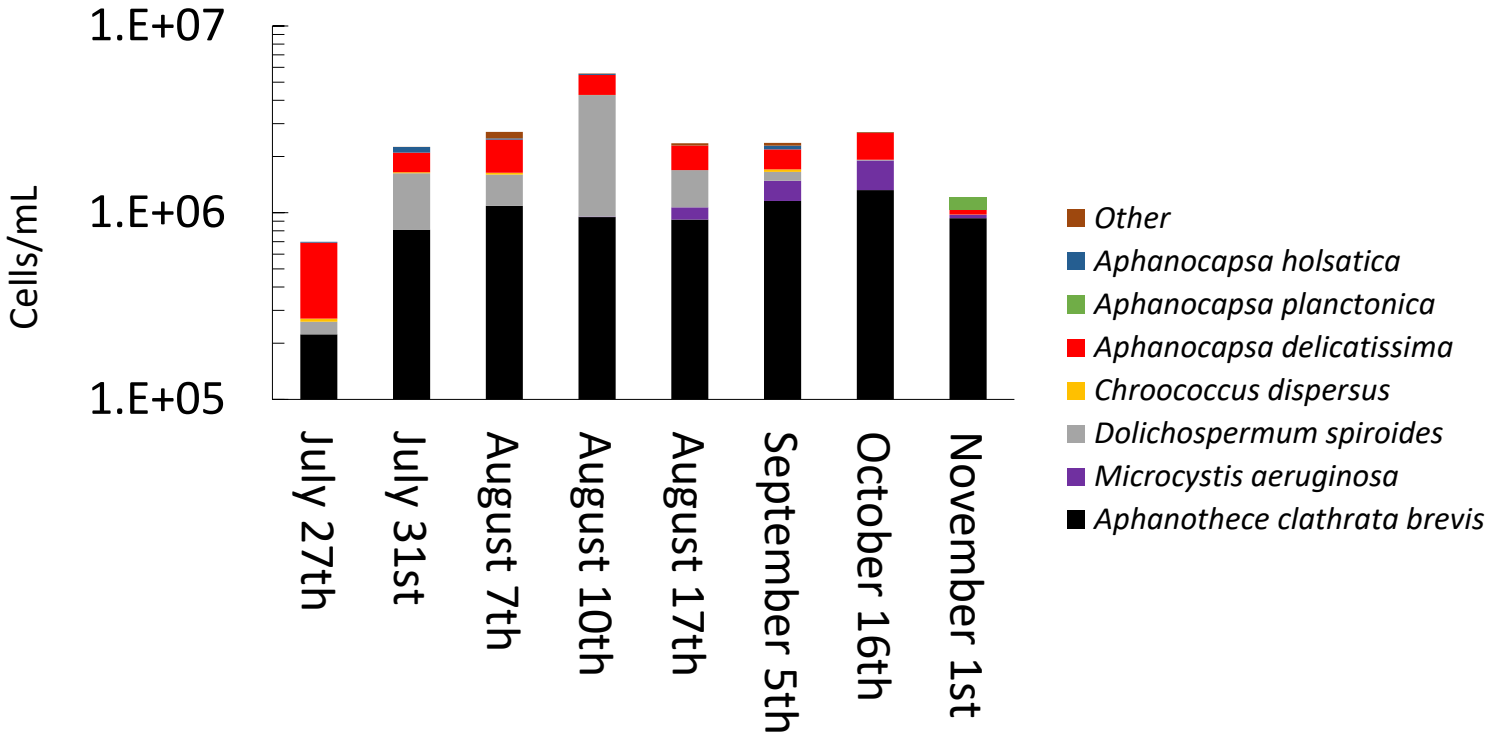

Figure 5-1 Cyanobacterial taxonomic cell counts in the sludge before stagnation. Other is less dominant species (< 5%) including *Aphanothece smithii*, *Microcystis wesenbergii*, *Dolichospermum circinale*, *Dolichospermum planctonicum*, *Merismopedia tenuissima*, *Merismopedia minima*, *Merismopedia punctata*, *Pseudanabaena limnetica*, *Coelosphaerium kuetzingianum*, *Aphanizomenon issatschenkoi*, *Aphanizomenon flos-aquae*.

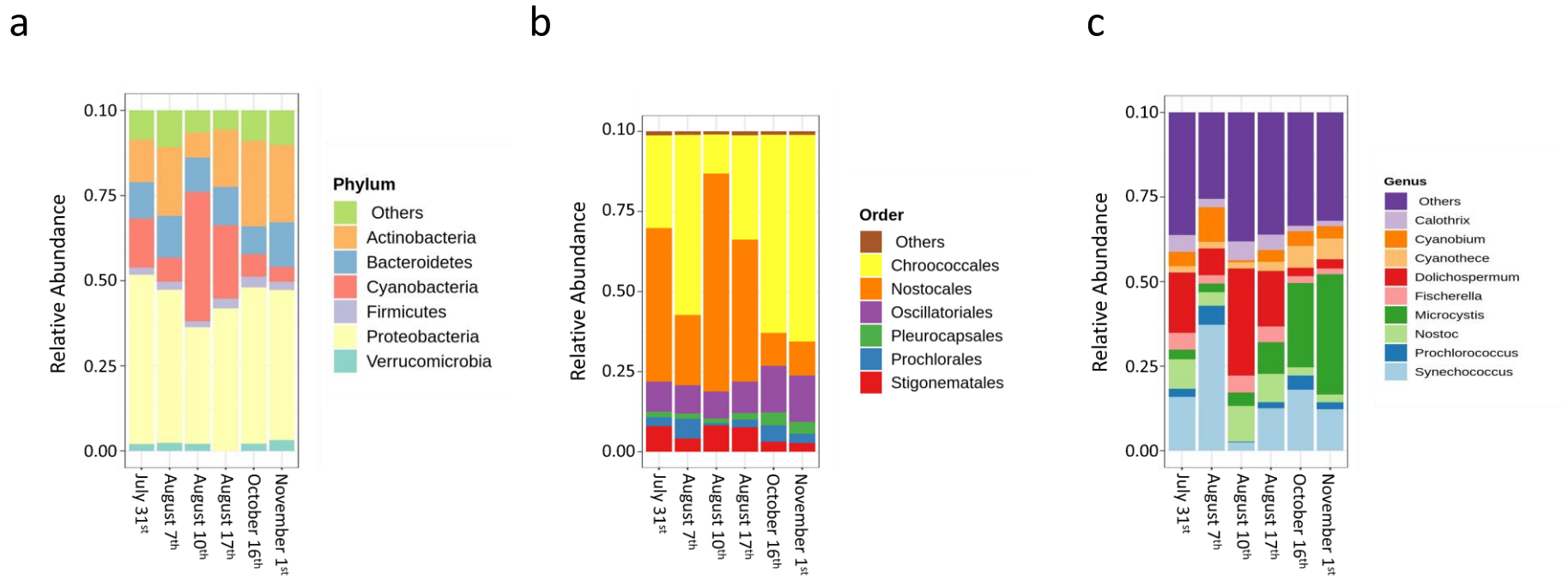

Figure 5-2 a) microbial communities at the phylum level, b) cyanobacterial communities at the order level, and c) cyanobacterial communities at the genus level in the sludge samples before stagnation.

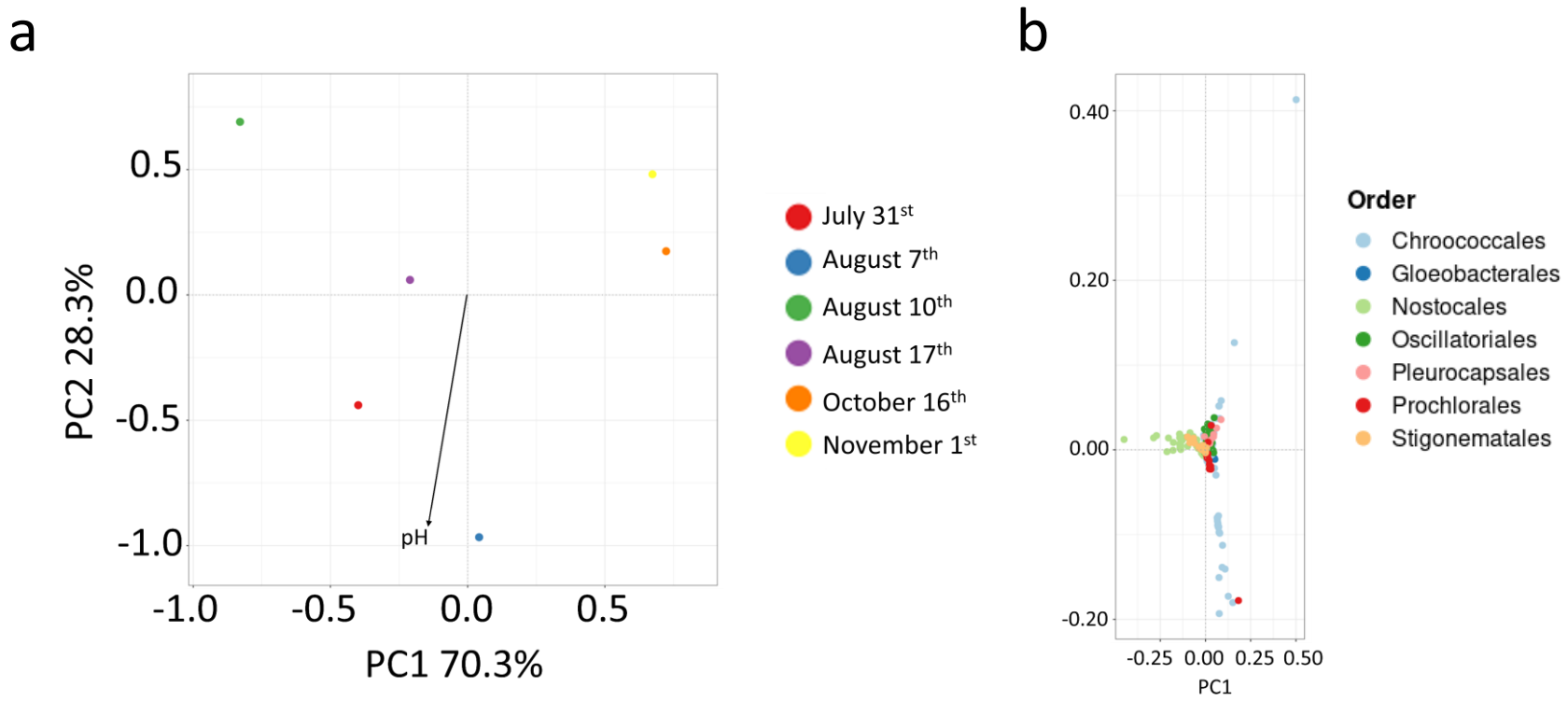

Figure 5-3 a) Impact of physico-chemical parameters on cyanobacterial communities at the order level. PC1: 70.3%, PC2: 28.3%. Only the significant parameter (pH) was shown (p <0.05), b) Cyanobacterial species grouped at the order level.

5.3.2 Fate of Cyanobacterial Cells in the Sludge during Stagnation

Figure 5-4 and Figure B- 1 show the dynamics of taxonomic cell counts of sludge stored in the dark for up to 38 days. Overall, total cell counts increased for 4 out of 8 sampling dates on July 31st, August 10th, September 5th and October 16th, although the dynamics showed inconsistent yet interesting trends. Taxonomic cell counts during stagnation do not follow a consistent trend neither by species nor by duration of stagnation. Considering the fate of cyanobacterial species during stagnation, results strongly suggest that several species grew during sludge stagnation on July 27th and 31st, August 10th and 17th, September 5th, October 16th and November 1st. This was observed for several species including *Microcystis aeruginosa, Aphanothece clathrata brevis, Aphanocapsa delicatissima* and *Aphanocapsa holsatica, Chroococcus dispersus* and *Dolichospermum spiroides.*

The highest cell count growth was observed on October 16th. For this sample, total cell counts clearly increased from 2.7 x 10^6 cells/mL (before stagnation) to 3.1 x 10^6 cells/mL (after 8 days of stagnation), 5.3 x 10^6 cells/mL (after 16 days of stagnation) and 4.3 x 10^6 cells/mL (after 30 days of stagnation). *Aphanocapsa holsatica* (4200%), *Dolichospermum spiroides* (582%), *Aphanocapsa delicatissima* (164%), *Microcystis aeruginosa* (134%) and *Aphanothece clathrata brevis* (35%) had the highest contribution to this trend (Figure 5-4). Observations of total cell counts on July 31st, August 10th, September 5th and October 16th provide clear evidence of growth during stagnation of the sludge in the dark for the first time. Concerns about cyanobacterial growth during stagnation have been recently raised by several authors; but has not been demonstrated (Dreyfus et al., 2016; Pestana et al., 2016; Water Research Foundation (WRF) and Water Research Australia, 2015). The fate of cyanobacterial cells in the sludge is complex and various

environmental conditions (e.g. presence of nutrients) can contribute to their dynamics (Dreyfus et al., 2016; Jalili et al., 2021; Pestana et al., 2016). In our previous study on the same DWTP, we hypothesized that sludge storage time is an important parameter affecting cyanobacterial diversity and dynamics in the sludge (Jalili et al., 2021). These findings point to the importance of sludge storage in terms of cyanobacterial growth potential.

Considering cell counts at the order level, cell survival, and growth were mostly observed within Synechococcales (order of *Aphanocapsa*) and Chroococcales (order of *Microcystis*) (Figure 5-5a). In contrast, Nostocales (order of *Dolichospermum*) most often markedly declined during stagnation, with some exceptions on July 31st (day 9 to 16), August 10th (day 9 to 17) and October 16th (before stagnation to day 9). Shotgun metagenomic sequencing showed that the relative abundance of Nostocales decreased in all stagnated samples as compared to the time zero point (before stagnation) (Figure 5-5b). The sole exception was October 16th where, the relative abundance of Nostocales increased slightly from the time zero point to day 8. The relative abundance of Chroococcales and Synechococcales remained either constant or increased in most of the dates except July 27th and August 17th (for Synechococcales) and September 5th (for Chroococcales). The only persistent trend is observed for Nostocales: When abundant, a clear decrease in the relative abundance was observed in almost all samples.

Overall, the variable trends are confirmed by both shotgun metagenomic sequencing and taxonomic cell counts at the order level revealing the persistence of Synechococcales and Chroococcales as well as the sensitivity of Nostocales during stagnation (Figure 5-5b). Since the

persistent genera are mostly MC producer (Jakubowska and Szeląg-Wasielewska, 2015; Mariani et al., 2015), these findings emphasize the necessity of cyanobacteria-laden sludge management.

Non-concordance of taxonomic cell counts and shotgun metagenomic sequencing results are observed at the genus level (Figure 5-4, Figure B- 2). For instance, *Aphanocapsa* including *delicatissima*, *holsatica*, and *planctonica* were detected by taxonomic cell counts, while they were not detected by shotgun metagenomic sequencing. Additionally, *Synechococcus* was detected by shotgun metagenomics, while it was not counted by taxonomic cell counts (Figure 5-4, Figure B-2). This non-concordance was reported in the previous investigations at the genus and species levels (American Water Works Association (AWWA), 2010; Jalili et al., 2021; Moradinejad et al., 2020). This can be due to either taxonomic cell count drawbacks such as misidentification of the morphologically similar species (American Water Works Association (AWWA), 2010; Hawkins et al., 2005; Jalili et al., 2021; Park et al., 2018) or, shotgun metagenomic challenges including DNA extraction, sequencing and library limitation (Bag et al., 2016; Gevers et al., 2012; Kuczynski et al., 2011; Teeling and Glockner, 2012). The observed concordance in diversity trends and shifts at the order level caused by stagnation are especially noteworthy. By combining taxonomic cell counts and shotgun metagenomic sequencing results, it is possible to conclude on abundance and cell counts, not only on relative shifts in diversity.

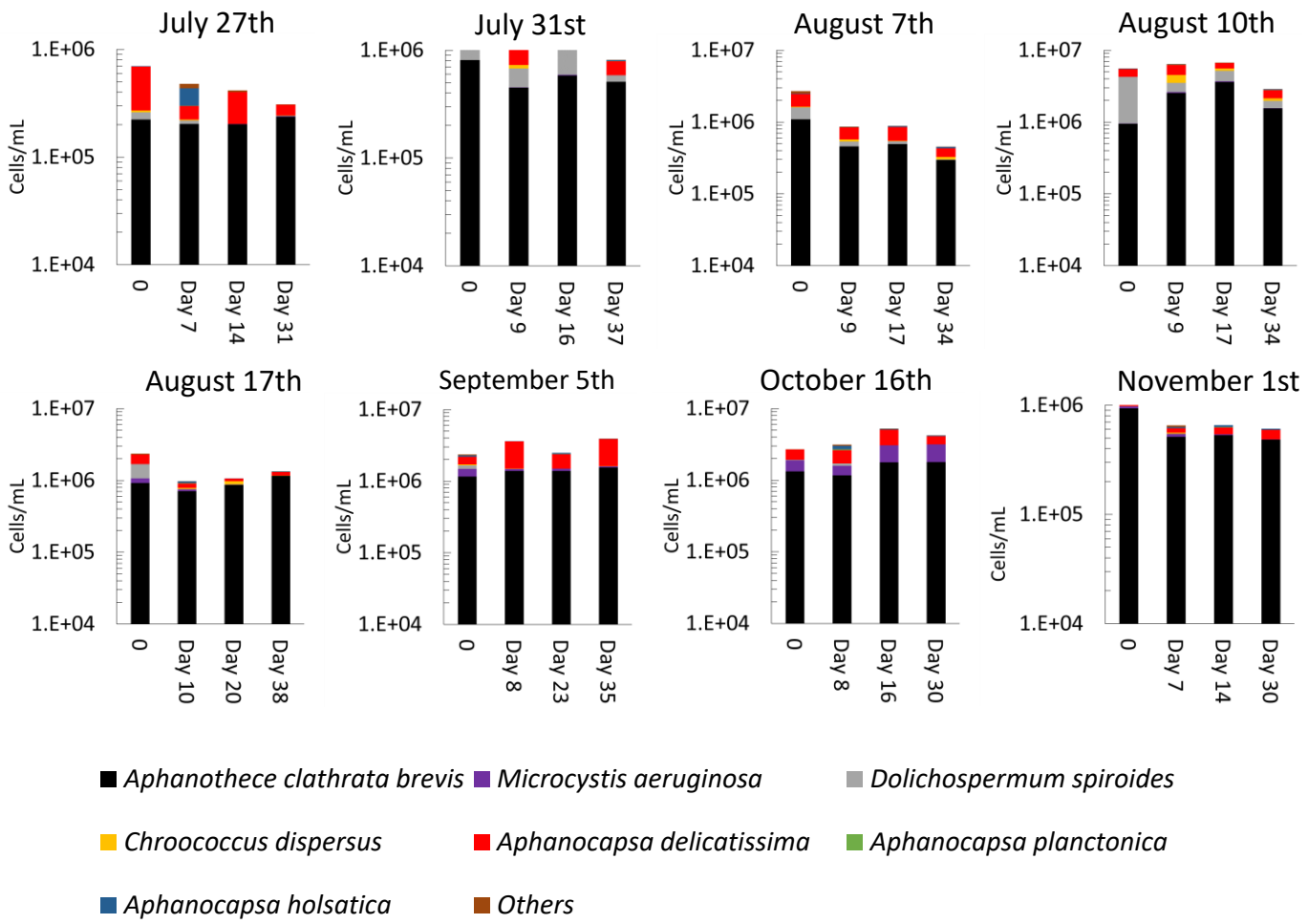

Figure 5-4 Taxonomic cell counts after sludge stagnation, 0: before stagnation. Other: see Figure 5-1.

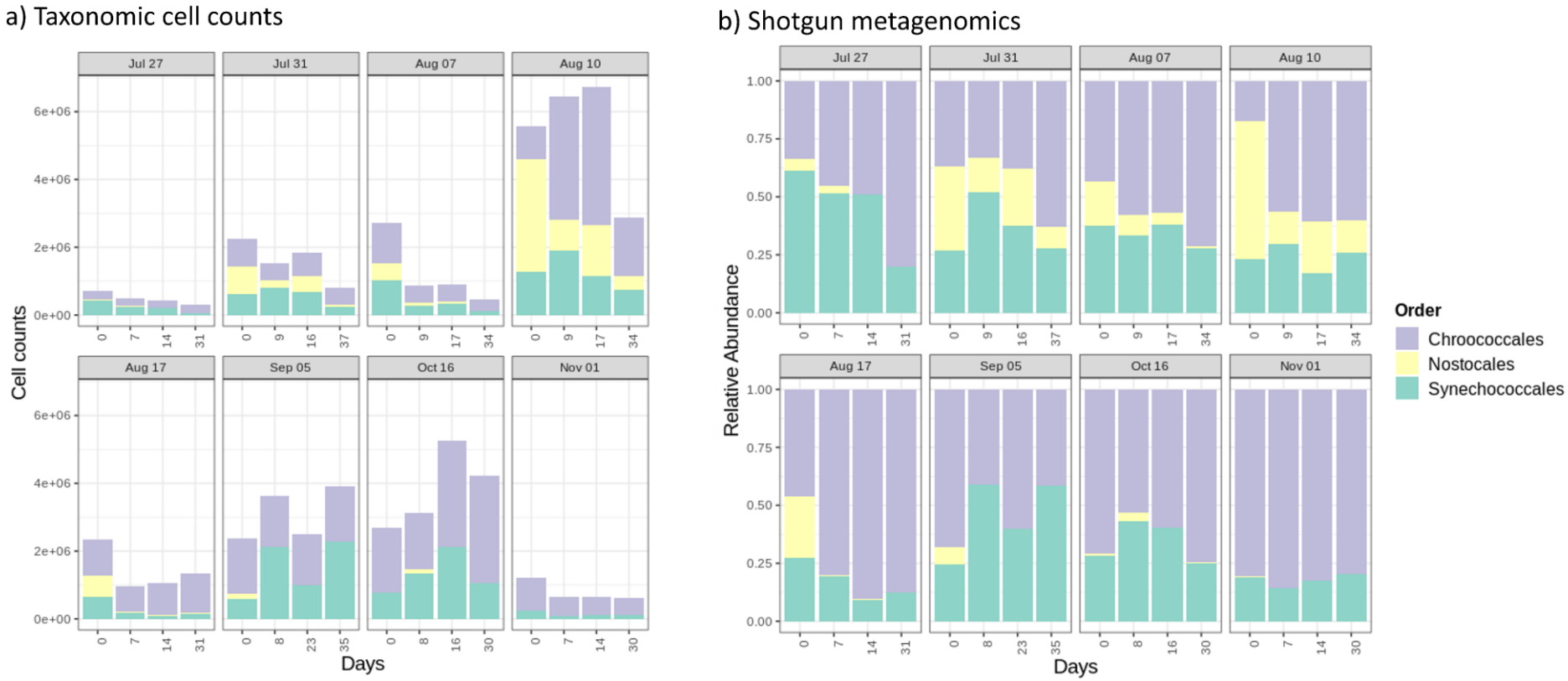

Figure 5-5 a) Taxonomic cell counts, b) Cyanobacterial community using shotgun metagenomic sequencing; before and after sludge stagnation at the order level, 0: before stagnation. Only the predominant orders as Chroococcales, Synechococcales and Nostocales are shown.

5.3.3 Fate of Cyanobacterial Functions during Sludge Stagnation

In order to track the functional cyanobacterial footsteps in response to stagnation, four cyanobacterial biomarkers (level 4 subsystems) were selected: “Cyanobacterial circadian clock”, “Heterocyst formation in Cyanobacteria”, “Transcription factors cyanobacterial *RpoD*-Like sigma factors”, and “Pentose phosphate pathway-*OpcA*” (Cohen and Golden, 2015; Imamura and Asayama, 2009; Kumar et al., 2010). The relative abundance of the selected biomarkers related to “Cyanobacterial circadian clock” and “*RpoD*-like sigma factors” remarkably increased during stagnation on October 16th (Figure 5-6). Accordingly, they persisted on certain dates. This could be related to cell survival or growth during stagnation. “Heterocyst formation” is a cyanobacterial biomarker related to filamentous genera with heterocysts such as *Dolichospermum* and *Nostoc* (representatives of Nostocales) (Herrero et al., 2016; Kumar et al., 2010). The relative abundance of this biomarker decreased during sludge stagnation. The exception was on the October 16th sample where its relative abundance increased slightly, coinciding with the increase of *Dolichospermum* cell counts during stagnation (Figure 5-4). These findings are in line with our hypothesis about the vulnerability of Nostocales (*Dolichospermum*) and resistance of Chroococcales (*Microcystis*) and Synechococcales (*Aphanocapsa*) during sludge stagnation. In fact, *Microcystis* and *Aphanocapsa* have the ability to form a glycoprotein S-layer (Rachel et al., 1997), protecting the cells against ecological stresses (Callieri, 2017; Sleytr et al., 1999; Šmarda et al., 2002). Interestingly, the “Pentose phosphate pathway (*OpcA*)” marker gene, which is specific to dark heterotrophic growth (Min and Golden, 2000), increases during stagnation suggesting growth of cyanobacteria in the sludge holding tank (a dark place).

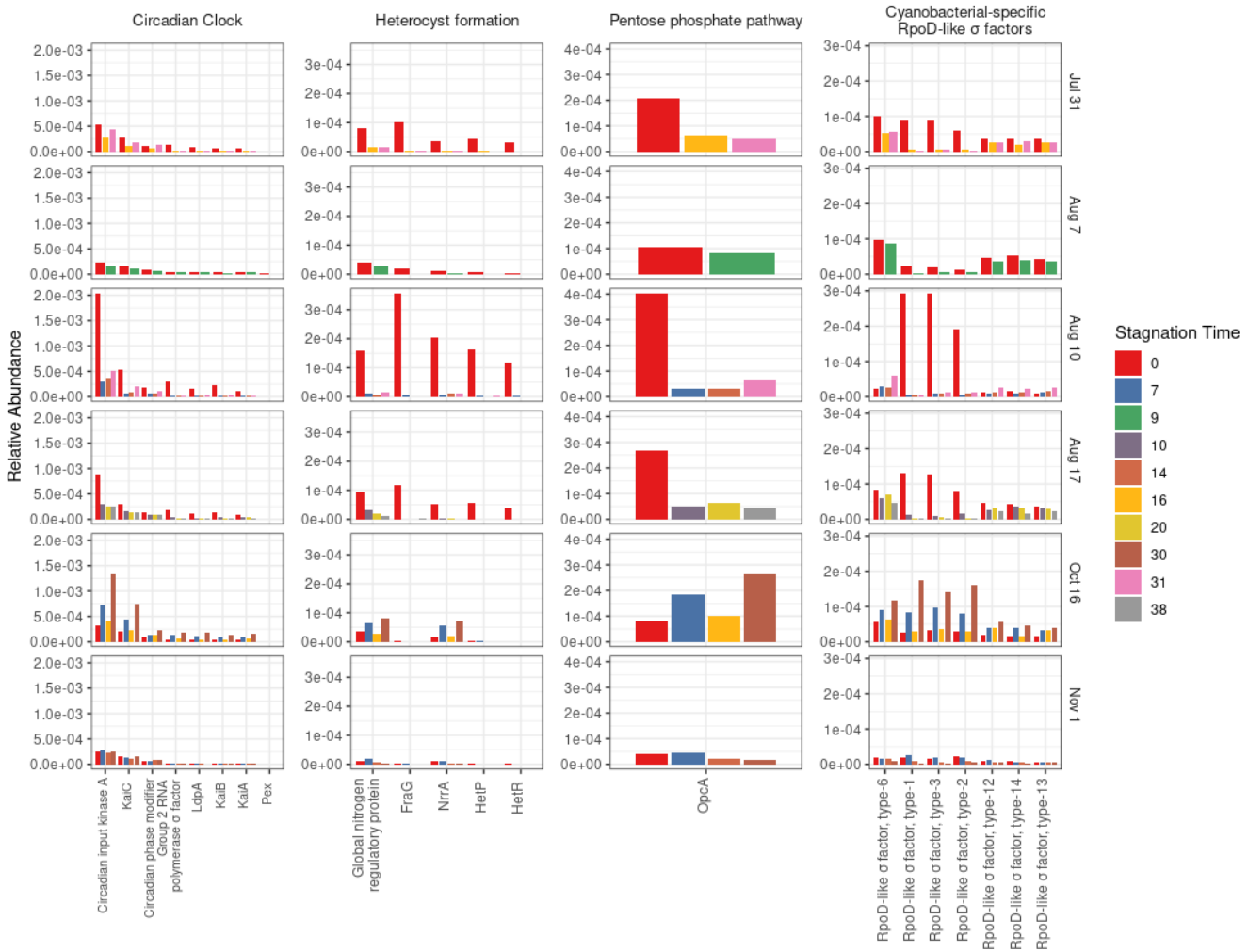

Figure 5-6 Relative abundance of selected cyanobacterial biomarker subsystems (level 4), before, and after stagnation.

5.3.4 Fate of MCs during Sludge Storage

Although MC-producer genera such as *Microcystis*, *Aphanocapsa*, *Chroococcus*, and *Dolichospermum* grew during stagnation, cell-bound MCs generally decreased during the stagnation time (Figure 5-7).

The dynamics of cell-bound and dissolved MCs are influenced by: i) activation of the *mcy* genes in existing and newly grown cyanobacterial cells, and ii) the rate of release and subsequent biodegradation of released cell-bound MCs. In our previous study, we showed that sludge oxidation by potassium permanganate can play a role as oxidative stress and cause an increase in *mcyD* gene copy numbers in the oxidized sludge during storage (Jalili et al., 2022b). Biodegradation of MCs has been shown for species representatives of Proteobacteria, Actinobacteria and Firmicutes (Kormas and Lymperopoulou, 2013; Maghsoudi et al., 2016). Moreover, some loss of MCs could be attributed to adsorption of dissolved MC onto the PAC injected into the intake water (Jalili et al., 2021). Since cell damage, cyanotoxin release and cyanotoxin degradation occur simultaneously in the stored sludge, the prediction of the cyanotoxin concentration in the sludge remains complex (Jalili et al., 2021; Water Research Foundation (WRF) and Water Research Australia, 2015).

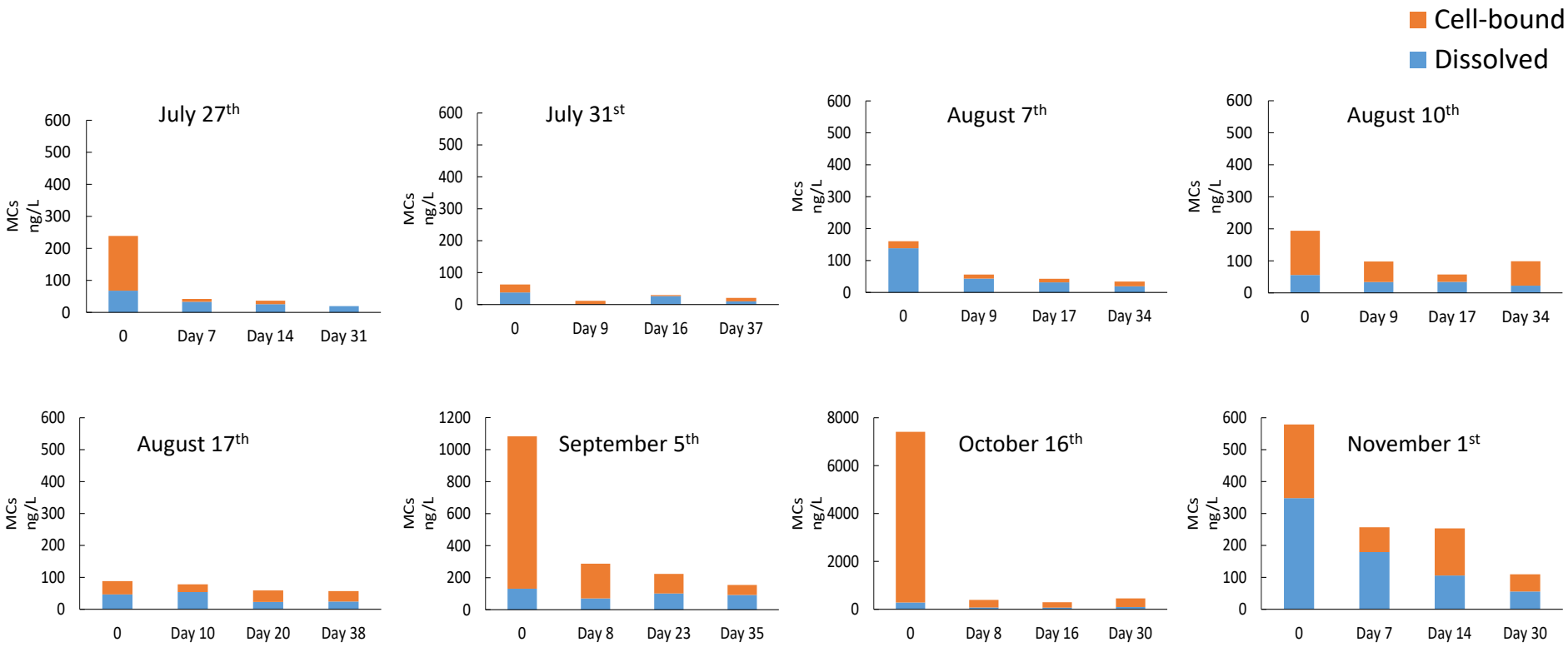

Figure 5-7 MC concentrations during sludge stagnation. 0: Before stagnation.

5.3.5 Cyanobacteria-laden Sludge Management

While DWTP's sludge is collected in a holding tank or lagoon to be disposed of, its supernatant can be discharged into the source water, in a sewer or be recycled to the head of the DWTP (Zamyadi et al., 2016b; Zamyadi et al., 2019). The presence of cyanobacteria and cyanotoxins in the recycle water can negatively affect the intake and treated water (Zamyadi et al., 2019). In this investigation, we showed that sludge storage can increase risks associated with survival/ proliferation of cyanotoxin producer species. This raise concerns in terms of the environmental and health risks associated with i) supernatant handling (discharge or recycling), and ii) residual (solids) disposal during periods of cyanobacterial blooms. Adjusting management strategies may include decreasing cyanobacterial loads in the sludge by optimizing the water treatment chain mainly through the addition of i) pre-oxidation, ii) supplementary treatment (e.g. separation and oxidation) to the recycling stream, iii) sludge treatment (e.g. sludge oxidation as well as PAC injection), and iv) restriction of land application of cyanobacteria-laden residuals.

5.4 Conclusions

- Cyanobacterial growth, survival, and decay were quantified during sludge stagnation in the dark under controlled conditions. Longitudinal monitoring in summer and fall 2018 was conducted on sludge from a DWTP sludge holding tank. For 4 out of 8 sampling dates, cyanobacterial cell growth was observed by total taxonomic cell counts during extended stagnation in the dark ranging from 7 to 38 days. The highest observed cell growth was 96% after 16 days of stagnation. Growth of cells was dominated by potential MC producers such as *Microcystis*, *Aphanocapsa*, *Chroococcus*, and *Dolichospermum*. Overall, up to 4200%, 1500%, 582% and 134% cell growth was observed in potential MC-producer genera such as *Aphanocapsa*, *Chroococcus*, *Dolichospermum*, and *Microcystis*, respectively, during stagnation. Additionally, up to 35% cell growth was observed in non-

toxic *Aphanothece*. Shotgun metagenomic sequencing revealed that sludge stagnation affected cyanobacterial diversity. Chroococcales (*Microcystis*) and Synechococcales (*Synechococcus*) were the most persistent orders, whereas Nostocales (*Dolichospermum*) was less resistant.

- Sludge characteristics including cyanobacterial cell counts and MCs dynamically changed in the sludge. Amongst studied physico-chemical parameters, only pH showed a significant correlation ($p < 0.05$) with the cyanobacterial community in sludge.
- Cyanobacterial biomarkers (level 4 subsystems) related to "Circadian clock", "*RpoD*-like sigma" and "Pentose phosphate pathway" increased during stagnation, confirming cyanobacterial growth even in the dark. In contrast, the relative abundance of the "Heterocyst formation" biomarker related to filamentous genera declined in most of the stagnated samples.
- Taxonomic cell counts, shotgun metagenomic sequencing, and cyanotoxin quantification provided consistent and complementary evidence regarding the quantification and dynamics of cyanobacteria in the stored sludge. This comprehensive investigation provides a sound basis to draft best cyanobacteria-laden sludge management practices. Under the conditions tested, persistence and/or growth of potential MC producers during storage raise the need to monitor cell counts and cyanotoxins in the sludge and its supernatant.
- Managing cyanobacteria-laden sludge is a challenge as the presence of cyanobacterial toxins raises limitations for their safe disposal. Furthermore, cyanotoxin-laden sludge represents a risk to raw and potentially treated water quality if the sludge supernatant is recycled to the head of the DWTP or is discharged into the source.

Author Contributions

Conceptualization, F.J., H.T., S.D., and M.P.; Methodology, F.J., H.T., J.F.G.M., N.F., B.J.S., Y.T., A.Z., S.D., S.S., and M.P.; Software, F.J., J.F.G.M., and Y.T.; Validation, F.J., H.T., J.F.G.M., Y.T., S.D., and M.P.; Formal analysis, F.J., H.T., J.F.G.M., and Y.T.; Investigation, F.J., H.T., J.F.G.M., S.D., A.Z., and M.P.; Resources: F.J., and H.T.; Data curation, F.J. and J.F.G.M.; Writing—original draft preparation, F.J., H.T., and J.F.G.M.; Writing—review and editing, F.J., H.T., N.F., J.F.G.M., S.S., S.D., and M.P.; Visualization, F.J., H.T., J.F.G.M., S.D., and M.P.; Supervision, A.Z., S.D., and M.P.; Project administration, A.Z., S.D., B.J.S., S.S., and M.P.; Funding acquisition, S.D., S.S., B.J.S., and M.P. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement

Data is contained within the article and supplementary material.

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Conflicts of Interest

The authors declare no conflict of interest.

CHAPTER 6 ARTICLE 3: OXIDATION TO CONTROL CYANOBACTERIA AND CYANOTOXINS IN DRINKING WATER TREATMENT PLANTS: CHALLENGES AT THE LABORATORY AND FULL-SCALE PLANTS

In this chapter, we discussed about the impact of oxidation on mitigation of cyanobacteria and cyanotoxin challenges in DWTP's sludge. According to our results presented in Chapter 4Chapter 5, accumulated cyanobacteria and cyanotoxins in the DWTP's sludge can raise technical and health concerns. Therefore, a treatment approach is necessary. Oxidation can be an available option in DWTPs. While, oxidation is widely applied to control cyanobacteria and cyanotoxins in source and intake waters, there is knowledge gap in cyanobacteria-laden sludge treatment. Results of this chapter will be used for development of a strategy for management and handling of sludge containing cyanobacteria and cyanotoxins. This chapter was published as a research article in the Water Journal on February 2022. Supplementary data is presented in Appendix C.

OXIDATION TO CONTROL CYANOBACTERIA AND CYANOTOXINS IN DRINKING WATER TREATMENT PLANTS: CHALLENGES AT THE LABORATORY AND FULL-SCALE PLANTS

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Abstract

The impact of oxidation on mitigation of cyanobacteria and cyanotoxins in drinking water treatment sludge was investigated at the laboratory and treatment plant scales. Two common oxidants, $KMnO_4$ (5 and 10 mg/L) and H_2O_2 (10 and 20 mg/L) were applied under controlled steady state conditions. Non-oxidized and oxidized sludge was left to stagnate in the dark for 7 to 38 days. Controlled laboratory trials show that $KMnO_4$ and H_2O_2 decreased cell counts up to 62% and 77%, respectively. The maximum total MC level reduction achieved after oxidation was 41% and 98% using 20 mg/L H_2O_2 and 10 mg/L $KMnO_4$, respectively. Stagnation caused cell growth up to 2.6 fold in 8 out of 22 oxidized samples. Microcystin (MC) producer orders as Chroococcales and Synechococcales were persistent while Nostocales was sensitive to combined oxidation and stagnation stresses. In parallel, two on-site shock oxidation treatments were performed in the DWTP's sludge holding tank using 10 mg/L $KMnO_4$. On-site shock oxidation decreased taxonomic cell counts by up to 43% within 24 h. Stagnation preceded by on-site shock oxidation could increase total cell counts by up to 55% as compared to oxidation alone. The increase of cell counts and *mcyD* gene copy numbers during stagnation revealed the impact of oxidation/ stagnation on cyanobacterial cell growth. These findings show the limitations of sludge oxidation as a strategy to manage cyanobacteria and cyanotoxins in sludge and suggest that alternative approaches to prevent the accumulation and mitigation of cyanobacteria in sludge should be considered.

Keywords

Cyanobacteria; Shotgun metagenomic sequencing; Sludge; Drinking water treatment plant; Oxidation; Storage; Stagnation; Microcystins

6.1 Introduction

Health concerns about the occurrence of cyanobacterial cells and their associated metabolites (cyanotoxins, taste and odor agents) in water sources have been raised in the past decade

(Chorus et al., 2021; Ho and Michalak, 2019; Ho et al., 2019; Kimambo et al., 2019; Pick, 2016; Winter et al., 2011).

Conventional treatment processes including coagulation, flocculation, sedimentation, and filtration are common approaches to respond to the presence of cyanobacteria and cyanotoxins in drinking water treatment plants (DWTPs) (Chorus and Welker, 2021; Drikas et al., 2001; Jalili et al., 2021; Newcombe et al., 2010; Zamyadi et al., 2013a). In spite of high efficiency of these processes to remove cyanobacterial cells and cell-bound metabolites (up to 99%), the accumulation of cells and metabolites in the sludge and backwash water is considered a challenge (Almuhtaram et al., 2018; Drikas et al., 2001; Shang et al., 2018; Sun et al., 2012; Teixeira and Rosa, 2007; Zamyadi et al., 2013b; Zamyadi et al., 2012c). In certain DWTPs, the supernatant of the sludge is discharged to the water source or is recycled to the head of the DWTPs (Jalili et al., 2021; Zamyadi et al., 2016b; Zamyadi et al., 2019). The quality of stored sludge impacts the load of cyanobacteria and toxins in the sludge supernatant (Jalili et al., 2021).

Several studies have reported that toxic cyanobacterial cells (eg. *Microcystis aeruginosa, Dolichospermum circinale, Oscillatoria sp*., and *Cylindrospermopsis raciborskii)* can survive and release cyanotoxins in the stored sludge for up to 12 days (Drikas et al., 2001; Ho et al., 2012; Li et al., 2018; Sun et al., 2013; Sun et al., 2012; Sun et al., 2018; Xu et al., 2016). Recent studies hypothesized toxic cyanobacterial cells can even grow in the stored sludge up to 16 days leading to cyanotoxin release up to 4 times higher than the expected concentration (Water Research Foundation (WRF) and Water Research Australia, 2015). Recent evidence documenting the growth of toxin producing species during controlled extended storage of sludge raises the need for additional treatment controls to be implemented to limit this risk (*this research*).

$KMnO_4$ and H_2O_2 have been successfully applied to manage cyanobacteria and cyanotoxins in DWTPs and source waters (Fan et al., 2013a; Fan et al., 2013b; Lusty and Gobler, 2020; Matthijs et al., 2012; Moradinejad et al., 2020; Piezer et al., 2020b). However, there are limited data on the efficacy of oxidants and adsorbents such as powdered activated carbon (PAC)

applied directly to DWTP sludge. Zamyadi et al. (2016b) showed that online continuous application of 10 mg/L $KMnO_4$ decreased cyanobacterial cell counts by up to 97% within 72 h in the sludge thickener. Using 20 mg/L powdered activated carbon (PAC) also decreased 21% of the 2-Methylisoborneol (MIB) concentration. The fate of cyanobacteria and cyanotoxins in oxidized sludge needs to be studied during subsequent storage in lagoons or sludge holding tanks before further processing or disposal (Jalili et al., 2021; Pestana et al., 2016).

The objectives of this study were to i) investigate the impact of oxidation on cyanobacteria and cyanotoxins in both lab- and full- scales, ii) determine the efficiency of oxidation during sludge storage and, iii) evaluate the impact of oxidation and stagnation on microbial and cyanobacterial communities. To the authors' knowledge, this is the first study on oxidation of DWTP's sludge containing natural cyanobacterial blooms followed by storage (stagnation) in a full-scale DWTP using taxonomic cell counts, shotgun metagenomic sequencing, MC and *mcyD* gene copy quantification.

6.2 Materials and Methods

6.2.1 Studied Drinking Water Treatment Plant and Sampling Description

The studied DWTP was located Southeast of Montreal on the Canadian side of Missisquoi Bay (Lake Champlain). The occurrence of cyanobacterial blooms has been reported in this DWTP in previous studies (Jalili et al., 2021; Zamyadi et al., 2013a; Zamyadi et al., 2012c), and detailed information on the diversity of the blooms and water quality at this site is presented in (Jalili et al., 2021).

The treatment chain comprised of powdered activated carbon (wood-based), coagulation, flocculation, sedimentation and filtration followed by post-chlorination. Sludge of the clarifier is collected in a sludge holding tank (200 m^3). The supernatant of the holding tank is discharged to the source and the solids are transferred to a wastewater treatment plant (WWTP) for treatment. Sludge storage time in the holding tank varied from 7 to 35 days. All details are presented in our previous study (Jalili et al., 2021). The samples for laboratory oxidation were

taken from the solids of the sludge holding tank on July 31st, August 7th and 17th. On-site oxidation was performed on sludge holding tank on August 17th and September 5th.

6.2.2 Oxidation and Stagnation Procedures

6.2.2.1 Oxidation

The experimental plan for the oxidation assays is presented in Table 6-1. The maximum dose of $KMnO_4$ (10 mg/L) was selected based on a previous study on the sludge dewatering tank of a DWTP (Zamyadi et al., 2016b). However, there was a need for data about sludge oxidation. Therefore, the decisions were based on the previous studies of oxidation of water samples containing cyanobacteria and cyanotoxins (Fan et al., 2014b; Moradinejad et al., 2020).

For the laboratory oxidation by $KMnO_4$, powdered $KMnO_4$ (> 99.9%, Sigma Aldrich, Oakville, ON, Canada) was dissolved in ultrapure water to prepare a 5000 mg/L stock solution. This stock solution was used to prepare the applied doses (5 and 10 mg/L $KMnO_4$). For H_2O_2, a 3,000 mg/L stock solution was prepared using stabilized H_2O_2 (30%, Sigma Aldrich, St. Louis, MO, USA). The applied H_2O_2 doses were 10 and 20 mg/L. All laboratory oxidation scenarios were performed at room temperature (20 ±2 ^oC). The contact time of $KMnO_4$ and H_2O_2 oxidation was 1 and 24 h, respectively. The volume of the sludge in the laboratory oxidation assays was 2 L.

On-site (full- scale) treatment was conducted as a shock oxidation. Powdered $KMnO_4$ (> 97.5%, Carus, LaSalle, IL, USA) was added into the sludge holding tank. Then, sludge was mixed manually to ensure that $KMnO_4$ was completely dissolved. During on-site oxidation, the clarifier's sludge was extracted into the sludge holding tank around four times per hour. The effective volume of the sludge holding tank was 200 m^3. The holding tank was full during on-site oxidation and the supernatant surplus was returned to the lake. After the shock oxidation, samples were taken daily from the sludge and corresponding supernatant.

Concentrations of $KMnO_4$ stock solution and residual were determined by the DPD-colorimetric (4500-Cl) standard method using DR5000 (HACH, Canada) spectrophotometer

and applying the stoichiometric ratio to convert the chlorine concentration to the $KMnO_4$ concentration (American Public Health Association (APHA) et al., 2012). The H_2O_2 residual was measured by Chemetrics K-5510 colorimetric test kit (Midland, VA, USA). At the end of the contact times, $KMnO_4$ and H_2O_2 residuals were quenched using sodium thiosulfate (Fisher Scientific, Whitby, ON, Canada) at stoichiometric ratio (American Public Health Association (APHA) et al., 2012; Fan et al., 2014a).

Oxidant exposure (CT) as residual concentration (mg/L) multiplied by contact time (min) was calculated for each oxidation assay according to the Equation 1 (Moradinejad et al., 2020).

$$CT = \int_0^t Oxidant\ dt = \frac{C_0}{k}\ (e^{kt} - 1) \qquad (1)$$

Where k (min^{-1}): is the first-order decay rate, t (min) is the exposure time (contact time) and C_0 is the initial concentration of the oxidant (mg/L).

For statistical analysis, CT values were normalized and reported as relative CT by dividing CT of each oxidation assay by the maximum observed CT of each oxidant (i.e. $KMnO_4$: 239 mg.min/L and H_2O_2: 7781 mg.min/L). Thus, the relative CT of both 10 mg/L $KMnO_4$ and 20 mg/L H_2O_2 oxidation on the August 7th sample were 1.0 (Table 6-2).

Table 6-1 Experimental plant of oxidation assays. DOC: dissolved organic carbon.

Date	Oxidant	Initial applied dose (mg/L)	Contact time (h)	Oxidant/ DOC ratio
July 31st	$KMnO_4$	5	1: Laboratory assay	1.4
		10		2.8
August 7th		5		1.6
		10		3.1
	H_2O_2	10	24: Laboratory assay	3.1
		20		6.3
August 17th	$KMnO_4$	5	1: laboratory assay	1.5
		10	24, 72 : On-site assay (10 mg/L)	3.1
September 5th	$KMnO_4$	10	24, 48 : On-site assay (10 mg/L)	1.0

Table 6-2 Apparent decay rate constant in cyanobacteria-laden sludge oxidation. Contact times: KMnO4: 1 h, H2O2: 24 h.

Date	July 31st		August 7th		August 17th		August 17th	
Oxidant	$KMnO_4$		$KMnO_4$		$KMnO_4$		H_2O_2	
Dose (mg/L)	5	10	5	10	5	10	10	20
k (min-1)	0.054	0.037	0.044	0.042	0.049	0.043	0.0024	0.0022
Half-life (min)	12.8	18.7	15.8	16.5	14.1	16.1	288.8	315.0
R^2	0.92	0.90	0.90	0.92	0.91	0.93	0.95	0.99
CT (mg.min/L)	89	239	105	218	96	214	4090	7781
Relative CT	0.37	1.0	0.44	0.91	0.40	0.90	0.53	1.0

6.2.2.2 Stagnation

Stagnation was applied to all the samples before (time zero) and after oxidation assays for 7-38 days. Stagnation time was selected based on the sludge storage time in the studied DWTP. The stagnated samples were stored in the dark and at room temperature (20 ±2 ^oC) to simulate sludge holding tank condition. To avoid interferences such as the presence of other microorganisms and air impact, the samples were stored in 1-L autoclaved polypropylene bottles and the bottles remained tightly capped (sealed) during stagnation.

6.2.3 Sample Preparation and Analysis

6.2.3.1 Taxonomic Cell Counts

Samples for taxonomic cell count analysis were preserved using Lugol's iodine. The analysis of taxonomic cell counts was conducted using inverted microscopy at magnification of 10X and 40X. All details of the analysis are presented in (Lund, 1959; Lund et al., 1958; Planas et al., 2000). The predominant species are presented and less dominant species (< 5%) are shown as "Other". In this study, cell removal is calculated based on the cell removal (disappearance) after oxidation.

6.2.3.2 Dissolved Organic Carbon (DOC)

Samples for DOC analysis were taken by filtration of the sludge samples using pre-rinsed 0.45-µm membranes (PALL, Port Washington, NY, USA). DOC analysis was conducted based on USEPA 415.1 method by total organic carbon analyzer (Sievers Analytical Instruments, Boulder, CO, USA) (United States Environmental Protection Agency (USEPA), 1974).

6.2.3.3 Microcystins (MCs)

Samples for MC (cell-bound and dissolved) analysis were prepared by filtration of the samples using pre-weighted 0.45-µm GHP membranes (Pall, Mississauga, ON). MC analysis was conducted by on-line solid-phase extraction ultra-high-performance liquid chromatography coupled to tandem mass spectrometry (on-line SPE-UHPLC-MS/MS). MC quantification was conducted on a Thermo Hypersil Gold C18 column (100 mm x 2.1 mm, 1.9 µm particle size). Potassium permanganate and sodium (meta) periodate (Sigma Aldrich, Oakville, ON, Canada) were used for the sample oxidation. Quenching was done by a 4 M sodium bisulfite solution (Sigma Aldrich, Oakville, ON, Canada). Standard solutions were 4-phenylbutyric acid (50 ng/L) (Sigma Aldrich, Oakville, ON, Canada) and erythro-2-Methyl-3-methoxy-4-phenylbutyric acid (D3-MMPB, 10 ng/L) (Wako Pure Chemicals Industries, Ltd., Osaka, Japan). The volume of the samples applied for MC measurement was 10 mL. Additional details including extraction steps are presented in Munoz et al. (2017) and Roy-Lachapelle et al. (2019).

6.2.3.4 DNA extraction, shotgun metagenomic sequencing protocols, bioinformatics description, and statistical analysis

Samples for shotgun metagenomic analysis were collected directly from the sludge samples without any filtration and were stored in a sterile falcon tube. A 10 mL of sludge samples were taken for DNA extraction. Sludge samples were homogenized before extraction. Extraction yields were evaluated using RT-qPCR and adding 200 µL of nuclease-free water and 5 µL of TATAA Universal DNA spike II (TATAA Biocenter AB). RNeasy PowerWater Isolation kit solution PM1 was used to lyse the cells along with Dithiothreitol (DTT), which prevents disulfide bonds forming residues of proteins. Total nucleic acid was extracted using RNeasy PowerSoil Total RNA Kit (Qiagen, Toronto, ON, Canada) and at room temperature. For the gDNA quantification, Qubit V2.0 fluorometer (Life Technologies, Burlington, ON, Canada) was applied. The Covaris E220 was used to shear gDNA.

Sequencing was done by Genome Quebec. Libraries were generated by the NEBNext Ultra II DNA Library Prep Kit for Illumina (New England BioLabs, USA) and according to the manufacturer's instruction. Adapters and PCR primers were provided from IDT (Coralville, Iowa, USA). SparQ beads (Qiagen, USA) were applied for the size selection of libraries. The Kapa Illumina GA with Revised Primers-SYBR Fast Universal kit (Kapa Biosystems, USA) was applied for library quantification. Metagenomic libraries were sequenced using NovaSeq 6000 S4 with a paired-end of 150 bp. Other details are presented in our previous work (Jalili et al., 2021). DNA extraction was done in triplicate, while one replicate was sequenced. Reproducibility and variability between replicates of our technique were evaluated by comparing results from triplicates (Moradinejad, 2021b).

Statistical analysis was conducted by R (3.6.2) and phyloseq (1.28.0) (McMurdie and Holmes, 2013). Normalization of the taxonomic data was performed using EasyCODA (0.31.1) and centered log-ratio transformation (Graffelman, 2019). Redundancy analysis (RDA) was applied for evaluation of the constrained variables. The variance homogeneity was validated prior to implementation of the model (Blanchet et al., 2008). Significant variables (> 95%) were selected by permutation test. All details are explained in our previous investigation (Jalili et al., 2021).

6.2.3.5 Droplet digital PCR (ddPCR)

Genomic DNA samples were normalized to 1.0 ng/µL. Initial concentrations below 1.0 ng/µL were concentrated to the ideal 1.0 ng/µL using a speed vacuum. Primers for the detection of the *mcyD* gene (microcystin production) were: mcyD(KS)F1: 5'-TGGGGATGGACTCTCTCACTTC-3' and mcyD(KS)R1: 5' GGCTTCAACATTCGGAAAACG-3' (Fortin et al., 2010). The details of the analysis are presented in Moradinejad et al. (2021a).

6.2.3.6 Solid Analysis

Solids analysis including total suspended solids (TSS) and volatile suspended solids (VSS) were performed based on the 2540- Standard Method (American Public Health Association (APHA) et al., 2012).

6.3 Results

6.3.1 Characteristics of Untreated Sludge

Characteristics of the sludge samples are presented in (Table S1). Total MC concentrations remained below 161 ng/L on July 31st, August 7th and 17th and increased to 1084 ng/L on September 5th with 88% being cell-bound MCs. DOC remained in the range of 3.19- 3.60 mg/L in with a peak concentration DOC concentration on September 5th (9.8 mg/L). Similarly, while pH remained in the range of 7.05- 7.54 on July 31st, August 7th and 17th (laboratory oxidation dates), it decreased to 6.81 on September 5th (on-site oxidation date). Higher turbidity, TSS and TVS were also recorded on 5 September as 701 NTU, 1957 mg/L and 1230 mg/L, respectively.

Taxonomic cell counts remained in the range of 2.25 x 10^6 cells/mL to 2.71 x 10^6 cells/mL during the studied dates (Table C- 1). The highest cell counts were reported on August 7th. *Aphanothece clathrata brevis, Microcystis aeruginosa, Dolichospermum spiroides, Aphanocapsa delicatissima, Aphanocapsa planctonica*, and *Aphanocapsa holsatica* were the predominant species in the sludge samples before oxidation or and stagnation (Figure C- 1).

Shotgun metagenomic sequencing showed that Proteobacteria was the predominant phylum in all studied dates. Cyanobacteria, Bacteroidetes and Actinobacteria were the next predominant phyla. Cyanobacteria reached its highest level on August 17th, while the lowest relative abundance of

Cyanobacteria was recorded on August 7th (Figure C- 2a). Investigation of Cyanobacteria at the order level revealed that Nostocales was predominant on July 31st and August 17th. In contrast, Chroococcales was the predominant order on 7 August (Figure C- 2b). Similarly, Dolichospermum (Nostocales) was predominant on July 31st and August 17th. Synechococcus was the predominant genus on August 7th (Figure C- 2c).

6.3.2 Oxidation of cyanobacteria-laden sludge in controlled conditions

The first-order rate constants and half-life of oxidants applied are presented in (Table 6-2). Rate constants varied from 0.044 min^{-1} to 0.054 min^{-1} using 5 mg/L KMnO4 in different oxidation assays. Increasing the $KMnO_4$ dose to 10 mg/L caused the rate constant range to drop to 0.037 min^{-1} to 0.43 min^{-1}. Finally, applying 10 mg/L and 20 mg/L H_2O_2 led to 0.0024 and 0.0022 min^{-1} rate constants, respectively. Amongst $KMnO_4$ assays, the highest CT (239 mg.min/L) was observed in the July 31st sample and after applying 10 mg/L $KMnO_4$. The highest CT of H_2O_2 (7781 mg.min/L) was reported by 20 mg/L H_2O_2 on August 7th.

Oxidation with $KMnO_4$ caused different shifts of diversity as revealed by taxonomic cyanobacterial cell counts. On July 31st, 5 mg/L $KMnO_4$ resulted in modest increases in cyanobacterial cell counts (16%), while 10 mg/L $KMnO_4$ slightly decreased cell counts (3%) (Figure 6-1). This apparent resistance to oxidation could be attributed to the presence of colonies leading to cell count uncertainty. In addition, oxidation could release cells from colonies resulting in an increase in cell counts. Similar increases after oxidation were observed in one of our pre-tests performed on sludge and water samples in 2017 (data not shown). In contrast, 5 and 10 mg/L $KMnO_4$ decreased cyanobacterial cell counts by 46-55% and 59-62%, on August 7th and 17th, respectively (Figure 6-2 and Figure 6-3). Applying 10 mg/L H_2O_2 removed 58% of cyanobacterial cells, while increasing the dose to 20 mg/L led to a removal of 77% of the cells (Figure 6-2).

At the phylum level, shotgun metagenomic sequencing showed that the relative abundance of Proteobacteria increased, while the relative abundance of Cyanobacteria decreased for both dates (August 7th and 17th) and oxidants ($KMnO_4$ and H_2O_2) (Figure 6-4a). The relative abundance trends are coherent with absolute abundance observations based on taxonomic cell counts (Figure 6-2, Figure 6-3 and Figure 6-4a).

At the cyanobacterial genus level, the relative abundance of *Synechococcus* increased to 3- 13% and 23- 25% after oxidation on August 7th and 17th, respectively. Additionally, *Dolichospermum* slightly decreased as 3- 9% and 13- 14% on August 7th and 17th, respectively. The relative abundance of the other genera (ex. *Microcystis*) remained almost constant (< 7%) (Figure 6-4c).

Overall, two different trends were observed in the microbial communities after oxidation: *i)* on August 7th, the diversity (Shannon index) remained almost constant after oxidation in all scenarios (Figure 6-5a), *ii)* on August 17th, the Shannon index increased after oxidation. This could be caused by the higher relative abundance of Cyanobacteria on August 17th (< 70%) as compared to August 7th (< 33%) (Figure 6-4a). Indeed, at the order level, Chroococcales was predominant on August 7th and was not impacted by oxidation (Figure 6-4b). On August 17th, Nostocales (*Dolichospermum*) was predominant and was most impacted by oxidation. Finally, the impact of oxidative stress on microbial communities can be assessed by considering the relative CT. The relative abundance of cyanobacteria is inversely associated with the relative CT (Figure 6-6a,b).

At the cyanobacterial level and on August 7th, 5 mg/L $KMnO_4$ did not affect diversity. However, 10 mg/L $KMnO_4$ and both doses of H_2O_2 reduced the Shannon index. Furthermore, on August 17th, both doses of $KMnO_4$ decreased cyanobacterial diversity (Figure 6-5b). The relative abundance of *Synechococcus*, *Microcystis*, *Cyanobium* and *Prochlorococcus* persisted during oxidation, while that of *Dolichospermum* decreased (Figure 6-4b,c). Precisely, on August 7th, the control (before oxidation) correlated with the relative abundance of Chroococcales followed by Nostocales. After oxidation with both $KMnO_4$ and H_2O_2, Chroococcales was the most abundant order within the cyanobacterial community. On August 17th, the control was dominated by Nostocales followed by Chroococcales. After oxidation, the cyanobacterial community shifted towards Chroococcales. Therefore, regardless of the initial cyanobacterial composition, oxidation shifted cyanobacterial communities to the resistant orders such as Chroococcales (Figure 6-6c,d). The initial microbial community before oxidation and the CT determine the extent of changes caused by oxidation within the microbial composition as reported by Moradinejad et al. (2020).

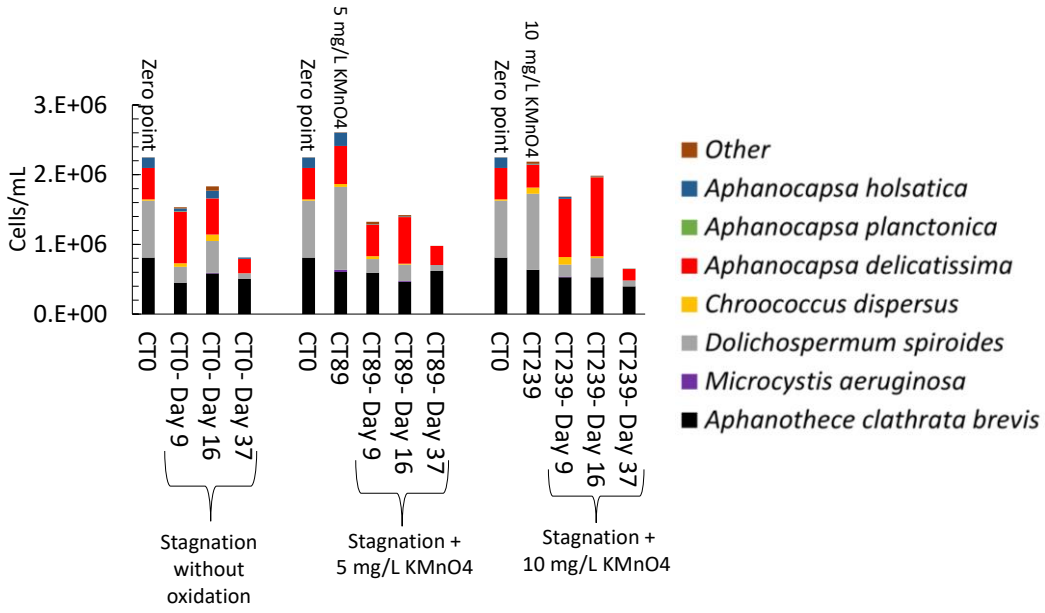

Figure 6-1 Fate of cyanobacterial cells (taxonomic cell counts) during sludge oxidation and stagnation in the laboratory on July 31st using 5 and 10 mg/L $KMnO_4$. Zero point: Before oxidation, CT: exposure (mg.min/L), Day: stagnation day.

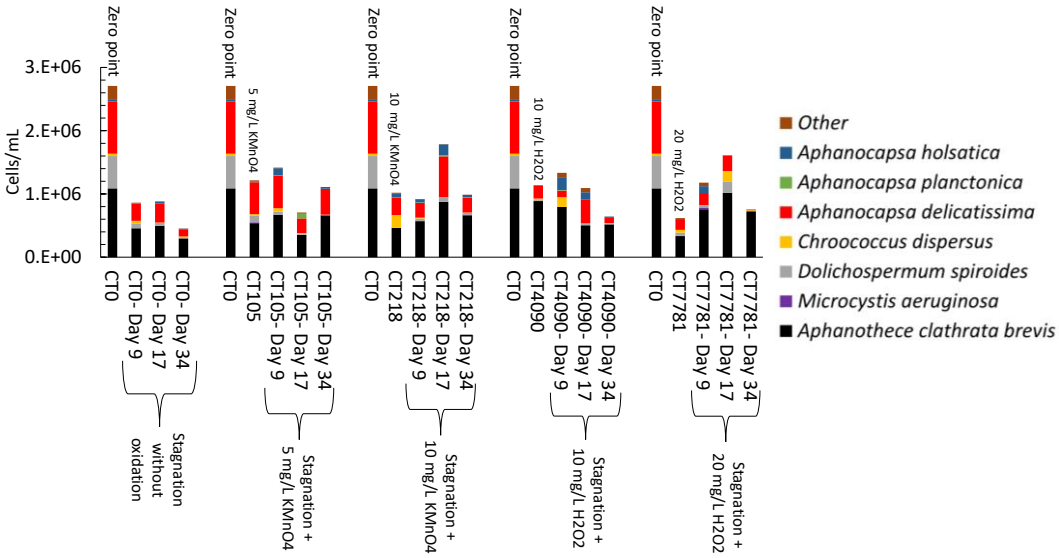

Figure 6-2 Fate of cyanobacterial cells (taxonomic cell counts) during sludge oxidation and stagnation in the laboratory on August 7th using 5, 10 mg/L $KMnO_4$ and 10, 20 mg/L H_2O_2. Zero point: Before oxidation, CT: exposure (mg.min/L), Day: stagnation day.

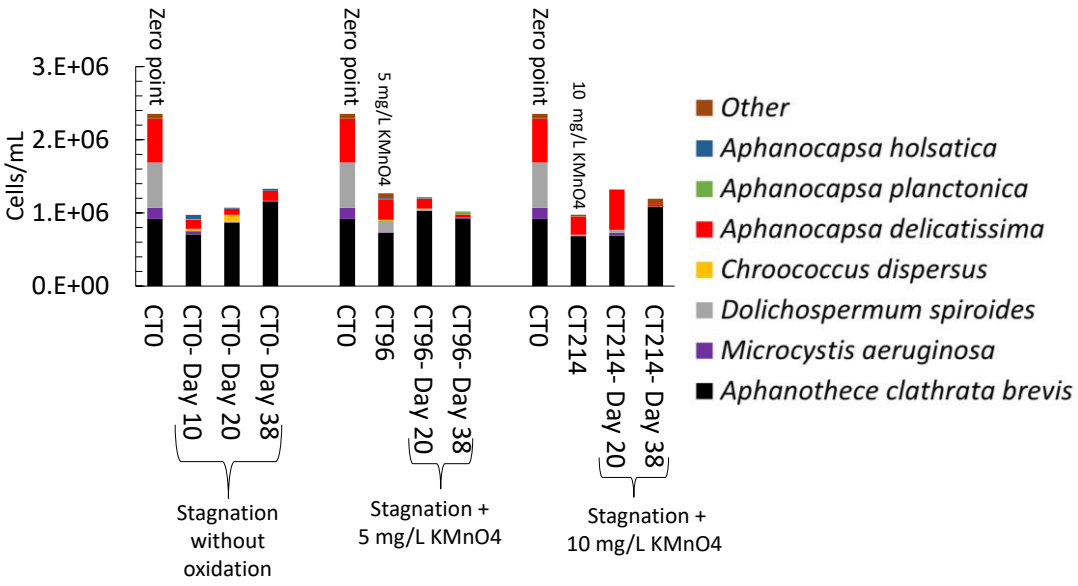

Figure 6-3 Fate of cyanobacterial cells (taxonomic cell counts) during sludge oxidation and stagnation in the laboratory on August 17th using 5 and 10 mg/L $KMnO_4$. Zero point: Before oxidation, CT: exposure (mg.min/L), Day: stagnation day.

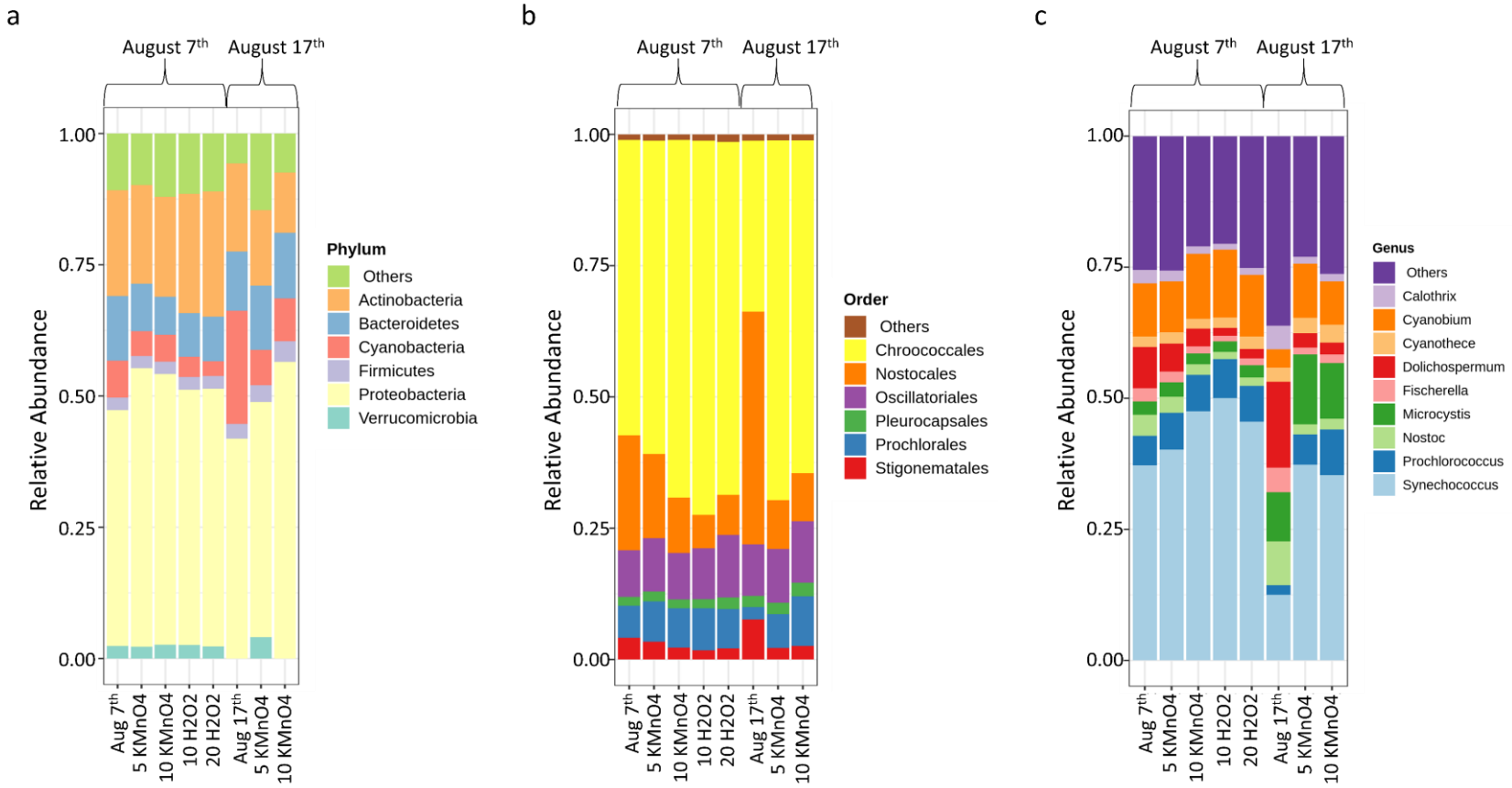

Figure 6-4 Impact of laboratory oxidation on a) microbial relative abundance at the phylum level, b) cyanobacterial relative abundance at the orders and c) cyanobacterial relative abundance at the genus level on August 7th and 17th. The relative abundance was calculated based on the number of reads of each phylum, order and genus divided by whole number of reads.

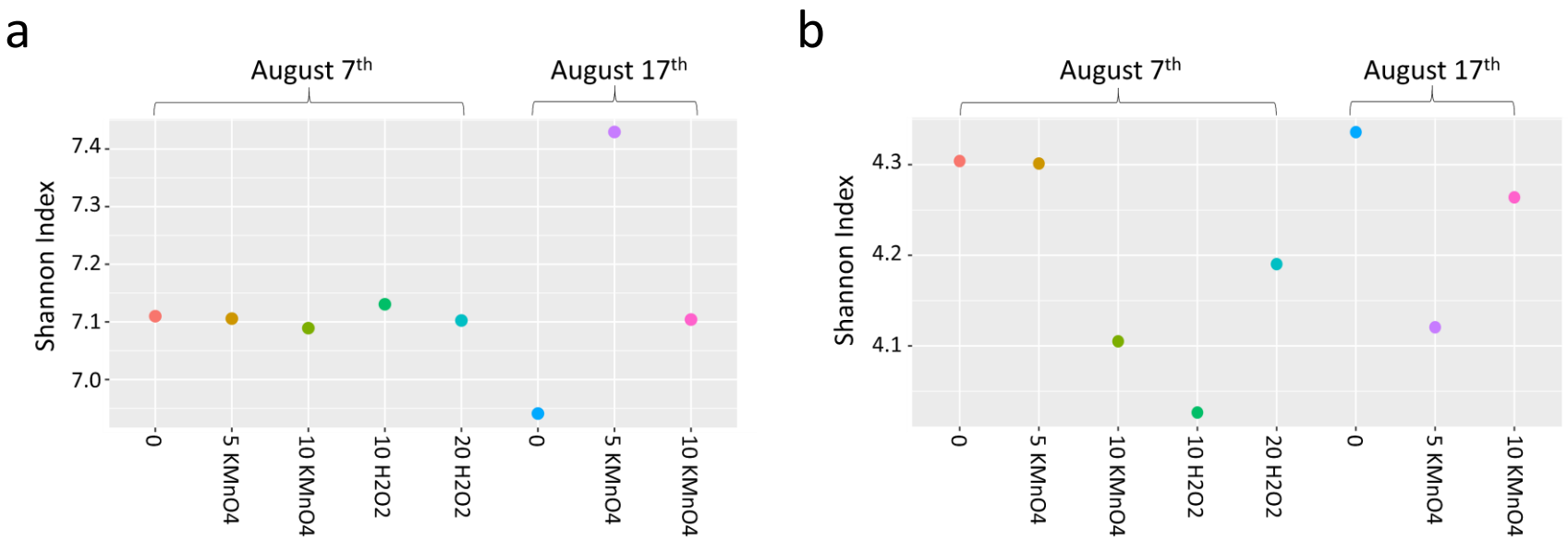

Figure 6-5 Impact of laboratory oxidation on a) Microbial and b) Cyanobacterial diversity (Shannon index) on August 7th and August 17th, 0: Sludge samples before oxidation.

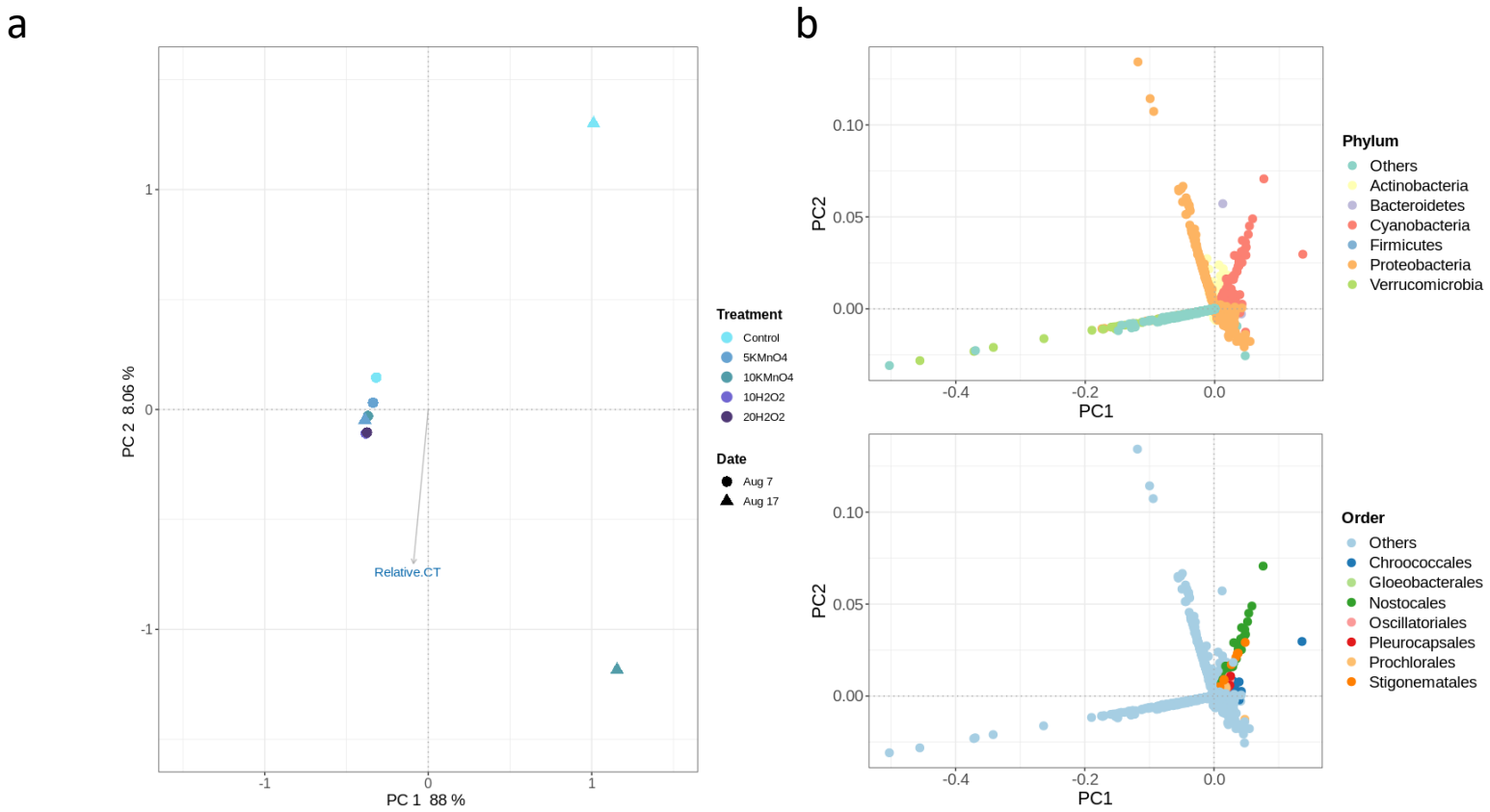

Figure 6-6 Principal component analysis (PCA) of a) Microbial communities, b) Microbial/ cyanobacterial communities grouped at the phylum and order levels, c) Cyanobacterial communities, d) Cyanobacterial species grouped at the order level on August 7th and 17th in the laboratory oxidation. The relative CT significantly affected microbial communities (p <0.05).

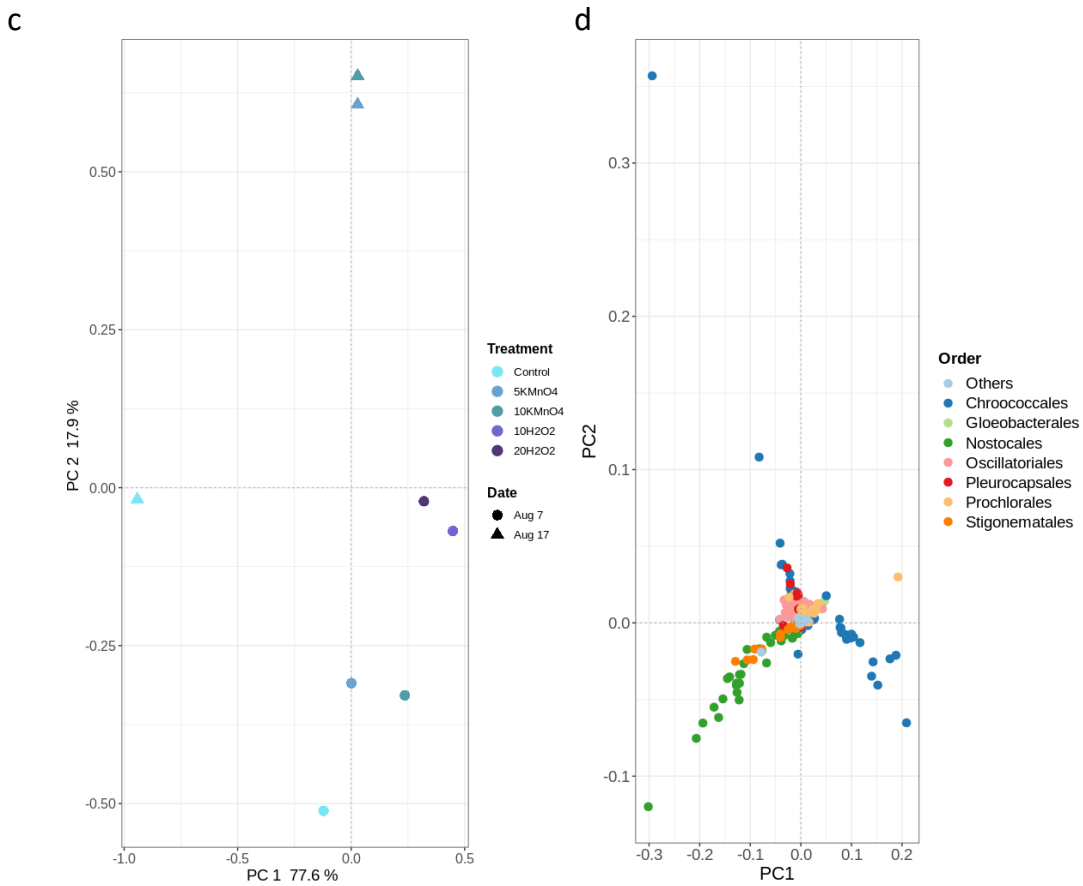

Figure 6-6 (Continue) Principal component analysis (PCA) of a) Microbial communities, b) Microbial/ cyanobacterial communities grouped at the phylum and order levels, c) Cyanobacterial communities, d) Cyanobacterial species grouped at the order level on 7 August and 17 August in the laboratory oxidation. The relative CT significantly affected microbial communities (p <0.05).

6.3.3 Oxidation of Cyanobacteria-laden Sludge in A Full-scale Plant

On-site sludge shock oxidation was performed by adding a single dose of 10 mg/L $KMnO_4$ into the sludge holding tank on 17 August and 5 September 5. This simulates a shock treatment in the sludge holding tank. This type of simple corrective response can easily be used by operators if toxicity was detected at the source or in the sludge holding tank.

On 17 August, a 42% decrease was observed in total cyanobacterial cell counts after 24 h. No cells of *Microcystis aeruginosa* were detected and 76% of *Dolichospermum spiroides* and *Aphanocapsa delicatissima* were removed. Total taxonomic cell counts increased by 33% between 24 and 72 h in the absence of $KMnO_4$ residual (*See 3.2*). This increase was driven mostly by *Aphanocapsa holsatica, Aphanocapsa delicatissima* and *Aphanothece clathrata brevis* but among potential toxin producers, *Microcystis aeruginosa* increased from non-detected to 3.3 x 10^4 cells/mL after 72 h. Additionally, *Dolichospermum spiroides* depletion continued (99% removal) (Figure 6-7a). On the 5 September and after 24 h, total taxonomic cell counts decreased by 34%. Precisely, 18- 99% reduction was observed in *Chroococcus dispersus*, *Aphanocapsa delicatissima*, *Microcystis aeruginosa,* and *Aphanothece clathrata brevis*. In contrast, cell counts of *Dolichospermum spiroides* increased by 79%. (Figure 6-7b). After 48 h, total cell counts increased by 72% as compared to the 24 h contact time. A full scale investigation of multiple repeated application of 10 mg/L $KMnO_4$ in a sludge thickener reported a similar range of total taxonomic cell count reduction ranging from 13- 98% (Zamyadi et al., 2016b).

The on-site oxidation testing was conducted during regular plant operation involving up to four sludge extractions per hour. Since $KMnO_4$ was applied as a single shock dose and because of regular inputs of new sludge, it was not possible to conduct a mass balance. Therefore, the observed increase in total taxonomic counts could be the result of: *i)* fresh sludge containing cyanobacterial cells entering the sludge holding tank from the clarifier, *ii)* cell settling from the sludge supernatant into the sludge in the sludge holding tank, and *iii)* cell growth of cyanobacterial cells after dissipation of $KMnO_4$.

On 17 August, the relative abundance of Proteobacteria increased slightly (> 7%) and the relative abundance of Cyanobacteria decreased (16%) after 24 and 72 h (Figure 6-8a), as observed for the laboratory testing. At the cyanobacterial genus level, the relative abundance of *Synechococcus* increased. Interestingly, on-site oxidation decreased the relative abundance of two potentially toxic

genera, *Microcystis* and *Dolichospermum*. However, *Microcystis* persisted more than *Dolichospermum* (Figure 6-8b).

On 17 August, cell-bound and dissolved MC levels were low (< 67 ng/L) before oxidation and after 24 h. After 72 h, cell-bound MCs increased to 552 ng/L (Figure 6-9a). This is in line with the increase of potential MC producer species such as *Microcystis aeruginosa* (3.3 x 10^4 cells/mL), *Chroococcus dispersus* (1.1 x 10^4 cells/mL) and *Aphanocapsa delicatissima* (3.8 x 10^5 cells/mL) (Figure 6-5a). On 5 September and before oxidation, total MCs were 1084 ng/L containing 952 and 132 ng/L cell-bound and dissolved MCs, respectively (Figure 6-9). After 24 and 48 h, cell-bound MCs reached 580 and 888 ng/L, respectively. Dissolved MCs increased to 230 and 168 ng/L after 24 h and 48 h, respectively.

$KMnO_4$ has been shown to cause cell-bound MCs release and degrade dissolved MCs efficiently, with oxidation rate constants ranging from 4.51- 22 $M^{-1} s^{-1}$ for cell bound MCs release to 118- 520 $M^{-1} s^{-1}$ for degradation of various dissolved analogues of MCs (Fan et al., 2014b; Kim et al., 2018b; Szlag et al., 2019). In fact, dissolved MCs are easily degraded and $KMnO_4$ compromises cell integrity. If a residual persists, it degrades released cell-bound MCs (Li et al., 2014b; Piezer et al., 2020b). However, in our work this trend was not observed on August 17th, as a sharp increase in cell-bound MCs after 72 h was detected due to either the impact of fresh MCs by producing species, an increased expression of MC synthesis genes (*mcy*) or growth of surviving MC producing species.

Taxonomic cell counts in the sludge supernatant increased by up to two fold after on-site oxidation, and by even more than 144X on August 17th after 72 h contact time (Figure C- 3a,b). Before oxidation, potential MC producing species accounted for 23 and 1% of the total cell counts on August 17th and September 5th, respectively. After oxidation, this percentage increased on 17 August to 36 and 51% after 24 and 72 h oxidation, respectively. On September 5th, up to 58% of total cell counts remained as potential MC producers for both contact times. Although total MC concentrations were lower than 160 ng/L before oxidation, they increased by 46- 140% in 3 out of 4 cases after oxidation (Figure C- 3c,d). In contrast to observations in the sludge (Figure 6-7), the proportion of dissolved to cell-bound MCs was greater. A dynamic exchange of cells and toxins can occur between sludge and supernatant in the holding tank (Jalili et al., 2021), posing an operational challenge during sludge management. These findings support other reports (Dreyfus

et al., 2016; Pestana et al., 2016; Zamyadi et al., 2016b), outlining the challenge of supernatant recycling to the head of the DWTPs during toxic cyanobacterial blooms.

Small facilities (eg. the studied DWTP) may not be able to install quickly and operate safely online dosage equipment for continuous on-site oxidation of sludge. Also, such an approach may be warranted if cyanobacterial accumulation in sludge is not a frequent problem at a given site. Sporadic shock oxidation treatment of sludge holding tanks is easy to implement and could provide a temporary response to the presence of toxins in the stored sludge, allowing for existing disposal methods to be maintained. In that case, the system will not be isolated at least for not for an extended period. Documenting the impact of on-site shock oxidation in this dynamic operating conditions provide valuable information to operators. The objective of on-site oxidation was the investigation of stagnation impact (*See 6.3.3.2*) on cyanobacteria-laden sludge after shock oxidation.

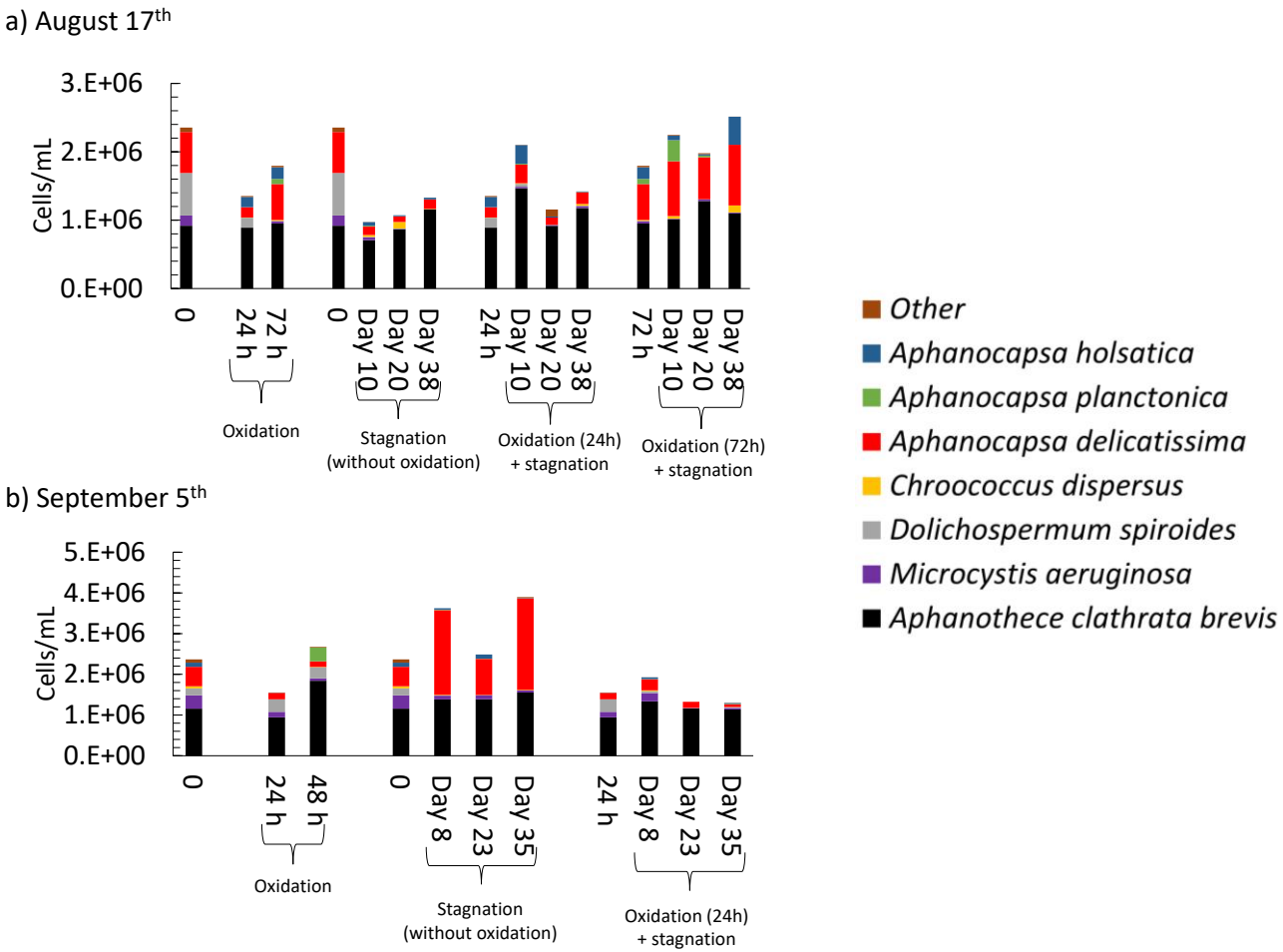

Figure 6-7 Taxonomic cell counts on a) August 17th, and b) September 5th after on-site oxidation using 10 mg/L $KMnO_4$ and stagnation. 0: Before oxidation, Day: Stagnation day.

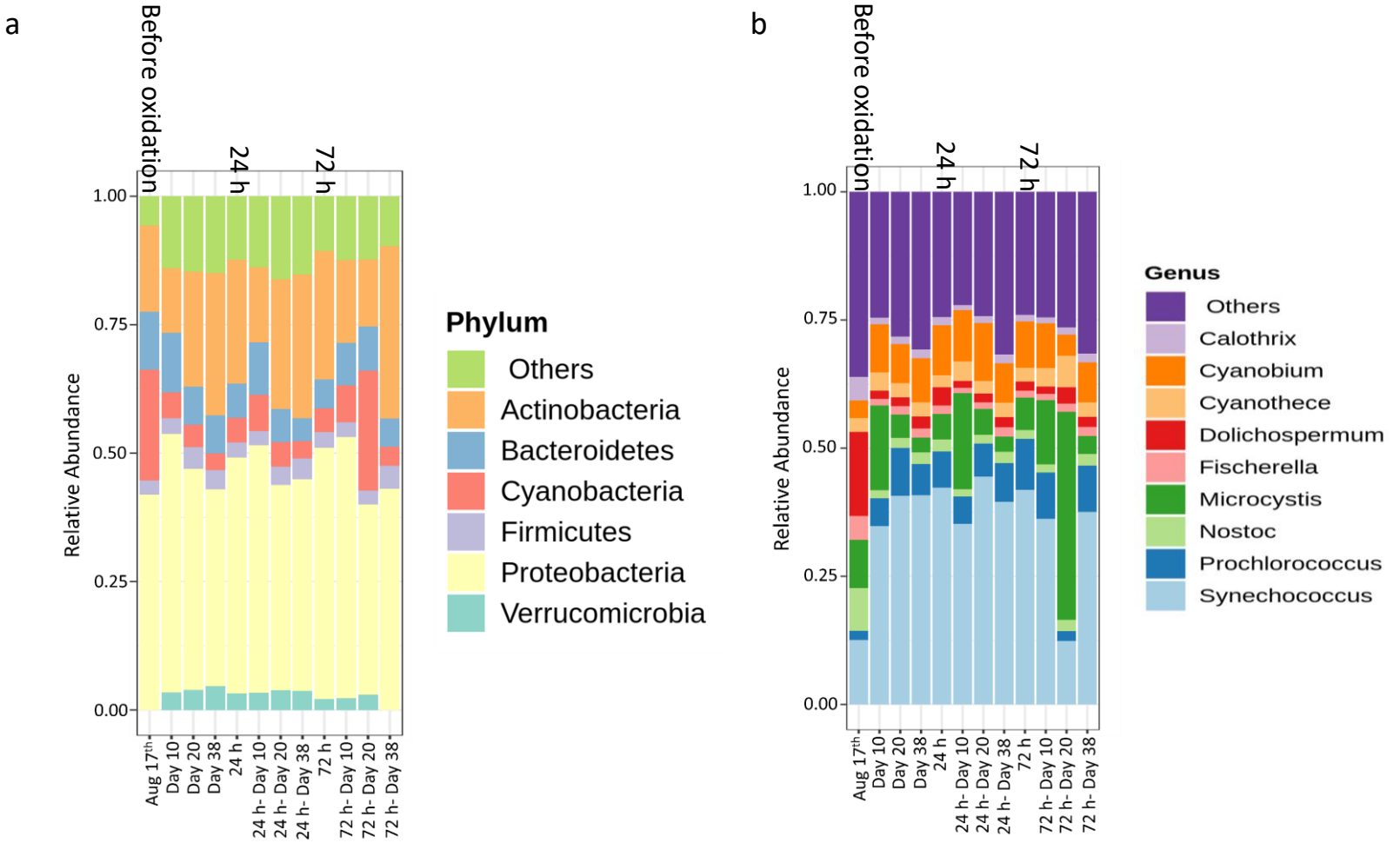

Figure 6-8 Taxonomic composition before/ after on-site oxidation by 10 mg/L KMnO4 and stagnation on August 17th: a) Microbial communities at the phylum level, b) Cyanobacterial communities at the genus level. 24 h and 72 h: oxidation contact time, Day: stagnation day.

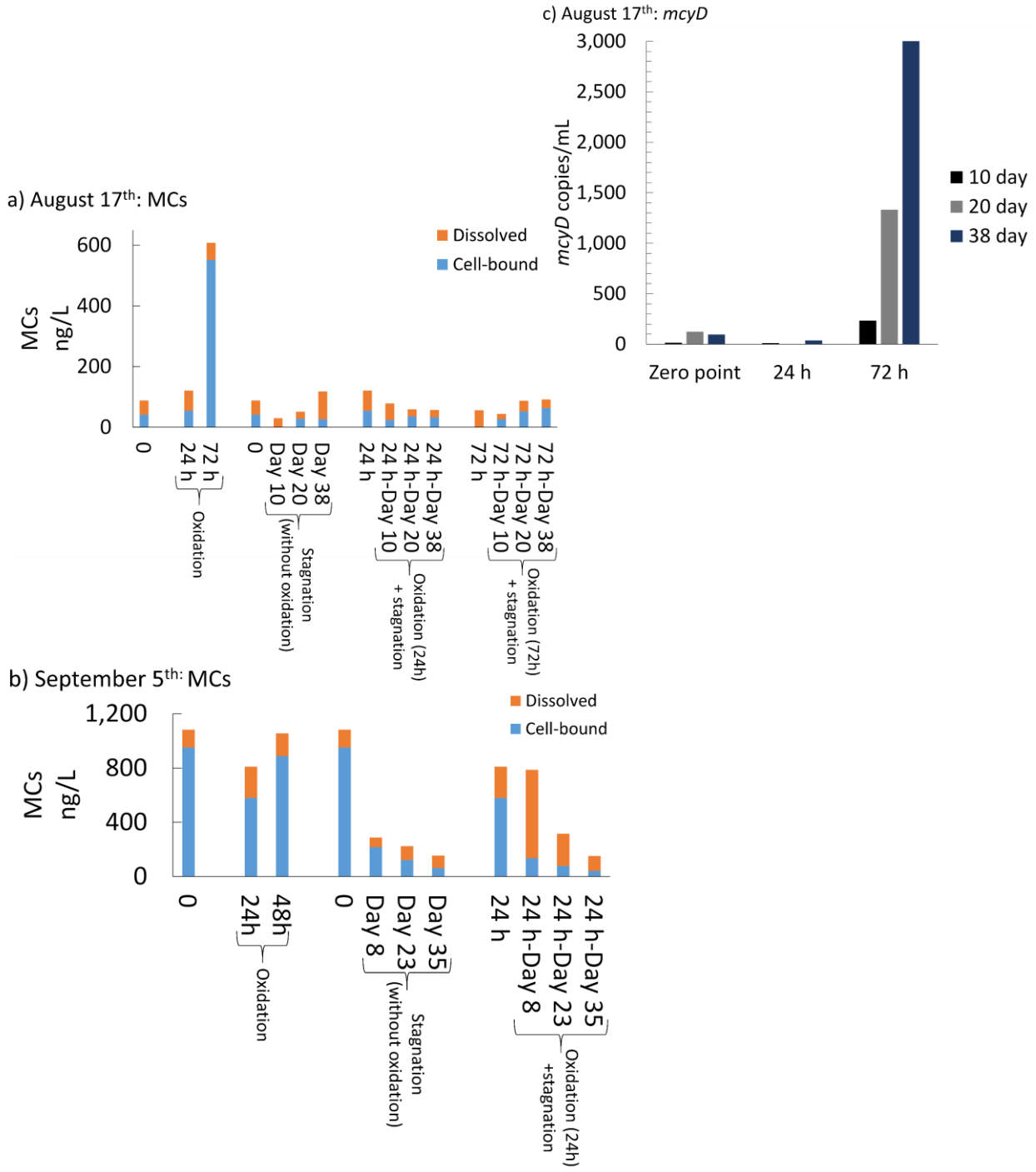

Figure 6-9 MC concentrations in the sludge after on-site oxidation and stagnation on a) August 17th, b) September 5th, c) *mcyD* gene copies during stagnation on August 17th.

6.3.4 Impact of Stagnation on Oxidized Cyanobacteria-laden Sludge

6.3.4.1 Stagnation after Laboratory Oxidation

This section provides results from a controlled system of cell decay or cell growth after sludge oxidation and stagnation. Overall, in most of the sludge samples (12 out of 16), $KMnO_4$ oxidation followed by stagnation resulted in a 4- 70% cell count reduction as compared to only oxidized samples (Figure 6-1-Figure 6-3). However, a 17- 75% increase in total counts was also observed in some samples (4 out of 16) during stagnation (Figure 6-1-Figure 6-3). H_2O_2 oxidation resulted in an important decrease in total taxonomic cell counts by 58% for 10 mg/L and 77% for 20 mg/L H_2O_2. However, during stagnation a 17-161% (1.2- 2.6 time) cell growth was observed in 4 out of 6 samples oxidized by H_2O_2. In the 10 mg/L H_2O_2 followed by stagnation, total taxonomic cell counts increased by 17% after 9 days, and then decreased by 4 and 44% after 17 and 34 days, respectively. In contrast, with 20 mg/L H_2O_2, taxonomic cell counts increased back by 22- 161% (1.2- 2.6 time) within 9 to 34 days (Figure 6-2). Results presented in section 3.3 and plotted on Figure 6-1 to Figure 6-3 show that stagnation alone causes a sharp and quite stable reduction of taxonomic cell counts. These findings reveal that the combination of oxidation and stagnation overall reduces taxonomic cell counts by 12-76% depending on the duration of stagnation, falling short of global removal observed without oxidation that range from 19-83%.

Combining oxidation with stagnation increased taxonomic cell counts by 7- 145% (1.1- 2.4 time) as compared to samples with only stagnation in 14 out of 22 cases (Figure 6-1-Figure 6-3). Specifically, 10 mg/L H_2O_2 followed by stagnation caused a 24- 55% increase in total cell counts within 9-34 days as compared to stagnated (non-oxidized) samples. A dynamic trend of taxonomic cell count decrease and increase was also observed in the sample oxidized by 20 mg/L H_2O_2 followed by stagnation.

These observations in controlled conditions clearly demonstrate an increased potential for regrowth when oxidation is used prior to stagnation. Regardless of the actual increase on cell counts, it may be more relevant to quantify what type of cells are likely to regrow after storage preceded by oxidation. Cell growth was mostly observed for *Aphanothece clathrata brevis* and for MCs producers, *Microcystis aeruginosa*, *Dolichospermum spiroides*, and *Aphanocapsa delicatissima.*

Despite rather low concentrations of measured MCs, MCs in the oxidized sludge generally decreased during stagnation, but less so than in only stagnated samples. In 14 out of 22 samples, MCs increased by 1.2- 10X, suggesting that oxidation followed by stagnation was not capable of controlling low levels of MCs in sludge (Figure C- 4).

The impact of $KMnO_4$ and H_2O_2 on cyanobacterial communities is quite different (Figure C- 5). Stagnation time significantly affected cyanobacterial communities ($p < 0.05$) in the sludge oxidized by $KMnO_4$ with a shift towards tolerant orders such as Chroococcales and Pleurocapsales. This shift was also observed for only stagnated samples. Similar to Moradinejad et al. (2020) findings about H_2O_2 oxidation of cyanobacterial bloom water, H_2O_2 oxidation of sludge in this study reveals selective reduction of Chroococcales. Distinct shift is obtained following stagnation only.

6.3.4.2 Stagnation After On-site Oxidation

On August 17th and as compared to oxidation alone, taxonomic cell counts increased by 55% in sludge collected 24 h after on-site oxidation and subjected to controlled stagnation for 10 days (Figure 6-7a). In contrast, taxonomic cell counts decreased by 15% after 20 days of stagnation. After 38 days, taxonomic cell counts increased by 5% as compared to oxidation alone. Taxonomic cell counts increased by 10- 40% in sludge collected 72 h after on-site oxidation and subjected to controlled stagnation as compared to oxidation alone (Figure 6-7a).

The relative abundance of Cyanobacteria increased during oxidation/ stagnation as compared to the non-oxidized/ stagnated samples (Figure 6-8a). Similarly, the relative abundance of *Microcystis* increased during oxidation/ stagnation. The highest increase was observed in the 24 h/10-day and the 72 h/20-day samples (Figure 6-8b). In accordance with our laboratory oxidation/stagnation results, depletion of Nostocales and persistence of Chroococcales order were observed (Figure C-6).

To better visualize the taxonomic composition after 38 days of stagnation following on-site $KMnO_4$ oxidation, differential heat trees were used to show pairwise comparisons at the microbial and the cyanobacterial level between T0 (oxidized sludge and not stagnated) and T38 (oxidized sludge after 72 h and stagnated for 38 days). Verrucomicrobia, Actinobacteria, and Proteobacteria (Gammaproteobacteria and Alphaproteobacteria) phyla were more abundant after stagnation. Within Cyanobacteria, the majority of Oscillatoriales, Gloebacterales were more abundant after

stagnation. Chroococcales and Nostocales were partitioned between representatives that persist after stagnation and others that do not persist (Figure C- 7).

Cell-bound MCs in the oxidized sludge (24 and 72 h) remained 1.2- 27 time higher than that of non-oxidized sludge during stagnation (Figure 6-9a). In the non-oxidized sludge, *mcyD* gene copy numbers gradually increased with stagnation from 14 copies/mL (day 10) to 123.7 copies/mL (day 20) and 95.6 copies/mL (day 38). Samples collected 24 h after oxidation showed a low level of *mcyD* gene copies (< 37 copies/mL). Surprisingly, *mcyD* gene copy numbers increased after 72 h oxidation/stagnation by 5.7X and 13.0X after 20 and 38 days, respectively as compared to the 10-day stagnated sample (Figure 6-9c). Interestingly, 58% of cell counts were potential MCs producers before oxidation, and on-site oxidation decreased this percentage to 33% and 46% after 24 and 72 h oxidation, respectively (Figure 6-7a). The percentage of potential MC producers remained relatively stable during stagnation without oxidation and in the sample taken 24 h after oxidation (13- 30%). Most importantly, this proportion increased up to 35- 56% in the samples collected 72 h after oxidation followed by stagnation (Figure 6-7a). This marked increase corresponds to 7.2 x 10^5 cells/mL out of which 82% were potential MC producers mainly *Aphanocapsa delicatissima, Aphanocapsa holsatica,* and *Chroococcus dispersus*.

On September 5th and as compared to oxidation alone, taxonomic cell counts in the sludge after on-site oxidation (24 h) / 8-day stagnation increased by up to 24% (Figure 6-7b). Then, taxonomic cell counts slightly decreased (15%) after 23 and 35 days of stagnation (Figure 6-7b). About 48% of cyanobacterial cells before oxidation were potential MC producers. In this case, oxidation decreased the percentage of potential MC producer species by 39%, and subsequent stagnation caused an additional 12- 31% decrease in cell counts (Figure 6-7b). On this date, slightly higher MC concentrations were detected (Figure 6-9b). On-site oxidation did not markedly decrease these MCs (3- 25%) as shown by the 24 h and 48 h results (Figure 6-9b). However, stagnation without oxidation decreased MCs by 73% within 8 days and up to 86% within 35 days. A different trend was observed for the oxidized sludge as total MCs were barely reduced by 8 days and then reduced by up to 81% within 35 days. Cell-bound MCs in the oxidized sludge remained around 0.7 times less than non-oxidized sludge during stagnation. However, dissolved MCs remained 1.2- 9.2X higher than non-oxidized/stagnated sludge within 8-35 days (Figure 6-9b).

6.3.5 Factors Affecting the Fate of Cyanobacteria and MCs in Sludge

Different phenomena should be considered to understand the fate of cyanobacteria and MCs in real sludge after oxidation and stagnation:

MCs detected in the sludge are present in dissolved and cell-bound forms and this partitioning is affected by storage and oxidation. Cyanobacterial cell integrity losses can lead to the release of cell-bound cyanotoxins after oxidation (Fan et al., 2013a; Fan et al., 2013b) and as revealed by this study, after stagnation. Released dissolved MCs can be oxidized (Piezer et al., 2020b), adsorbed onto PAC or flocs (Jalili et al., 2021; Ma et al., 2016b) or can be biodegraded by species belonging to Proteobacteria, Actinobacteria and Firmicutes (Kormas and Lymperopoulou, 2013; Maghsoudi et al., 2016).

Oxidation causes significant changes in microbial diversity leading to the selective persistence of some cyanobacterial species over other communities (Lusty and Gobler, 2020; Moradinejad et al., 2020) and nutrient availability (Jalili et al., 2021) as obtained here by the increase of DOC (data not shown). These factors could explain the observed growth of some cyanobacterial species proceeded by oxidation and stagnation.

Oxidative stress (ex. H_2O_2 and nutrient depletion) may cause gene expression regulation of *mcy* genes, leading to MCs production (Pimentel and Giani, 2014; Schatz et al., 2007). There are no data about gene expression regulation in the presence of $KMnO_4$ in water or sludge samples. In our investigation, the combination of oxidation and stagnation represents an increase of oxidative stress.

6.4 Conclusions

- Controlled laboratory oxidation with $KMnO_4$ and H_2O_2 decreased total taxonomic counts as well as potential MC producers. The highest observed cell count decrease was from 2.7 x 10^6 cells/mL to 6.2 x 10^5 cells/mL (77%) when applying 20 mg/L H_2O_2. However, stagnation after controlled oxidation (laboratory scale) led to an increase of cyanobacterial cells in 8 out of 22 samples as compared to oxidation alone. The highest cell count increase after stagnation was from 6.2 x 10^5 cells/mL to 1.6 x 10^6 cells/mL (2.6-fold increase) observed in the sludge sample oxidized by 20 mg/L H_2O_2 and stagnated for 17 days. $KMnO_4$ (10 mg/L) and H_2O_2 (20 mg/L) could decrease MC concentration up to 98% (from

63 ng/L to below detection limit) and 41% (from 139 ng/L to 77 ng/L), respectively. Laboratory oxidation did not lead to higher production of MCs during stagnation. When comparing H_2O_2 and $KMnO_4$ oxidation results in the same day, H_2O_2, with greater half-lives than $KMnO_4$, was more effective than $KMnO_4$ for decrease of cyanobacterial cells and cyanotoxin concentrations. Similarly, oxidation/ stagnation caused cell growth in 14 out of 22 samples by up to 145% as compared to stagnation alone.

- Laboratory oxidation shifted cyanobacterial diversity from Nostocales (*Dolichospermum*) towards Chroococcales (*Microcystis*) and Synechococcales (*Synechococcus*) as the persistent orders (genus). Opposite to $KMnO_4$ oxidation, in which no selective removal within the cyanobacterial community was observed, H_2O_2 selectively reduced Chroococcales.
- Short-term (24 h) reduction on total taxonomic cell counts was observed during shock on-site oxidation. Indeed, on-site shock oxidation could decrease total taxonomic cell counts up to 43% (after 24 h). In contrast, it did not deplete cyanobacteria in the sludge supernatant. However, stagnation led to exceeding total cell counts by up to 55% in 6 out of 9 samples as compared to on-site oxidation alone. Partial cell growth was also observed in MC producer genera as *Microcystis*, *Chroococcus*, and *Aphanocapsa*.
- Total MCs after stagnation preceded by shock on-site oxidation (10 mg/L $KMnO_4$) remained below initial MCs concentrations. However, *mcyD* gene copies increased during stagnation, suggesting the growth of potential MC producer species.
- As compared to storage (stagnation) only, sludge oxidation with $KMnO_4$ and H_2O_2 at the dosages studied did not bring remarkable additional benefits for the mitigation of cyanobacterial cells and cyanotoxins during subsequent storage. In some cases, oxidation prior to storage led to enhanced growth of potential MC producers in the sludge. Continuous application of oxidants or higher concentrations may prove to be more effective.

- These results demonstrate the interest in developing strategies that minimize cyanobacteria and cyanotoxin accumulation in the stored sludge, such as pre-oxidation, PAC application, and source treatment.

Author Contributions

Conceptualization, F.J., H.T., S.D., and M.P.; Methodology, F.J., H.T., J.F.G.M., N.F., B.J.S., Y.T., A.Z., S.D., S.S., and M.P.; Software, F.J., J.F.G.M., and Y.T.; Validation, F.J., H.T., J.F.G.M., N.F., Y.T., S.D., and M.P.; Formal analysis, F.J., H.T., J.F.G.M., and Y.T.; Investigation, F.J., H.T., N.F., J.F.G.M., S.D., A.Z., and M.P.; Resources: F.J., H.T., and N.F.; Data curation, F.J. and J.F.G.M.; Writing—original draft preparation, F.J., H.T., and J.F.G.M.; Writing—review and editing, F.J., H.T., N.F., J.F.G.M., S.S., S.D., and M.P.; Visualization, F.J., H.T., J.F.G.M., N.F., S.D., and M.P.; Supervision, A.Z., S.D., and M.P.; Project administration, A.Z., S.D., B.J.S., S.S., and M.P.; Funding acquisition, S.D., S.S., B.J.S., and M.P. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest

The authors declare no conflict of interest.

CHAPTER 7 ARTICLE 4: EVIDENCE-BASED FRAMEWORK TO MANAGE CYANOBACTERIA AND CYANOTOXINS IN WATER AND SLUDGE FROM DRINKING WATER TREATMENT PLANTS

This chapter combines our previous achievements (Chapter 4-Chapter 6) with other studies to suggest a strategy for controlling of cyanobacteria and cyanotoxins in DWTPs and associated sludge. The result was published as a review article in the *Toxins* Journal on June 2022.

EVIDENCE-BASED FRAMEWORK TO MANAGE CYANOBACTERIA AND CYANOTOXINS IN WATER AND SLUDGE FROM DRINKING WATER TREATMENT PLANTS

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Abstract:

Freshwater bodies and, consequently, drinking water treatment plants (DWTPs) sources are increasingly facing toxic cyanobacterial blooms. Even though conventional treatment processes including coagulation, flocculation, sedimentation, and filtration can control cyanobacteria and cell-bound cyanotoxins, these processes may encounter challenges such as inefficient removal of dissolved metabolites and cyanobacterial cell breakthrough. Furthermore, conventional treatment processes may lead to the accumulation of cyanobacteria cells and cyanotoxins in sludge. Pre-oxidation can enhance coagulation efficiency as it provides the first barrier against cyanobacteria and cyanotoxins and it decreases cell accumulation in DWTP sludge. This critical review aims to: i) evaluate the state of the science of cyanobacteria and cyanotoxin management throughout DWTPs, as well as their associated sludge, and ii) develop a decision framework to manage cyanobacteria and cyanotoxins in DWTPs and sludge. The review identified that lab-cultured-based pre-oxidation studies may not represent the real bloom pre-oxidation efficacy. Moreover, the

application of a common exposure unit CT (residual concentration x contact time) provides a proper understanding of cyanobacteria pre-oxidation efficiency. Recently, reported challenges on cyanobacterial survival and growth in sludge alongside the cell lysis and cyanotoxin release raised health and technical concerns with regards to sludge storage and sludge supernatant recycling to the head of DWTPs. According to the review, oxidation has not been identified as a feasible option to handle cyanobacterial-laden sludge due to low cell and cyanotoxin removal efficacy. Based on the reviewed literature, a decision framework is proposed to manage cyanobacteria and cyanotoxins and their associated sludge in DWTPs.

Keywords:

Cyanobacteria; cyanotoxins; pre-oxidation; sludge; accumulation; management; water treatment plant

Key Contribution:

Cyanobacteria and cyanotoxins management in DWTPs is a triangular activity including monitoring, treatment and sludge handling and interrelation.

7.1 Introduction

A cyanobacterial bloom occurrence may result in metabolite (cyanotoxins and taste and odor agents) production and release, which is considered a widespread problem in drinking water resources around the world (Akyol et al., 2021; Chorus et al., 2021; Churro et al., 2012; Huisman et al., 2018; Kosten et al., 2012; Mishra et al., 2019; Mokoena and Mukhola, 2019; O'Keeffe, 2019; Paerl and Paul, 2012b; Zamyadi et al., 2021).

Conventional treatment processes, including coagulation, flocculation, sedimentation, and filtration, are widely applied to remove cyanobacterial cells and cell-bound cyanotoxins (Drikas et al., 2001; Szlag et al., 2015; Westrick et al., 2010; Zamyadi et al., 2013a). However, conventional treatment processes may not be able to remove dissolved metabolites (e.g. cyanotoxins) efficiently (Gonzalez-Torres et al., 2014; Henderson et al., 2010; Newcombe, 2009; Newcombe and Nicholson, 2004; Pivokonsky et al., 2015; Wert and Rosario-Ortiz, 2013; Zamyadi et al., 2013a; Zamyadi et al., 2020; Zamyadi et al., 2012c). Moreover, toxic cyanobacterial breakthrough has been reported in the effluent of conventional treatment processes and even after post-oxidation (Zamyadi et al., 2012c). Therefore, additional treatment such as oxidation or powdered

(PAC)/granular activated carbon (GAC) may be required to control dissolved metabolites (Abbas et al., 2020; He et al., 2016; Merel et al., 2013; Villars et al., 2020).

Pre-oxidation enhances cyanobacteria cell removal during the coagulation/sedimentation process (Chu et al., 2017; Lapsongpon et al., 2017; Lin et al., 2018; Naceradska et al., 2017; Petrusevski et al., 1996; Wang et al., 2015b; Xie et al., 2016; Xie et al., 2013) and may decrease the cell breakthrough potential from the downflow processes. However, it is reported that pre-oxidation may cause cyanobacteria cell damage (decrease in cell viability) and cell-bound cyanotoxin release (Fan et al., 2013a; Fan et al., 2013b; Zamyadi et al., 2013c). The level of cell lysis/damage and cyanotoxin degradation/release following pre-oxidation depends on the oxidation exposure (CT as residual concentration x contact time), and it is the driver to find the best pre-oxidation practice against cyanobacteria and cyanotoxins (Moradinejad et al., 2020; Moradinejad et al., 2021b).

Furthermore, conventional treatment processes cause cell accumulation in drinking water treatment plants' (DWTPs) sludge, even in DWTPs with low cyanobacterial cell numbers in the intake water (Almuhtaram et al., 2018; Chorus and Bartram, 1999a; Drikas et al., 2001; Sun et al., 2012; Teixeira and Rosa, 2007; Zamyadi et al., 2013b; Zamyadi et al., 2012c). Several studies have demonstrated that cyanobacterial cells could survive in the stored sludge and release cyanotoxins for up to 12 days (Drikas et al., 2001; Ho et al., 2012; Li et al., 2018; Li et al., 2015; Ma et al., 2016b; Sun et al., 2015; Sun et al., 2013; Sun et al., 2012; Xu et al., 2017; Xu et al., 2016). Recent studies revealed a new challenge on the probability of extended survival time and even cyanobacterial growth during sludge storage (Dreyfus et al., 2016; Pestana et al., 2016; Water Research Foundation (WRF) and Water Research Australia, 2015). Thus, recycling the supernatant of stored cyanobacteria-laden sludge to the head of the DWTPs can increase health-related concerns (Dreyfus et al., 2016; Jalili et al., 2021). Such challenges highlight the importance of the treatment and management of cyanobacteria-laden sludge (Jalili, 2022; Jalili et al., 2022b; Zamyadi et al., 2016b).

The objectives of this study are to: (1) critically review shreds of evidence of the pre-oxidation impact on the cultured-based and natural bloom studies, (2) perform a critical review of the fate of cyanobacteria and cyanotoxins in conventional treatment plants' sludge and during sludge storage, and (3) develop an operational decision framework to determine the best practice to minimize risks associated with cyanobacteria and cyanotoxin presence in DWTPs.

This critical review provides insight into the fate of cyanobacteria and their associated metabolites throughout DWTPs and their sludge; furthermore, a practical decision framework to mitigate health and operational risks is developed.

7.2 Impact of Conventional Treatment on Cyanobacteria and Cyanotoxin accumulation in the Sludge

Different studies have reported that conventional treatment processes can remove 62–99% of the cyanobacterial cells in DWTPs (Chorus and Bartram, 1999a; Drikas et al., 2001; Jalili et al., 2021; Sun et al., 2012; Teixeira and Rosa, 2007; Zamyadi et al., 2012c). It has been demonstrated that potential toxic cyanobacterial cells such as *Microcystis*, *Dolichospermum*, and *Aphanocapsa* can be removed using conventional processes (Jalili et al., 2021; Water Research Foundation (WRF) et al., 2009; Zamyadi et al., 2013a; Zamyadi et al., 2019).

The long-term monitoring of a high-risk DWTP (Lake Champlain—Quebec) during cyanobacterial bloom seasons from 2008 to 2011 showed an extreme accumulation of cyanobacteria cells (up to 10^7 cells/mL) and cyanotoxins (up to 60 μg/L microcystin-LR (MC-LR)) in the sludge of the clarifier (Zamyadi et al., 2013a; Zamyadi et al., 2012c). Monitoring of the same DWTP in 2017 showed that cyanobacteria cell accumulation in the sludge holding tank was up to 31-fold higher than taxonomic cell counts in the intake water (Jalili et al., 2021). An investigation of four DWTPs in the Great Lakes (Ontario) with low cyanobacterial cell influx (< 1000 cells/mL) revealed that cyanobacterial cells and cyanotoxins accumulated in the sludge by up to 100 and 12 times higher than the raw water, respectively (Almuhtaram et al., 2018). Zamyadi et al. (2016b) reported a 406% and 2600% cell count increase in the thickened and centrifuged sludge, respectively, in a DWTP equipped with dissolved air flotation (DAF). A similar high accumulation was also reported for the backwash of the direct filtration process (Ho et al., 2012).

Pre-oxidation may decrease the risk of cyanobacterial accumulation in the clarifier and sludge (Zamyadi et al., 2013b). Two DWTPs (the same source for intake water with low cyanobacteria cells; maximum < 500 cells/mL) with chemically enhanced conventional treatment processes were studied, but only one of the DWTPs was equipped by pre-ozonation (Zamyadi et al., 2013b). Pre-ozonation (initial concentration: 0.3–0.8 mg/L, contact time: 6.3 min) decreased cell accumulation in the surface of the clarifier and filters by up to 1450 times as compared to the DWTP without

pre-ozonation. Accordingly, an up to 7 times lower cell accumulation was observed in the sludge of the DWTP with pre-ozonation (Figure 7-1).

In many DWTPs, the supernatant of the stored sludge is recycled to the head of the plant as the spent filter backwash water (Ho et al., 2013; Ho et al., 2012; Zamyadi et al., 2016b). A full-scale study on a low-risk DWTP (3400 cells/mL at the intake water) documented that cyanobacterial cell counts in intake water increased by up to 43% after recycling the supernatant. Surprisingly, 80% of the transferred cells from the supernatant water were viable (Zamyadi et al., 2019). A recent laboratory investigation on intake water that contained 1×10^6 cells/mL of cultured *M. aeruginosa* reported that although conventional treatment maintained the treated effluent parameters at below WHO and USEPA guidelines, recycling of the sludge supernatant resulted in an additional increase in cells and cyanotoxins levels in the influent by up to 7×10^4 cells/mL and 0.26 µg/L MC-LR, respectively (Pinkanjananavee et al., 2021).

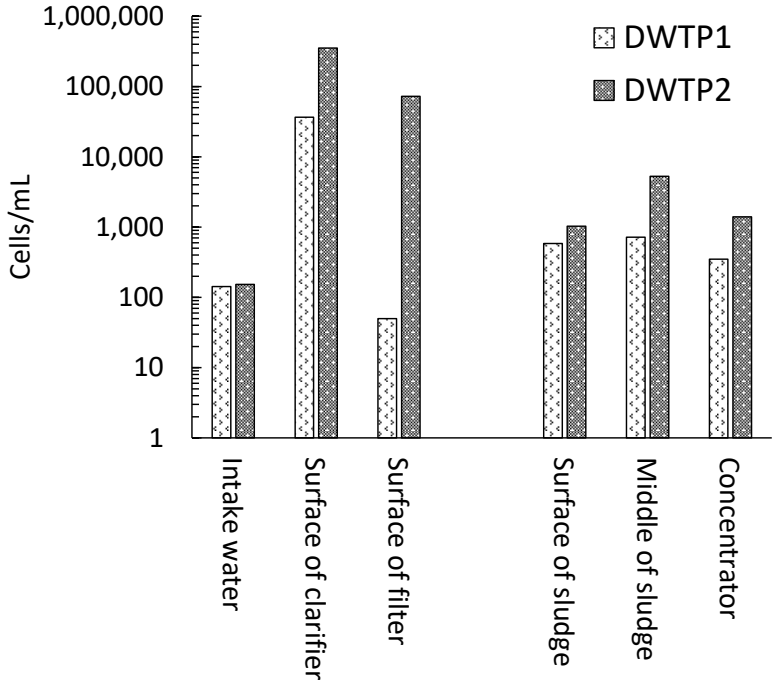

Figure 7-1 Cyanobacterial accumulation in two low risk DWTPs (Max. influx cell: < 500 cells/mL). Only DWTP1 has pre-ozonation. Average values from August to October 2011, adapted from (Zamyadi et al., 2013b).

7.3 Pre-oxidation Impact on Cyanobacteria Cells, Viability and Cyanotoxins

7.3.1 Impact of Pre-oxidation on Cyanobacteria Cell Counts

Cyanobacterial entry into DWTPs can be dampened by using pre-oxidation. Pre-oxidation may cause cell lysis, damage, and cyanotoxin release and degradation. Several studies were conducted to evaluate the pre-oxidation impact on cyanobacteria (e.g., cell viability and lysis) and cyanotoxins (release and degradation). A recent study tried to map the treatment barriers against cyanobacteria cells and cyanotoxins in drinking water facilities (Zamyadi et al., 2021). The results showed that the efficiency of the multi-barrier approach depends on the species present, metabolite concentration, and pre-oxidation dose (Zamyadi et al., 2021). Table 7-1-Table 7-4 summarize the literature on the impact of pre-oxidation on cyanobacteria (cultured-based and natural blooms) for four common oxidants (chlorine, ozone, potassium permanganate, and hydrogen peroxide). Table 7-1-Table 7-4 show that CT (residual concentration x contact time) is a main driver of cyanobacterial cell lysis, damage, cyanotoxin release, and degradation.

Although some studies have reported a reduction of more than 90% in taxonomic cell counts following pre-oxidation in the lab-cultured cells, Zamyadi et al. (2013c) reported a cell reduction of 70% at high chlorine exposure (CT 296 mg min/L). Fan et al. (2013b) showed a limited impact of chlorine exposure (CT 104 mg min/L) on the taxonomic cell counts of *Microcystis aeruginosa* (logarithmic phase). These observations might be related to the cyanobacteria stage of life and agglomeration. Furthermore, comparing the taxonomic cell count percentage in cultured-based and natural bloom studies demonstrates the lower impact of pre-oxidation during natural blooms. Figure 7-2 exhibits a lower impact of pre-ozonation (2 mg/L) on cell number reduction in a natural bloom in comparison with lab-cultured cyanobacteria.

Table 7-1-Table 7-4 and Figure 7-2 show that pre-oxidation, even at high CTs, may not be able to cause complete cell lysis. Consequently, it is important to clarify how far the pre-oxidation can cause viability loss and cyanotoxin release.

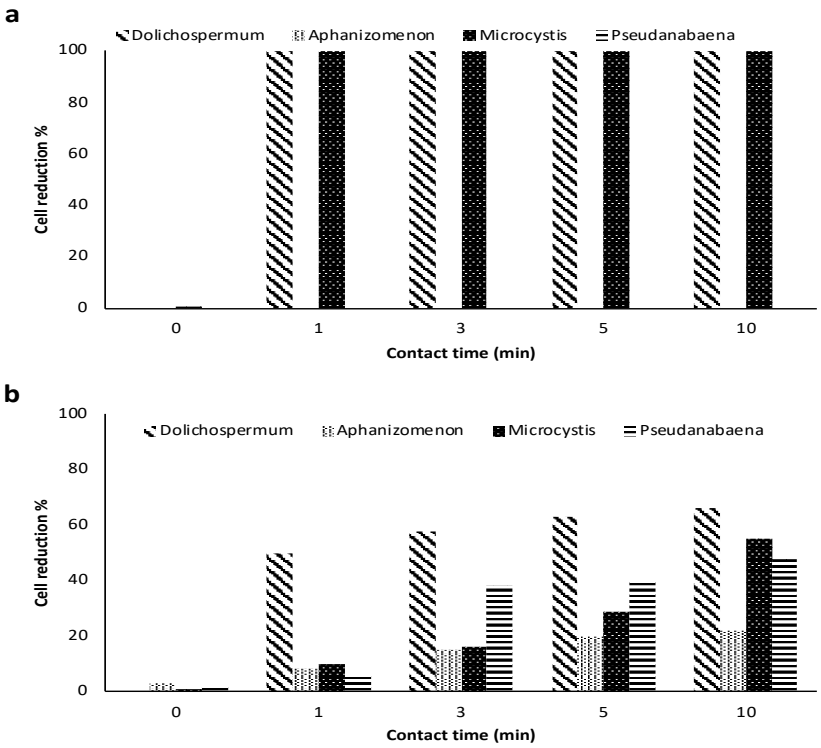

Figure 7-2 Comparison of cell count reduction following ozonation (2 mg/L) in the, a) Cultured *Dolichospermum*, *Microcystis*, (Coral et al., 2013b), b) Natural bloom (Zamyadi et al., 2015a).

Table 7-1 Summary of the literature on the impact of pre-chlorination on the cyanobacteria (culture and natural blooms), HV: high viability, LV: low viability, DV: Development stage, MA: maintenance stage.

Dominant Cyanobacteria (Cell counts)	Lab/ Field	Cl_2 Dose (mg/L)	Contact Time (min)	CT (mg.min/L)	Cell count reduction %	Cell Viability %	Toxins	Reference	Comment
Microcystis ($2x10^6$ cells/mL)	Lab	1-2	-	min. 15 Max. 90	-	min. 83 Max. 18.4	99% degradation	(Ding et al., 2010)	Saline solution, Exact dose and contact time were not provided. No residual. CT evaluation weak No cell-bound
D. circinalis (46,000 cells/mL)	Lab	2 3	0-60	min. 1.8 Max. 50	-	min. 15% 0 for CT 5.8	>100% release (CT 5.8) >90 degradation (CT 50)	(Zamyadi et al., 2010)	River Water, Using Fluorescein diacetate (FDA) for viability
Microcystis ($6x10^4$ cells/mL ($2.5x10^5$ cells/mL) ($5x10^5$ cells/mL)	Lab	2 4.5 10	0-60	min. 3 Max. 296.1	Max. 76%	-	>100% release (CT 5) >90 degradation (CT 35)	(Zamyadi et al., 2013c)	River water, ultrapure water, No viability was reported
Microcystis ($7x10^5$ cells/mL)	Lab	3, 4, 5	1, 2, 5, 10, 20, 30, 60	min. 2.8 Max. 104	Limited impact	< 5% (CT 4)	25% degradation (CT 2.8) Complete degradation (CT 104)	(Fan et al., 2013b; Fan et al., 2014b)	Ultrapure water
Microcystis ($2x10^6$ cells/mL)	Lab	0.5 0.7 1.5	5, 11, 50, 60, 120	min. 2.5 Max 180	-	<5% (CT 180)	10% degradation 40% increase in released	(Zhang et al., 2017a)	Lake water, No CT reported,
Microcystis (10^6 cells/mL)	Lab	0.2, 0.4, 0.8	Range 0-480	min. 12 Max. 396	-	18% (at CT 12) 0.1% (at CT 396)	-	(Qi et al., 2016)	Lake water, No CT reported No cell count, No toxin

Table 7-1 Summary of the literature on the impact of pre-chlorination on the cyanobacteria (culture and natural blooms) (continue)

Dominant Cyanobacteria (Cell counts)	**Lab/ Field**	**Cl_2 Dose (mg/L)**	**Contact Time (min)**	**CT (mg.min/L)**	**Cell count reduction %**	**Cell Viability %**	**Toxins**	**Reference**	**Comment**
Microcystis (10^6 cells/mL)	Lab	1, 2, 4, 8	1, 2, 4, 8, 16, 32, 60	HV min 0.98 Max 361 LV min. 0.98 Max 200	-	HV 95% - 0% (CT>15) LV 44% - 0% (CT>15)	HV CT↑ - degradation↑ Complete (CT 108) CT↑ - degradation↑ >50% release CT>7 >62% degradation at highest CT	(Li et al., 2020b)	Ultrapure water Two viability range
Microcystis ($1x10^6$ cells/mL) ($2x10^6$ cells/mL)	Lab	1, 2, 4, 8	1, 2, 4, 8, 16, 32, 60	DV min. 3.8 Max 356 MA Min 3.7 Max 293	>95% reduction (CT> 13.3) >95% reduction (CT> 11.9)	No cell viability after oxidation	Same as cell death	(Li et al., 2020a)	Ultrapure water Two stage of life
Microcystis-Colony (10^5 cells/mL)	Lab	0.3, 0.5, 1, 2	Range 0-20 min	min. 0.97 Max. 52	-	Depends on colony size (0-95%)	Release and degradation Colony size dependent	(Fan et al., 2016)	Lake water Different colony size No cell count
Natural Bloom	Field	Cl_2/DOC: 0.05-3.6	0-20 min	min. 0.15 Max 6.8	>80% increase (CT 6.8)	88% reduction**	Complete release CT:4 (Cl_2/DOC: 0.3)	(He and Wert, 2016)	No CT provided, CT estimated Chl-a measured as cell damage surrogate

Table 7-1 Summary of the literature on the impact of pre-chlorination on the cyanobacteria (culture and natural blooms) (continue)

Dominant Cyanobacteria (Cell counts)	Lab/ Field	Cl_2 Dose (mg/L)	Contact Time (min)	CT (mg.min/L)	Cell count reduction %	Cell Viability %	Toxins	Reference	Comment
Natural Bloom US ($3x10^6$ cells/mL)- *Planktothrix agardhii* CA ($3x10^5$ cells/mL)- *D.spiroides*	Field	Cl_2/DOC: 0.05, 025, 0.15, 0.1, 1	0- 20 min	US Min 0.13 Max 15 CA Min 0.3 Max 21	-	Complete degradation	Complete degradation CT 11 (US), CT 7.5 (CA)	(Greenstein et al., 2020a)	No cell viability No cell count Chl-a measured as cell damage surrogate
Natural Bloom ($3.3x10^5$ cells/mL) *D.spiroides* ($5.4x10^4$ cells/mL) *M.aeruginosa*	Field	0.2, 0.6	0- 120 min	Min 0.15 Max 3.84	min. CT 5% decrease Max. CT 34% decrease	Min CT: 82% Max CT:55%	CT 3.84: 23% decrease	(Moradinejad, 2021a)	Soft chlorination (low dose)
Natural Bloom	Field	2, 5	0- 60 min	Min 1.14 Max 14.8	min. <5% reduction Max. >50% reduction	-	2 mg, CT 10, >200% release 5 mg, CT 20, >200% release	(Zamyadi et al., 2012a)	No cell viability

Table 7-2 Summary of the literature on the impact of pre-ozonation on the cyanobacteria (culture and natural bloom)

Dominant Cyanobacteria (Cell counts)	**Lab/Field**	**O_3 Dose (mg/L)**	**Contact Time (min)**	**CT (mg.min/L)**	**Cell count reduction %**	**Cell Viability % (for CT)**	**Toxins**	**Reference**	**Comment**
Microcystis ($2x10^6$ cells/mL)	Lab	1 2	-	min. 12 Max 16	-	CT > 54, complete loss	CT=12 Complete degradation	(Ding et al., 2010)	Saline solution, Exact dose and contact time were not provided. No residual. CT evaluation weak
Microcystis ($7x10^5$ cells/mL)	Lab	2, 4, 6	5	min. <0.22 Max. 2.29	-	Min CT: 50% Max CT: 8.5%	>100% release (high CT) 50% degradation	(Fan et al., 2013b; Fan et al., 2014b)	Ultrapure water
Microcystis *D. flos-Aquae* ($2.5x10^4$ cells/mL ($1.5x10^5$ cells/mL)	Lab	0.5, 2 ,4	0.5 -10	min. <0.2 Max. 22	32% for 2 mg/L 41% for 4 mg/L	Complete loss, CT<0.2	-	(Coral et al., 2013a)	Ultrapure No flow cytometry
Microcystis ($2x10^5$ cells/mL) *Oscillatoria* (2800 cells/mL) *Lyngbya sp.* (1600 cells/mL)	Lab	0.63 - 5	24 h	min. 0.5 Max 17	100% reduction (CT 0.5)	**Complete loss (CT>2)	-	(Wert et al., 2013)	River water Chl-a measured as cell damage surrogate, No toxin measurement
Microcystis, *Dolichospermum* ($4x10^5$ cells/mL)	Lab	0.5, 1 , 2	5, 10	Max. 2.5	>95% reduction	Complete loss	-	(Moradinejad et al., 2019b)	Natural water No toxin measurement

Table 7-2 Summary of the literature on the impact of pre-ozonation on the cyanobacteria (culture and natural bloom) (continue)

Dominant Cyanobacteria (Cell counts)	**Lab/Field**	**O_3 Dose (mg/L)**	**Contact Time (min)**	**CT (mg.min/L)**	**Cell count reduction %**	**Cell Viability % (for CT)**	**Toxins**	**Reference**	**Comment**
Microcystis, *Dolichospermum* ($1.2x10^5$ – $2x10^6$ cells/mL)	Field	2,3,4,5	0-10	min. 1.4 Max 16.8	75% reduction (CT 16.8)	CT 3.2: 45% CT 16.8: 15%	CT<2, more than 100% release	(Zamyadi et al., 2015a)	Natural bloom
Natural Bloom US ($3x10^6$ cells/mL)- *Planktothrix agardhii* CA ($3x10^5$ cells/mL)- *D.spiroides*	Field	O_3/DOC: 0.05 – 0.75	0-20	US-min. 1.5 Max. 3 CA-Min 0.2 Max. 4.1	-	-	>80% degradation CT 4.1(CA)	(Greenstein et al., 2020a)	No cell viability, No cell count, Chl-a measured as cell damage surrogate
Natural Bloom ($3.3x10^5$ cells/mL) *D.spiroides* ($5.4x10^4$ cells/mL) *M.aeruginosa*	Field	0.1, 0.3	0-10	Max: 0.86	Max CT 14% decrease	Max CT: 79%	14% degradation No release	(Moradinejad, 2021a)	Soft ozonation (low dose)

Table 7-3 Summary of the literature on the impact of potassium permanganate on the cyanobacteria (cultured and natural bloom)

Dominant Cyanobacteria (Cell counts)	**Lab/Field**	**$KMnO_4$ Dose (mg/L)**	**Contact Time (h)**	**CT (mg.min/L)**	**Cell count reduction %**	**Cell Viability % (for CT)**	**Toxins**	**Reference**	**Comment**
Microcystis ($2x10^6$ cells/mL)	Lab	1 - 2	-	min. 15 Max. 600	-	Min CT: 60%, CT>60: complete loss	CT: 30 Complete dissolved degradation	(Ding et al., 2010)	Saline solution, Exact dose and contact time were not provided. No residual. CT evaluation weak
Microcystis ($7x10^5$ cells/mL)	Lab	1, 5, 10	0.25– 7	min. 28.7 Max. 2642	14% cell number reduction(CT max)	CT 2600: complete loss	Release at CT> 70 Complete degradation CT 2600	(Fan et al., 2013b; Fan et al., 2014b)	Ultrapure water
Microcystis, *Dolichospermum* ($4x10^5$ cells/mL)	Lab	2, 5	20	Max. 456	10% reduction at highest CT	CT 456: 18% viability	-	(Moradinejad et al., 2019a)	Natural water No toxin measurement
Microcystis Bloom Lake Erie	Lab Field	0.5 - 8	1– 5	min. 120 Max. 1920	-	Cell, CT 1920: 2% Bloom, CT 1920: 40%	-	(Piezer et al., 2020a)	No cell count and toxin No CT CT with lower doses was unable to decrease viability

Table 7-3 Summary of the literature on the impact of potassium permanganate on the cyanobacteria (cultured and natural bloom) – (continue)

Dominant Cyanobacteria (Cell counts)	**Lab/Field**	**$KMnO_4$ Dose (mg/L)**	**Contact Time (h)**	**CT (mg.min/L)**	**Cell count reduction %**	**Cell Viability % (for CT)**	**Toxins**	**Reference**	**Comment**
Natural Bloom US-($3x10^6$ cells/mL)- *Planktothrix agardhii* CA-($3x10^5$ cells/mL)- *D. spiroides*	Field	$KMnO_4$/DOC: 0.1 – 5.3	0-20	US min. 13 Max. 741 CA min. 17 Max. 782	-	-	US, CT 741: 40% degradation CA, CT 748: 30% degradation, 40% MC release	(Greenstein et al., 2020a)	No cell viability, No cell count, Chl-a measured as cell damage surrogate
Natural Bloom ($3.3x10^5$ Cells/mL)- *D. spiroides* ($5.4x10^4$ cells/mL) *M. aeruginosa*	Field	5	0.5 2	Max. 565	Max. CT: 57% decrease	Max. CT: 20%	Degradation dissolved (27%), Cell-bound (36%) No release	(Moradinejad, 2021a)	

Table 7-4 Summary of the literature on the impact of hydrogen peroxide on the cyanobacteria (culture and natural bloom), h: hour, d: day.

Dominant Cyanobacteria (Cell counts)	**Lab/ Field**	**H_2O_2 Dose (mg/L)**	**Contact Time**	**CT (mg.h/L)**	**Cell count reduction %**	**Cell Viability % (for CT)**	**Toxins**	**Reference**	**Comment**
Microcystis ($3.7x10^6$ cells/mL)	Lab	3.4, 17	4h, 2d, 4d	min. 13.6 Max 1632	min. CT: 8% reduction Max. CT: 89% reduction	K^+ release Min CT: 81% Max CT: 5%	CT>816 26% MC release	(Zhou et al., 2013)	K release as a surrogate for cell damage No CT provided
Microcystis ($7x10^5$ cells/mL)	Lab	10.2, 51, 102	0.1d – 7h	min. 189.3 Max 17678	Limited change	min. CT: 86% CT 4770: 7%	No release, CT 364: >95% degradation	(Fan et al., 2013b; Fan et al., 2014b)	Ultrapure water
Pseudanabaena (10^7 cells/mL)	Lab	3, 5, 10, 20	2h, 4h , 8h, 2d, 4d	min. 6 Max. 960	min. CT: No change Max. CT: >90% reduction	CT 120: 2%	-	(Xu et al., 2019)	Reservoir water No toxins
Microcystis ($6x10^6$ cells/mL)	Lab	1-15	0.1d – 7d	min. 2.4 Max 2520	CT 1680: 95% reduction	Max CT 3% viability	CT>1512, 82% degradation	(Zhou et al., 2020)	Culture No CT provided
Microcystis, Dolichospermum ($4x10^5$ cells/mL)	Lab	5, 10	6h	min. 13.9 Max 96.1	<5% reduction	Min CT: 39% Max CT: 30%	-	(Moradinejad et al., 2019b)	Natural water
Natural Bloom -($3.3x10^5$ Cells/mL) *D. spiroides* ($5.43x10^4$cells/mL) *M.aeruginosa*	Field	10	6h – 1d	min. 47 Max 140.7	Max CT 52% reduction	Min CT: 60% Max CT: 40%	No release Max 15% MC degradation	(Moradinejad , 2021a)	-

7.3.2 Chlorination

Figure 7-3 is a reconstructed graph from the cell viability results following pre-chlorination based on the oxidant exposure (CT). Parameters such as background water quality (e.g., pH and dissolved organic carbon (DOC)) which have an impact on the oxidant demand are included in the CT concept. Therefore, a comparable level of damage should be found by comparing cell viability results using oxidant exposure (CT) for lab-cultured and natural blooms. Figure 7-3 demonstrates that at the same level of chlorine exposure, the natural bloom is more resistant to pre-chlorination as compared to lab-cultured cells. In other words, lab-cultured studies are not representative of natural bloom pre-chlorination. Fan et al. (2016) reported that the level of cell damage and toxin release depends on the colony size. Figure 7-3b shows the cultured-based studies fitted with the Chick–Watson equation. Although the results from different unicellular studies are aligned with each other, the colonial *Microcystis* chlorination shows a different cell damage rate. This could be related to the agglomeration of cyanobacteria cells and increasing the mucilage sheath in colonial cyanobacteria (Fan et al., 2016). Despite using the CT calculation to compare the results, Figure 7-3c demonstrates different cell damage rates for each study of real bloom after chlorination, and the same level of chlorine exposure may not result in the same level of cell damage. Figure 7-3c shows that cyanobacterial bloom oxidation could be site- and bloom-specific, depending on the agglomeration, cyanobacteria (bloom) stage of life, and metabolic functions. Higher cell damage following pre-oxidation (especially with higher CTs) can lead to higher cyanotoxin release, which cannot be removed during conventional treatment. Soft chlorination showed cell damage by up to 45% and total microcystin (MC) degraded by up to 23%, while no cyanotoxin release was observed (Moradinejad, 2021a). In addition, soft chlorination may cause lower disinfection by-products as a lower oxidant concentration is used in this approach.

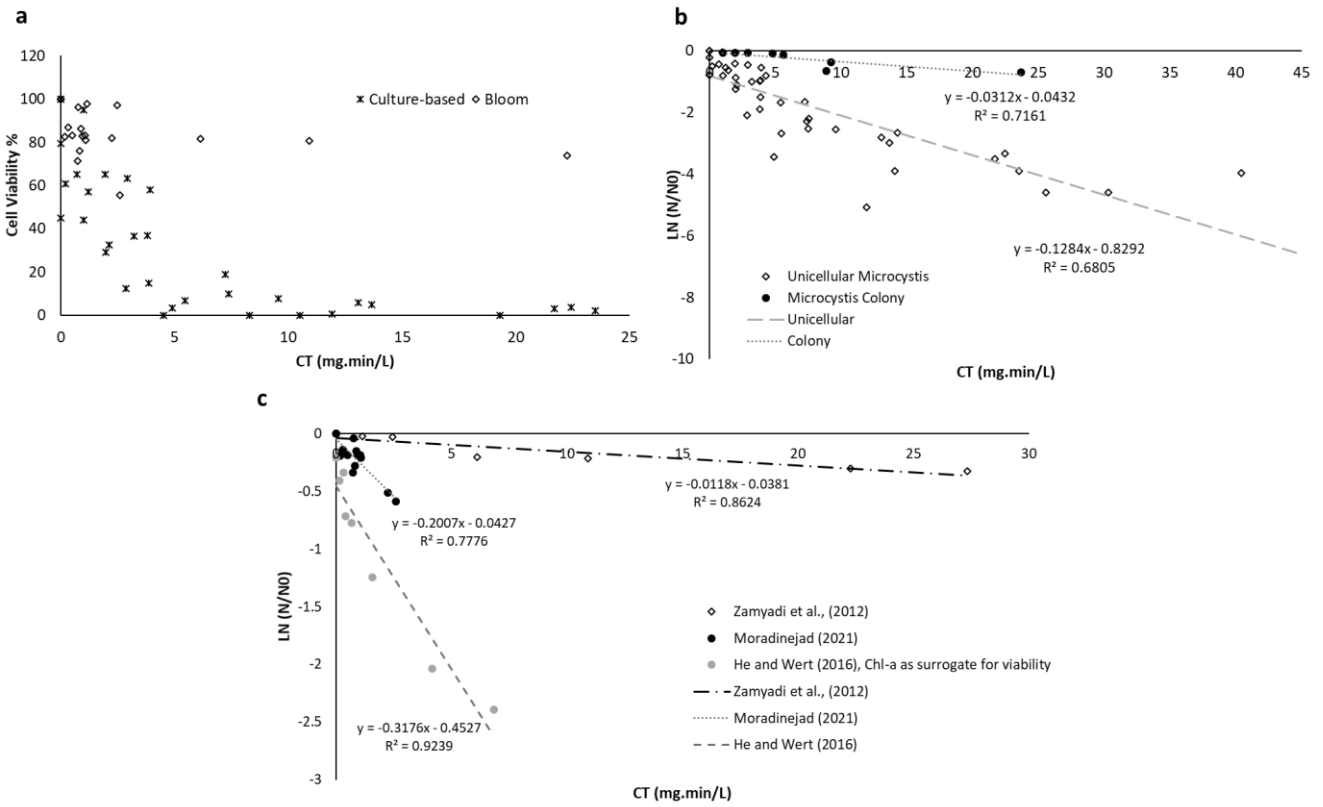

Figure 7-3 a) Comparison of cell viability results of culture-based *Microcystis* and, *Dolichospermum* (Daly et al., 2007; Fan et al., 2013b; Fan et al., 2014b; Li et al., 2020a; Li et al., 2020b) and natural cyanobacterial blooms (He and Wert, 2016; Moradinejad, 2021a; Zamyadi et al., 2012a) following pre-chlorination. (He and Wert, 2016) used Chl-a as a proxy for cell viability, b) Cell viability experimental data and fitted model of unicellular (Daly et al., 2007; Fan et al., 2013b; Fan et al., 2014b; Li et al., 2020a; Li et al., 2020b) and colonial *Microcystis* (Fan et al., 2016) following pre-chlorination, c) cell viability experimental data and fitted model for three different cyanobacterial blooms following pre-chlorination.

7.3.3 Ozonation

Figure 7-4 shows the impact of pre-ozonation on cyanobacteria cell damage for cultured-based and natural bloom studies. Figure 7-4a demonstrates lower cyanobacteria cell damage for a specific ozone exposure for natural blooms as compared to the lab-cultured cyanobacteria. The model fit results (Figure 7-4b) show a higher cell damage rate for the lab-cultured cyanobacteria in comparison to the natural bloom. As per soft oxidation, soft pre-ozonation was reported to cause up to 21% of cell damage and 14% of MC degradation, while no MC release was observed simultaneously (Moradinejad, 2021a). Such an observation implies the effectiveness of soft pre-ozonation to damage the cells without cyanotoxin release.

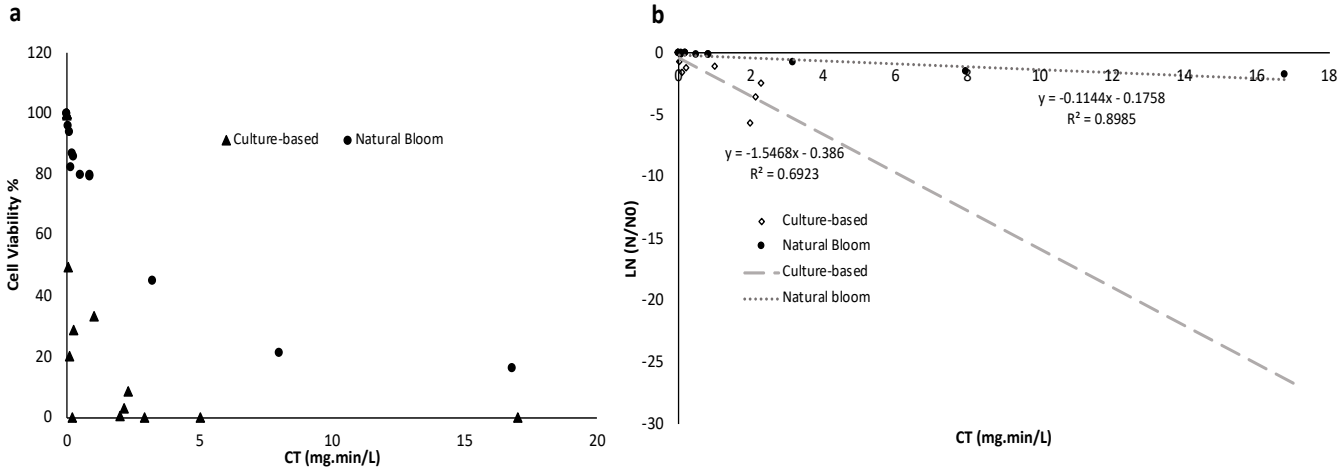

Figure 7-4 a) Comparison of cell viability results of culture-based *Microcystis, Dolichospermum, Oscillatoria, Lyngbya sp*. (Coral et al., 2013a; Fan et al., 2013b; Fan et al., 2014b; Moradinejad et al., 2019b; Wert et al., 2013) and natural blooms (Moradinejad, 2021a; Zamyadi et al., 2015a) following pre-ozonation, b) Cell viability experimental data and fitted model for culture-based and natural blooms following pre-ozonation.

7.3.4 Potassium Permanganate

Figure 7-5 demonstrates that the viability loss of the lab-cultured studies harvested in the logarithmic phase is lower than those that harvested in the stationary phase. This observation implies the impact of the cyanobacteria stage of life on pre-oxidation efficiency. A comparison of the cell viability results of the lab-cultured with natural bloom studies following potassium permanganate pre-oxidation confirms the higher resistance of real cyanobacterial bloom cells (Figure 7-5). In addition, the degradation rate constant of dissolved MCs was higher than that released by MCs for high potassium permanganate doses (Fan et al., 2013a; Piezer et al., 2020a).

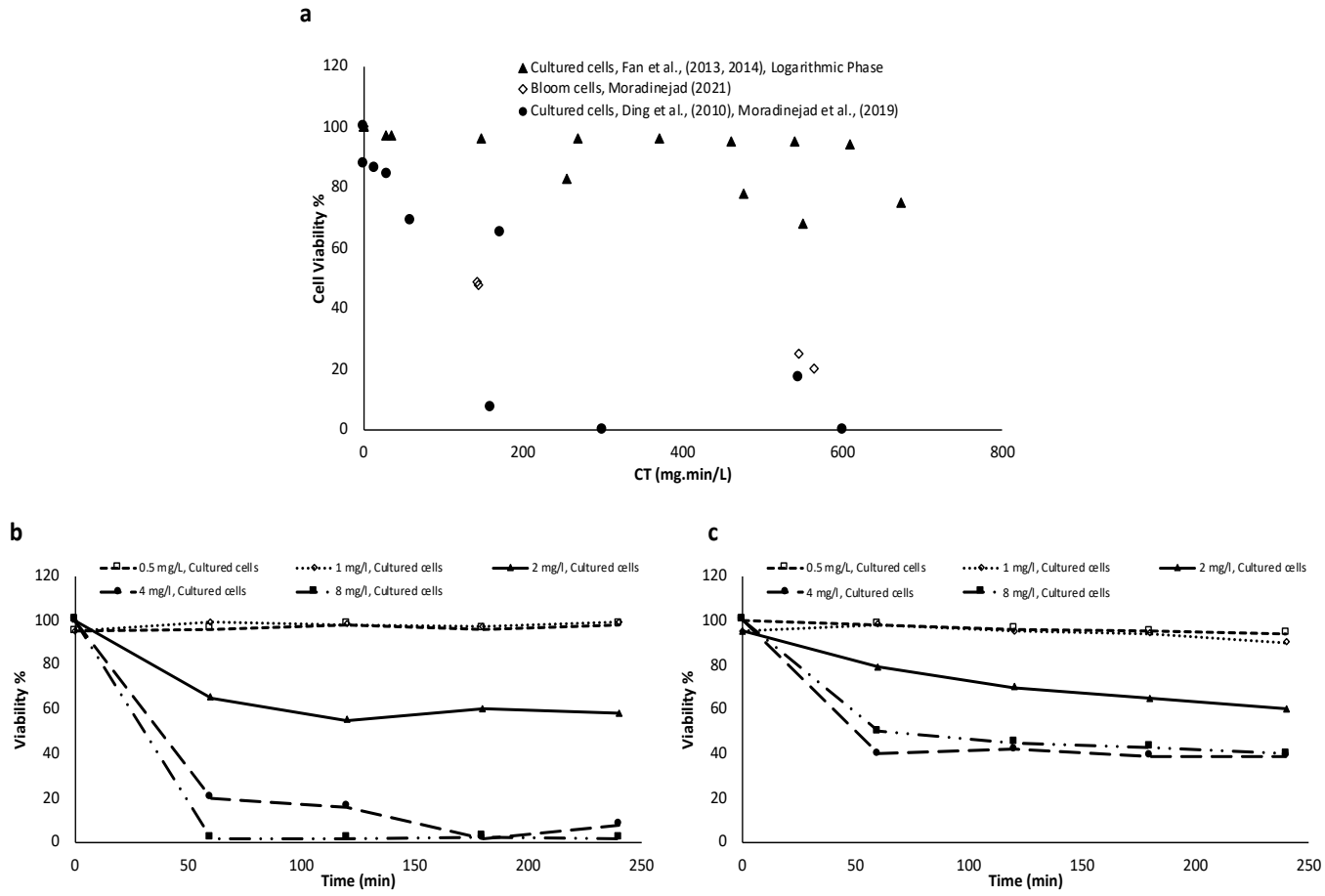

Figure 7-5 a) Comparison of cell viability results of culture-based and natural blooms for different studies following potassium permanganate peroxidation: *Microcystis* (Fan et al., 2013b; Fan et al., 2014b), *Microcystis*, *Dolichospermum* (Moradinejad et al., 2019b) and natural blooms (Moradinejad, 2021a) , b) Comparison of cell viability results of culture-based (*Microcystis*) following potassium permanganate peroxidation adapted from (Piezer et al., 2020a), *c)* Comparison of cell viability results of natural blooms (Lake Erie) adapted from (Piezer et al., 2020a).

7.3.5 Hydrogen Peroxide

Matthijs et al. (2012) reported that a concentration of 2 mg/L H_2O_2 was able to decrease cyanobacteria (natural bloom) by two logs within 3 days. In addition, cyanobacteria remained at a low abundance level for 7 weeks following H_2O_2 addition. Figure 7-6 demonstrates that natural blooms are more resistant to H_2O_2 than the lab-cultured cyanobacteria, as observed for other oxidants (Figure 7-2-Figure 7-5). Foo et al. (2020) reported that the impact of H_2O_2 on cyanobacteria is dependent on the residual concentration (C) and contact time (T). In addition, the authors concluded that toxic and non-toxic *Microcystis aeruginosa* are impacted by H_2O_2 with the same trend. Zhou et al. (2020) stated that a low dose of H_2O_2 (< 5 mg/L) would have a low and recoverable impact on the lab-cultured *Microcystis*. On the other hand, the higher the H_2O_2 dose (> 8 mg/L), the higher necrosis, cell death, and consequent cyanotoxin release. A medium dosage of H_2O_2 with low to medium contact time can activate apoptosis-like programmed cell death (AL-PCD) (Zhou et al., 2020). The cellular energy required for AL-PCD is provided from the transcriptional, biochemical, and structural changes. Zhou et al. (2020) documented the maximum cell death with low MC production by AL-PCD activation. Zamyadi et al. (2020) studied the impact of H_2O_2 on blooms and lab-cultured cyanobacteria (M*icrocystis aeruginosa*). The results highlighted a delayed impact of H_2O_2 on cyanobacteria cells after complete depletion of H_2O_2 during stagnation (up to one week) (Zamyadi et al., 2020). Chl-a and phycocyanin (PC) fluorescence were significantly declined by 93% and 74% in natural bloom and lab-cultured samples, respectively. Additionally, the lab-cultured results revealed delayed MC release during stagnation (Zamyadi et al., 2020).

Besides the current oxidants, peracetic acid (PAA) has been used in wastewater treatment facilities as a disinfection alternative for chlorine (Luukkonen et al., 2014). Almuhtaram and Hofmann (2022) studied the impact of PAA and PAA/UV on cyanobacteria and cyanotoxin removal. The results show that 10 mg/L of PAA with 60 min contact time was able to degrade MC-LR by 80% (3.46 $M^{-1}\ s^{-1}$ lower reaction rate as compared to HOCl $1.2 \times 10^2\ M^{-1}s^{-1}$). In addition, the results elaborated that PAA alone can barely remove cyanobacteria, except at a high dose (10 mg/L) and with lower cyanobacterial cell counts (10^5 cells/mL).

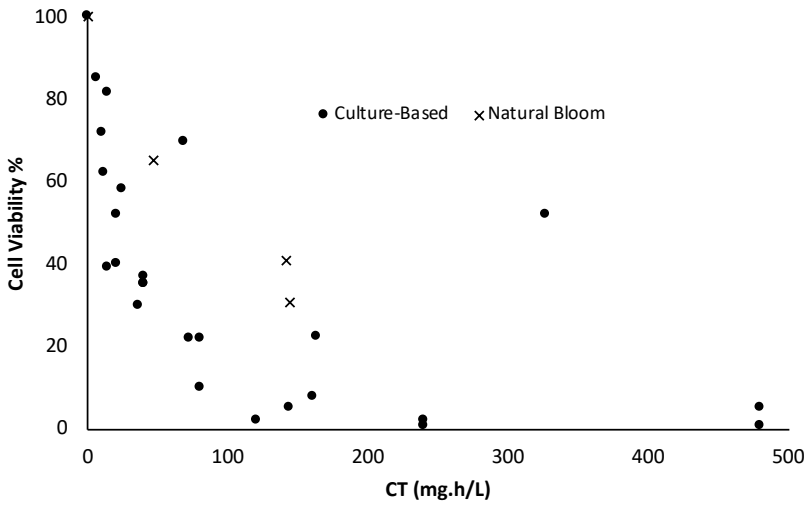

Figure 7-6 Comparison of cell viability results of culture-based cyanobacterial cells (*Microcystis*, *Pseudanabaena*) (Fan et al., 2013b; Fan et al., 2014b; Xu et al., 2019; Zhou et al., 2020) and natural bloom (Matthijs et al., 2012; Moradinejad, 2021a) after oxidation by hydrogen peroxide.

7.3.6 Considerations on the Impact of Pre-Oxidation on Downflow Processes

The impact of pre-oxidation on downflow processes should also be considered as it may influence the removal of cyanobacteria by coagulation, flocculation, and sedimentation. Previous studies have been reported that pre-oxidation has a positive impact on enhancing cyanobacterial removal through coagulation/flocculation and sedimentation (Lapsongpon et al., 2017; Lin et al., 2018; Naceradska et al., 2017; Wang et al., 2015a; Xie et al., 2016; Xie et al., 2013). Pre-oxidation can cause morphological deformation (Moradinejad et al., 2019b) and changes in the surface charge of the cells, leading to increased cell removal efficiency during coagulation/flocculation (Fan et al., 2013b).

$KMnO_4$ increases the binding potential to the coagulant by oxidizing organic matter (extracellular and released cell-bound) to lower molecular weight fractions, as well as forming colloids (by MnO_2) to be adsorbed to the cyanobacterial cells and forming larger flocs (Naceradska et al., 2017; Piezer et al., 2020a; Xie et al., 2016). Xie et al. (2013) reported that $KMnO_4$ exposure (CT: 10 mg min/L, estimated) could increase cyanobacteria cell removal by 22% during coagulation/flocculation. In addition, pre-ozonation with CT: 4, 10, and 20 mg min/L (estimated) led to an increase in cyanobacteria cell removal during coagulation by 14%, 20%, and 24%,

respectively (Xie et al., 2013). Cyanobacteria cell removal during coagulation was improved in a full-scale DWTP equipped by pre-ozonation systems (CT: 2.52–3.78 mg min/L (estimated)) (Zamyadi et al., 2013b). Pre-oxidation may cause metabolite release (organic matter and cell-bound cyanotoxins) following cyanobacterial cell damage. Besides the challenge to remove dissolved cyanotoxins, coagulation efficiency can be compromised by high algal organic matter release following pre-oxidation. Xie et al. (2013) showed that due to pre-ozonation with CT > 4 mg min/L (estimated), cyanobacteria cell viability was completely degraded, and consequently, organic matter concertation increased. Further, Barešová et al. (2020) demonstrated that pre-ozonation (CT < 40 mg min/L (estimated)) could interrupt the coagulation (Al/Fe-based) efficiency of DOC removal (in comparison with higher CTs) due to the degradation of high molecular weight algal organic matter to low molecular weight compounds.

It is noteworthy to recall that H_2O_2 can have a delayed impact on cyanobacteria and, potentially, cyanotoxin release after complete degradation of the oxidant (Zamyadi et al., 2020). This delayed cyanotoxin release should be considered in the downstream processes, as well as in sludge handling.

The oxidant exposure must be adjusted to maximize cell damage and cyanobacteria cell removal (directly or after coagulation) and minimize cyanotoxin release and cell accumulation in the sludge, simultaneously. Figure 7-7 summarizes the pre-oxidation (soft and normal) advantages/disadvantages of cyanobacteria and cyanotoxins during water treatment. In fact, soft pre-oxidation (low CT of Cl_2 and O_3) can (1) partially degrade cyanobacteria cells, (2) cause low cyanotoxin release, (3) improve coagulation efficiency to remove cells, and (4) cause low cell accumulation in the downflow processes.

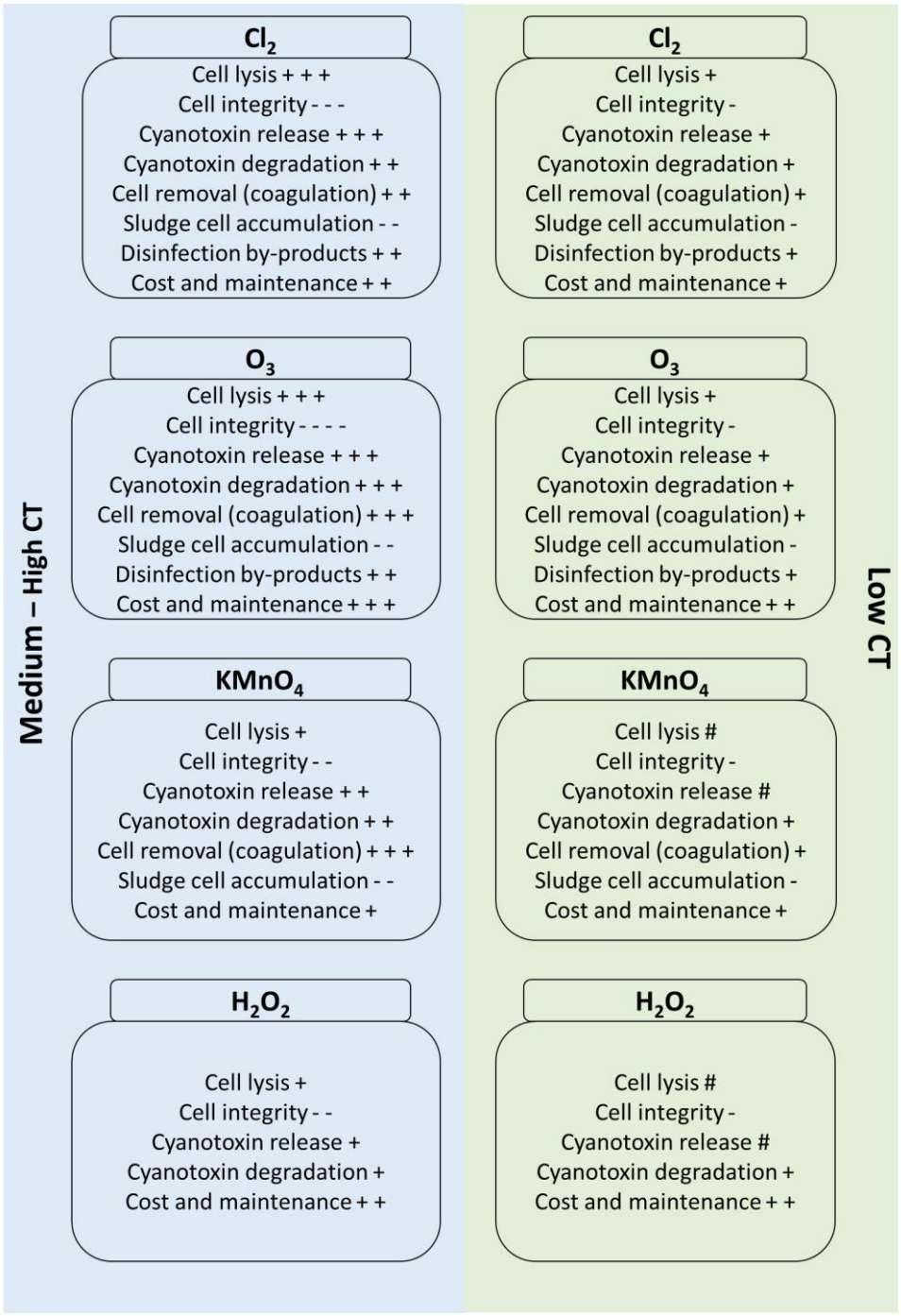

Figure 7-7 Summary of pre-oxidation (low and medium-high CT) impact on cyanobacteria/cyanotoxins and downflow processes (#: very low impact, +: increase, and -: decrease). Low CT for Cl_2 = CT < 4 mg min/L, low CT for O_3 = CT < 1 mg min/L, low CT for $KMnO_4$ = CT < 50 mg min/L, and low CT for H_2O_2 = CT < 50 mg h/L.

7.4 Sludge Storage, Oxidation, and Handling

Cyanobacteria and cyanotoxins (cell-bound) accumulate in the sludge of clarifiers throughout the flocculation/coagulation/sedimentation processes. This cyanobacteria-laden sludge remains in the sludge holding tank before disposal. In addition, potential options to treat cyanobacteria-laden sludge need to be considered. Furthermore, safe (healthy, both operationally and environmentally) cyanobacteria-laden sludge handling approaches are required.

7.4.1 Fate of Cyanobacteria and Cyanotoxins during Sludge Storage

Several studies (Table 7-5) demonstrated that cyanobacteria cells could stay viable within 2–12 days in the stored sludge. The loss of viability and consequent cyanotoxin release caused an increase in dissolved cyanotoxin concentrations during sludge storage (Drikas et al., 2001; Ho et al., 2012; Li et al., 2018; Li et al., 2015; Ma et al., 2016b; Sun et al., 2015; Sun et al., 2013; Sun et al., 2012; Xu et al., 2017; Xu et al., 2016). However, dissolved cyanotoxins in stored sludge can be adsorbed onto the remained PAC injected into the intake water (Jalili et al., 2021), flocs (Ma et al., 2016b) or it can be biodegraded by cyanotoxin degrader species (Jalili, 2022; Kormas and Lymperopoulou, 2013).

Besides cell survival potential during sludge storage, some studies have hypothesized that cyanobacteria can also grow in stored sludge (Dreyfus et al., 2016; Pestana et al., 2016; Water Research Foundation (WRF) and Water Research Australia, 2015). Water Research Foundation (WRF) and Water Research Australia (2015) documented that concentrations of DOC, MC-LR, and cylindrospermopsin in stored coagulated sludge contained *M. aeruginosa* and *C. raciborskii* exceeded the expected concentrations by 4–10-fold based on the cell quota (if all cell-bound metabolites are released) within 7–16 days, respectively. Dreyfus et al. (2016) studied the fate of stored sludge that contained cultured *M. aeruginosa*, *D. circinale*, and *C. raciborskii* within 18 days. The authors demonstrated that DOC, MC-LR, MC-LA, and CYN concentrations increased by up to 5-, 2.2-, 1.2-, and 2.5-fold during storage, respectively. Another investigation on stored sludge containing cultured *M.aeruginosa* and *D. circinale* reported that taxonomic cell counts increased by up to 4.2-fold in sludge stored in a lagoon within 7 days (Pestana et al., 2016). The authors also reported that the concentrations of cyanobacterial metabolites increased by up to 5 times in the sludge supernatant within 20 days. In the worst case, cyanobacteria could survive by

up to 35 days in the stored sludge (Pestana et al., 2016). Despite the important findings of the previous studies on cell survival and metabolite release during sludge storage, cyanobacterial cell growth during sludge storage is yet to be explored in detail. In these studies, cell and metabolite increase during sludge storage might be due to the cell growth or to either the (1) underestimation of cell quota, (2) increase of metabolite production per cell during storage, or (3) additional cell settling from the supernatant to the sludge during the storage (Dreyfus et al., 2016; Pestana et al., 2016; Water Research Foundation (WRF) and Water Research Australia, 2015).

Our recent study on the cyanobacteria-laden sludge of a DWTP documented cell depletion, survival, and growth in different sludge samples (Jalili, 2022). Cell growth was observed in four out of eight sludge samples (different sampling dates) stored in the dark for 7–38 days. In the worst-case scenario, taxonomic cell counts increased from 2.7×10^6 to 5.3×10^6 cells/mL within 16 days (96% cell growth). Cell growth was also confirmed by increasing cyanobacterial biomarkers such as the "Pentose phosphate pathway" marker, which is responsible for the heterotrophic growth of cyanobacteria (Min and Golden, 2000).

7.4.2 Cyanobacteria-Laden Sludge Treatment

A summary of studies on the treatment of cyanobacteria-laden sludge is presented in Table 7-6. The available data demonstrated that sludge oxidation could not completely remove cyanobacteria cells and metabolites from the sludge (Jalili et al., 2022b; Zamyadi et al., 2016b). Sludge is often stored after oxidation, while its supernatant can be recycled to the head of the DWTP. Thus, the impact of oxidation on sludge storage should be investigated. Recent findings showed no remarkable benefits in sludge oxidation followed by sludge storage as compared to only sludge storage (Jalili et al., 2022b). The maximum additional taxonomic cell count decreased by a combination of oxidation ($KMnO_4$ or H_2O_2) and storage was 32% as compared to storage only. However, oxidation/storage could cause a remarkable cell growth (by up to 145%) and toxic gene copy numbers of *mcyD* increase (by up to 13.0×) in some sludge samples (Jalili et al., 2022b). This phenomenon can be attributed to gene expression regulation due to the presence of oxidative stresses (Jalili, 2022; Jalili et al., 2022b; Pimentel and Giani, 2014; Schatz et al., 2007). Similarly, sludge oxidation could not completely remove cyanobacteria and cyanotoxins from the supernatant sludge (Jalili et al., 2022b). Finally, the costs and by-product formation during the oxidation of organic-matter-rich sludge should be considered (Jalili, 2022).

7.4.3 Sludge Handling Challenges

In general, the sludge supernatant is recycled to the head of the DWTP or is discharged into the source (Chow et al., 1999; Drikas et al., 2001; Jalili et al., 2021). The solid phase either can be transferred to the WWTP or is applied for landfilling (Qrenawi and Rabah, 2021; Turner et al., 2019; United States Environmental Protection Agency (USEPA), 2011). Less environmentally friendly approaches such as untreated residual discharge into lakes or ponds can be also applied in some circumstances (Ahmad et al., 2016). The re-use of DWTPs' residuals is growing (Jung et al., 2016; Suman et al., 2017; Turner et al., 2019; Xu et al., 2015). However, an investigation demonstrated that the half-life of MC analogs varies from 8 to 18 days in soil (Chen et al., 2006). Since there is a risk of soil and groundwater contamination, landfill and field applications of cyanobacteria-laden sludge should be avoided. Overall, cyanobacteria-laden sludge should be treated before disposal either in situ or via sending it to wastewater treatment plants.

Flocs may have a protective role during sludge storage for *M. aeruginosa* (Li et al., 2018; Li et al., 2015; Sun et al., 2015; Sun et al., 2013; Sun et al., 2012). In contrast, Li et al. (2018) documented that polyaluminium ferric chloride (PAFC) can stimulate the lysis of *C. raciborskii* and CYN release by up to 94% during sludge storage. This may occur in the sludge and lead to cyanotoxin release. However, all studies have been conducted in laboratory conditions and on cultured-based cyanobacteria. In fact, due to complex parameters such as the presence of various cyanobacterial cells in various forms (aggregated, multicellular), ages, and viabilities, the design of such experiments in full scale is complex.

Stresses such as oxidation and storage can shift cyanobacterial communities towards resistant genera (e.g., *Microcystis* and *Aphanocapsa*), which can produce MCs (Jalili, 2022; Jalili et al., 2021; Jalili et al., 2022b). Thus, the survival probability of MC producer species can increase during sludge oxidation or storage. The fate of cyanotoxins in the sludge is complex due to the simultaneous occurrence of various phenomena such as cell survival, growth, lysis, cell-bound cyanotoxin release, and released cyanotoxin degradation (Clemente et al., 2020; Dreyfus et al., 2016; Jalili et al., 2021; Jalili et al., 2022b; Pestana et al., 2019; Pestana et al., 2016; Water Research Foundation (WRF) and Water Research Australia, 2015). Based on the increased risk of cell lysis and cyanotoxin release during sludge storage, some studies have suggested that cyanobacteria-laden sludge should be disposed of prior to 4 days to avoid metabolite release (Pei et al., 2017; Sun

et al., 2015; Xu et al., 2017). However, these studies only focused on metabolite release and not on cell survival/growth phenomena. Additionally, the possibility of sludge disposal can be a technical and financial challenge in large DWTPs.

Table 7-5 Impact of sludge storage on cyanobacteria and cyanotoxins, STX: Saxitoxin, PACl: Polyaluminium chloride, CTSAC: Chitosan-aluminum chloride

Initial characteristics of Cyanobacteria/ Coagulation/sedimentation process	**Initial condition of cyanobacteria and cyanotoxins in the stored sludge**	**Observation**	**Reference**
Cultured *M. aeruginosa* (1×10^6 cells/mL) (Jar test, 70 mg/L alum)	8×10^6 cells/mL, 2,500 µg MC-LR /L	Cell survival (2 days) Cell lysis and cyanotoxin release (2 days) Degradation of dissolved cyanotoxins (8-10 days)	(Drikas et al., 2001)
Cultured *D. circinale* and *C. raciborskii* (1.0×10^5 cells/mL) (Jar test, 40 mg/L alum)	Sludge supernatant: *D. circinale*: 1300 cells/mL STX: 0.4 µg/L	Cells remained viable up to 7 days Cell lysis and toxin release within 3 days	(Ho et al., 2012)
Cultured *M. aeruginosa* (2×10^6 cells/mL) (Jar test, 15 mg/L $AlCl_3$)	18 µg/L dissolved MCs	Cell lysis and cyanotoxin release after 6 days	(Sun et al., 2012)
Cultured *M. aeruginosa* (1×10^6 cells/mL) (Jar test, 4 mg/L PACl- optimum dose)	20 µg/L dissolved MCs	Cell lysis and cyanotoxin release within 6-12 days	(Sun et al., 2013)
Microcystis flos aquae (5.2×10^5 cells/mL) (Jar test, 100 mg/L alum)	Sludge supernatant: MC-RR, MC-YR: < 2 µg/L	Cell survival (5 days) Cell lysis and cyanotoxin release (5-10 days) Degradation of dissolved cyanotoxins (up to 15 days)	(Ho et al., 2013)
Cultured *M. aeruginosa* (1×10^6 cells/mL) (Jar test, 15 mg/L $ALCl_3$, 4 mg/L PACl)	-0.9 bar vacuum pressure for dewatering the sludge 23 µg/L total MCs	Cell lysis and cyanotoxin release within 4- 6 days Optimum sludge storage time for $AlCl_3$ and PACl was suggested 4 and 2 days, respectively.	(Sun et al., 2015)
Cultured *M. aeruginosa* (1×10^6 cells/mL) (Jar test, 0-70 mg/L $FeCl_3$)	~1 µg/L dissolved MCs	Cell lysis and cyanotoxin release (2-8 days) Degradation of dissolved cyanotoxins (> 10 days)	(Li et al., 2015)

Table 7-5 Impact of sludge storage on cyanobacteria and cyanotoxins, STX: Saxitoxin, PACl: Polyaluminium chloride, CTSAC: Chitosan-aluminum chloride (continue)

Initial characteristics of Cyanobacteria/ Coagulation/sedimentation process	**Initial condition of cyanobacteria and cyanotoxins in the stored sludge**	**Observation**	**Reference**
Myponga reservoir Cultured *M. aeruginosa* (2.3×10^5 cells/mL) Cell-bound MC-LR: 4.7 µg/L Dissolved MC-LR: 2.0 µg/L (Jar test-80 mg/L alum)	Sludge supernatant after 1 day storage: Cells: 4300 cells/mL Cell-bound MC-LR: 0.5 µg/L Dissolved MC-LR: 2.5 µg/L	Cell survival (4 days) Cell lysis and cyanotoxin release (4-7 days) Degradation of dissolved cyanotoxins (> 4 days)	(Water Research Foundation (WRF) and Water Research Australia, 2015)
Myponga reservoir Cultured *M. aeruginosa* (3.1×10^5 cells/mL) DOC: 10.1 mg/L Cell-Bound MC-LR: 5.0 µg/L Dissolved MC-LR: 2.9 µg/L (Jar test-80 mg/L alum)	Sludge supernatant after 1 day storage: DOC: 5.2 mg/L, Cell: 2760 cells/mL Cell-bound MC-LR: < DL Dissolved MC-LR: 4.0 µg/L	Cell growth (within 7-16 days) confirmed by DOC and MC-LR cell quota.	(Water Research Foundation (WRF) and Water Research Australia, 2015)
Myponga reservoir Cultured *C. raciborskii* (3.1×10^5 cells/mL) DOC: 10 mg/L Cell-bound CYN: 2.5 µg/L Dissolved CYN: 0.7 µg/L (Jar test-80 mg/L alum)	Sludge supernatant after 1 day storage: DOC: 6.0 mg/L Cell: 7080 cells/mL Cell-bound CYN: 1.0 µg/L Dissolved CYN: 0.8 µg/L	Cell growth (within 7-23 days) confirmed by DOC and CYN cell quota.	(Water Research Foundation (WRF) and Water Research Australia, 2015)
River Murary Cultured *C. raciborskii* (3.1×10^5 cells/mL) DOC: 8.63 mg/L Cell-bound CYN: 2.7 µg/L Dissolved CYN: 0.3 µg/L (Jar test-80 mg/L alum)	Sludge supernatant after 1 day storage: DOC: 4.9 mg/L Cell: 4140 cells/mL Cell-bound CYN: 0.3 µg/L Dissolved CYN: 0.9 µg/L	Cell growth (within 15-23 days) confirmed by DOC and CYN cell quota.	(Water Research Foundation (WRF) and Water Research Australia, 2015)

Table 7-5 Impact of sludge storage on cyanobacteria and cyanotoxins, STX: Saxitoxin, PACl: Polyaluminium chloride, CTSAC: Chitosan-aluminum chloride (continue)

Initial characteristics of Cyanobacteria/ Coagulation/sedimentation process	**Initial condition of cyanobacteria and cyanotoxins in the stored sludge**	**Observation**	**Reference**
Cultured *M. aeruginosa* (1×10^6 cells/mL) (Jar test- 15 mg/L $AlCl_3$, 50 mg/L $FeCl_3$, 15 mg/L PAFC)	20 µg/L dissolved MCs 1- 4.2 mg/L dissolved polysaccharides 4 mg/L chla	Cell lysis and toxin release (2-10 days)	(Xu et al., 2016)
Cultured *M. aeruginosa* (2×10^6 cells/mL) (Jar test, 2.6 mg/L chitosan- 7.5 mg/L $AlCl_3$ (CTSAC)	9 µg/L dissolved MCs (after coagulation) 18 µg/L dissolved MCs (without coagulation) The difference is due to adsorption in CTSAC	Toxin release (0-4 days) Degradation of dissolved cyanotoxins (6-10 days)	(Ma et al., 2016b)
M. aeruginosa, D. circinale, C. raciborskii (3.0×10^5 cells/mL) (Jar test, 80 mg/L Alum)	Sludge supernatant after 1 day storage: DOC: 5.2-6.5 mg/L Cell: 2162-7080 cells/mL Cell-bound MC-LR: < 0.5 µg/L Dissolved MC-LR: 2.5-4.0 µg/L Cell-bound CYN: 1.0 µg/L Dissolved CYN: 0.8 µg/L	Increased DOC, MC-LR, MC-LA and CYN to higher the expected values. (Hypothesis: Increase of the metabolite production, cell growth or both)	(Dreyfus et al., 2016)
M. aeruginosa and *D. circinale* (8.6×10^4 cells/mL) (Jar test, 80 mg/L Alum)	Non-coagulated sludge: 5.0×10^6 cells/mL Coagulated sludge: 5.4×10^5 cells/mL	Cell survival (up to 35 days), 4.2X increase in cell counts in the sludge lagoon within 7 days Increased metabolites to higher the expected values (up to 5X) Increased cell counts in the sludge (Hypothesis: cell growth, additional settling or both)	(Pestana et al., 2016)
Cultured *M. aeruginosa* $\times 10^5$ cells/mL) (Jar test, 15 mg/L $AlCl_3$, 50 mg/L FeCl3, 15 mg/L PAFC)	1 µg/L dissolved MCs	Cell lysis and toxin release (4-6 days); Degradation of dissolved cyanotoxins (6-10 days)	(Xu et al., 2017)

Table 7-5 Impact of sludge storage on cyanobacteria and cyanotoxins, STX: Saxitoxin, PACl: Polyaluminium chloride, CTSAC: Chitosan-aluminum chloride (continue)

Initial characteristics of Cyanobacteria/ Coagulation/sedimentation process	**Initial condition of cyanobacteria and cyanotoxins in the stored sludge**	**Observation**	**Reference**
Cultured *Oscillatoria sp.* (1.0×10^4 cells/mL) (Jar test, 5 and 10 mg/L PAFC)	1.0 mg/L chla 2.3 µg/L cell-bound protein 8.6-11.4 µg/L dissolved CYN	Increase in chla level after 4 days suggesting cell growth, Loss of cell integrity after 2 days, while cells remained viable up to 8 days Increase in dissolved CYN showing toxin release within 4 days	(Sun et al., 2018)
Cultured *C. raciborskii* (1×10^6 cells/mL at late exponential phase) (Jar test, 10 mg/L PAFC)	1.1 µg/L dissolved CYN 2 mg/L cell-bound protein	Cell lysis and toxin release after 6 days Degradation of dissolved cyanotoxins after 10 days	(Li et al., 2018)
n/a	Sledge of a DWTP containing natural cyanobacterial blooms stored for 7-35 days in the darkness (8 samples). 0.7 x 105- 5.6 x 106 cells/mL 25-7130 ng/L cell-bound MCs 38-349 ng/L dissolved MCs	Cell growth in 4/8 samples after 9-35 stagnation days, Cell death in the rest 4/8 samples, Degradation of dissolved cyanotoxins after 8 days.	(This study)

Table 7-6 Data of cyanobacteria-laden sludge treatment

Source of sludge	Scale	Treatment agent / dosage	contact time	Initial conditions	Cell count reduction	Metabolite reduction	Reference
Sludge thickener	Laboratory	3 mg/L $KMnO_4$	2 h	5.0 x 10^4 cells/mL *Pseudanabaena*	> 95%	-	(Zamyadi et al., 2016b)
	Laboratory	10-100 mg/L PAC	1 h	100 ng/L MIB	-	42-100% MIB	
	Full-scale	10 mg/L $KMnO_4$	15 h (Max.)	4.3x10^5 cells/mL	13-98% total and *Pseudanabaena* cell counts	-	
	Full-scale	10 mg/L $KMnO_4$ 20 mg/L PAC	$KMnO_4$: 24-72 h PAC: 1 h	3.7 x 10^5 cells/mL 120 ng/L MIB	40-52% in total and *Pseudanabaena* cell counts	20-22% MIB	
Sludge holding tank	Laboratory	5 mg/L $KMnO_4$	60 min	2.3-2.7 x10^6 cells/mL 63-161 ng/L MCs	46-55% total cell counts	2- 24% MCs	(Jalili et al., 2022b)
		10 mg/L $KMnO_4$			59-62% total cell counts	2- 32% MCs	
		10 mg/L H_2O_2	24 h		58% total cell counts	27% MCs	
		20 mg/L H_2O_2			77% total cell counts	41% MCs	
	Full-scale (shock oxidation)	10 mg/L $KMnO_4$	24- 72 h	2.4 x10^6 cells/mL 88-1083 ng/L MCs	24-43% total cell counts (31% cell count increase after 48 h in one sample)	MCs: 3-25% decrease in one sample 37-589% increase in one sample	

7.5 Decision Framework to Manage Cyanobacteria and Cyanotoxins in Drinking Water Treatment Plants Framework basis

7.5.1 Framework Basis

Since cyanobacterial cells and their associated metabolites, including cyanotoxins, as well as taste and odor agents such as geosmin and 2-Methylisoborneol (MIB), affect water and sludge quality, monitoring should be applied for the evaluation of the water treatment chain and sludge handling.

Microscopy taxonomic cell count techniques have been widely applied to evaluate the water and sludge in previous studies (Almuhtaram et al., 2018; Jalili et al., 2021; Zamyadi et al., 2013a; Zamyadi et al., 2012a, 2013b; Zamyadi et al., 2012c). Previous cyanobacterial monitoring guidelines were prepared based on taxonomic cell counts and biovolumes (Chorus and Bartram, 1999b; Chorus and Welker, 2021; Ellis, 2009). A recent study suggested 0.3 mm^3/L biovolumes as the vigilance level (Chorus and Welker, 2021). However, bias related to human error (America Water Works Association (AWWA), 2010), the negative impact of Lugol's iodine on biovolumes (Hawkins et al., 2005), cell underestimation/overestimation due to the presence of aggregated cells (Park et al., 2018), and the presence of debris and sediments, especially in the sludge samples (Jalili et al., 2021), may affect the results. More importantly, the significant time required for taxonomic cell counts is a major barrier in using them for a real-time/practical approach.

In situ fluorometry using on-line probes is a compromising technique for measurement of PC based on relative fluorescence units (RFU) in water resources (Bowling et al., 2016; Gregor et al., 2007; McQuaid et al., 2011; Zamyadi et al., 2016a; Zamyadi et al., 2012c; Zamyadi et al., 2012d). However, the correlation between RFU and biovolume is complex and site-specific (Bowling et al., 2016; Cotterill et al., 2019; Thomson-Laing et al., 2020). Previous investigations have reported that various RFUs ranging from 0.7 to 1.8 could correlate with a 0.3 mm^3/L biovolume in different sources and bloom events (Bowling et al., 2016; McQuaid et al., 2011). Therefore, it is recommended to perform a correlation between on-line probe readings (RFU) and biovolumes for each water resource. It is noteworthy that the limits of detection and quantification of on-line probes should be considered (McQuaid et al., 2011; Zamyadi et al., 2016a).

MC concentration has been introduced in several guidelines such as those of the WHO (1.0 μg/L MC-LR) ((WHO), 2020c; World Health Organization (WHO), 1998) and Health Canada (1.5 μg/L) (Health Canada and Federal-Provincial-Territorial Commitee on Drinking Water, 2016). Geosmin and MIB negatively affect water quality, raise complaints about taste and odor, and decrease the public's confidence about the treated water safety (Hobson et al., 2010; Kim and Park, 2021; Ridal et al., 2001; Smith, 2011; Zamyadi et al., 2015b). Thus, they should be monitored throughout the treatment chain and the recycled sludge supernatant. Using enzyme-linked immunoassay (ELISA) tests for cyanotoxins measurement has been accepted for cyanotoxin monitoring (Almuhtaram et al., 2018; Picardo et al., 2019; Sanseverino et al., 2017; United States Environmental Protection Agency (USEPA), 2016). The reported thresholds for geosmin and MIB are 1.3–4.0 ng/L and 6.3–15 ng/L, respectively (Watson, 2004; Wert et al., 2014; Young et al., 1996). Since the taste and odor agents are not harmful, but increase complaints and concerns about the water quality (Hobson et al., 2010; Smith, 2011; Watson, 2004; Zamyadi et al., 2015b), olfactory detection can be considered for monitoring and detection (Chong et al., 2018; Kim and Park, 2021).

7.5.2 Decision Framework

A decision framework to manage cyanobacteria and cyanotoxins in DWTPs is presented in Figure 7-8. The objective of this framework is to minimize cell breakthrough and accumulation throughout DWTPs and sludge. The three steps, (i) source water risk assessment, (ii) treatment breakthrough assessment and management, and (iii) sludge and supernatant risk assessment and management, should be taken for cyanobacteria and cyanotoxin control in DWTPs and sludge. This framework can help water utilities to understand appropriate approaches/strategies against cyanobacteria and cyanotoxins in DWTPs.

Overall, taxonomic cell counts, MCs, and taste and odor agents should be monitored in the i) intake water, ii) treatment chain, and iii) sludge supernatant. These points are subjected to cyanobacteria and cyanotoxin accumulation, leading to a negative impact on water quality.

The optimization of conventional processes may include coagulant dose adjustment, applying aid-flocculants, and lowering the sedimentation and filtration rate during cell breakthrough (Agence Française de Sécurité Sanitaire des Aliments (AFSSA) and Agence Française de Sécurité Sanitaire de l'Environnement et du Travail (AFSSET), 2006a; Bernhardt and Clasen, 1993; Pietsch et al., 2002; Zamyadi et al., 2013a). Secondary barriers such as pre-oxidation, PAC injection, and GAC,

in case of metabolite breakthrough, should be applied (Abbas et al., 2020; Lusty and Gobler, 2020; Moradinejad et al., 2020; Newcombe, 2002; Newcombe et al., 2002; Newcombe and Drikas, 1996; Newcombe and Nicholson, 2004; United States Environmental Protection Agency (USEPA), 2019; Zamyadi et al., 2015a). The impact of supernatant recycling or discharging on the source/intake water quality should be considered during toxic cyanobacterial blooms. Supernatant treatment may be required in the presence of cyanotoxins or taste and odor agents. MC concentration levels should be monitored in the sludge (solids) in case of landfilling or land application. In the case of elevated concentrations of MCs, sludge treatment is required.

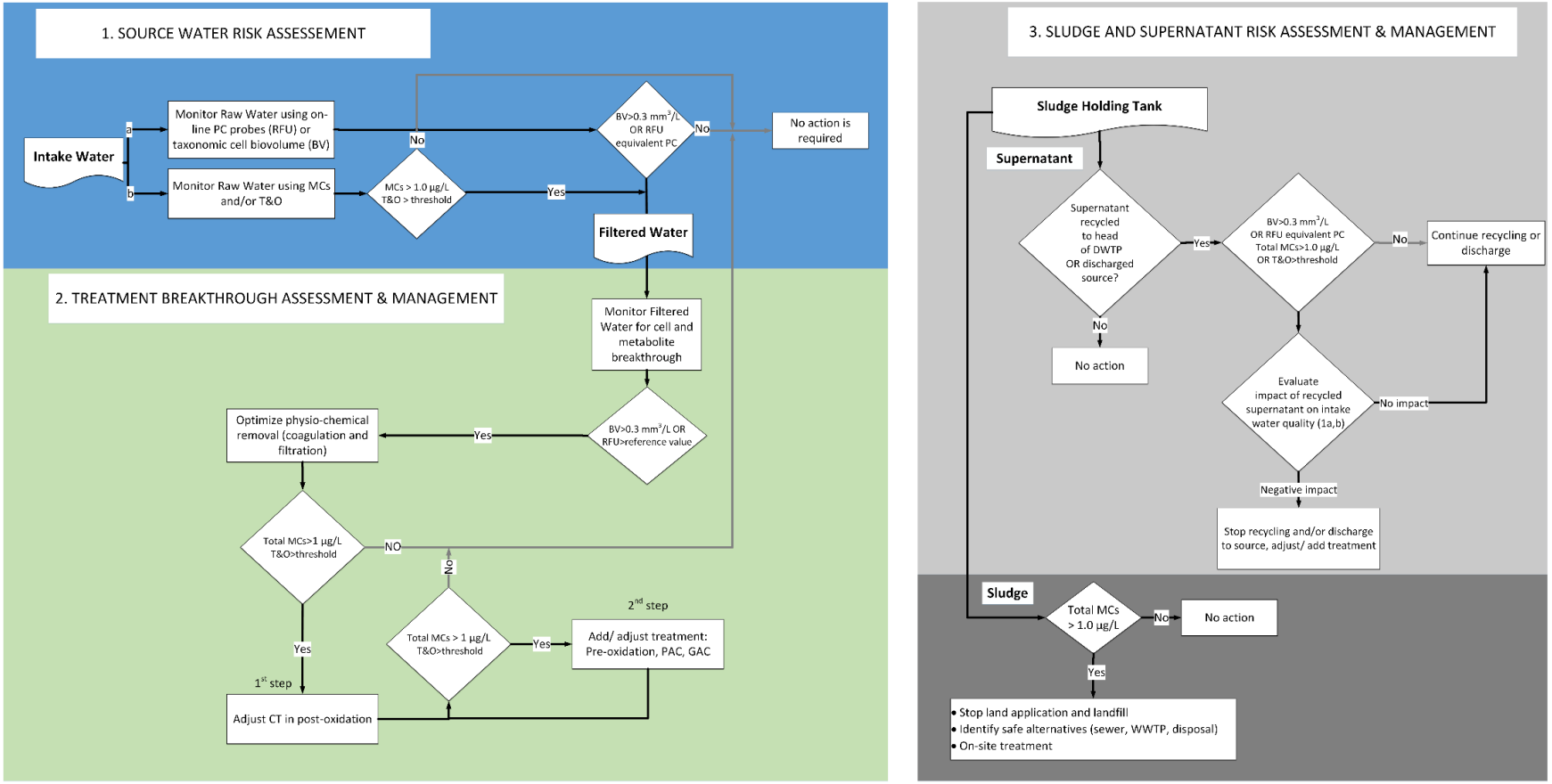

Figure 7-8 Decision framework for cyanobacterial bloom management in drinking water treatment utilities.

7.6 Conclusions

- Using the exposure unit (CT) is recommended for cyanobacteria and cyanotoxins oxidation studies, rather than using dose or contact time individually.
- Regardless of the oxidant type, lab-cultured studies cannot depict the complete picture of natural cyanobacterial bloom behavior during oxidation and may overestimate the oxidation efficiency. In addition, cyanobacterial bloom oxidation is site- and bloom-specific, which could be related to the level of agglomeration, cyanobacteria (bloom) stage of life, and metabolic functions.
- Soft pre-chlorination and pre-ozonation can compromise cell viability with no or limited cyanotoxin release. Overall, soft pre-oxidation may cause lower disinfection by-products compared to normal pre-oxidation.
- The cyanobacteria in stored sludge can not only survive, but also grow and release cyanotoxins, even in the dark. Although dissolved cyanotoxins can be degraded during sludge storage, the potential risk of growth and cyanotoxin release should be considered. In fact, the cell growth/depletion in stored sludge is complex and not easy to predict. Therefore, the worst-case scenario should be considered during sludge handling.
- Due to the low efficacy of sludge oxidation as compared to only stored sludge, as well as the occurrence of cell growth, and gene expression regulation during oxidation/storage, oxidation cannot be a reliable approach in sludge treatment and management.
- Management of cyanobacteria and cyanotoxins in sludge should be initiated with the minimization of cyanobacteria and cyanotoxin accumulation throughout DWTPs.
- To control the negative impacts of cyanobacterial accumulation in DWTPs, recycling sludge supernatant to the head of the DWTPs should be regulated during cyanobacterial seasons. Suitable treatment and disposal approaches should be set into guidance and regulations for sludge-containing cyanotoxins.

Author Contributions

Conceptualization, F.J., S.M., S.D., A.Z., S.S. and M.P.; methodology, F.J., S.M., S.D. and M.P.; validation, F.J., S.M., A.Z., S.D. and M.P.; formal analysis, F.J., S.M. and M.P.; investigation, F.J.,

S.M., S.D., A.Z. and M.P.; writing—original draft preparation, F.J. and S.M.; writing—review and editing, F.J., S.M., S.D., A.Z., S.S. and M.P.; visualization, F.J. and S.M.; supervision, S.D., S.S. and M.P.; project administration, S.S., S.D. and M.P.; funding acquisition, S.D. and S.S. All authors have read and agreed to the published version of the manuscript."

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Institutional Review Board Statement

Not applicable.

Informed Consent Statement

Not applicable.

Conflicts of Interest

The authors declare no conflict of interest.

CHAPTER 8 GENERAL DISCUSSION

The main achievements, novelties, and new challenges suggested by this research study are presented in this chapter.

The main objective of this research study was to investigate the fate of cyanobacteria and cyanotoxins in the sludge during storage and handling. The pathway of cyanobacteria and cyanotoxins from the source to the treatment chain and the sludge is illustrated in (Figure 8-1). Overall, cyanobacteria and cyanotoxins enter from the source to DWTPs. Although conventional processes can remove the majority of cyanobacterial cells and cell-bound cyanotoxins, cell and cyanotoxin breakthrough is probable during these processes. Meanwhile, cell accumulation and further phenomena (cell survival, growth, lysis and cyanotoxin release) occur in the stored sludge. Sludge supernatant recycling to the head of the DWTP increases the risks attributed to cyanobacteria and cyanotoxin. Therefore, a management strategy for the handling of cyanobacteria-laden sludge is necessary.

The result and scientific contribution of this research is summarized in Table 8-1.

The achievements of this research study are:

- Method validation, reproducibility and variability of the analyses
- Critical accumulation of cyanobacteria and cyanotoxins in DWTPs and sludge
- Oxidation of cyanobacteria-laden sludge
- Important parameters on management and handling of cyanobacteria-laden sludge
- Implementation of sludge management in DWTPs

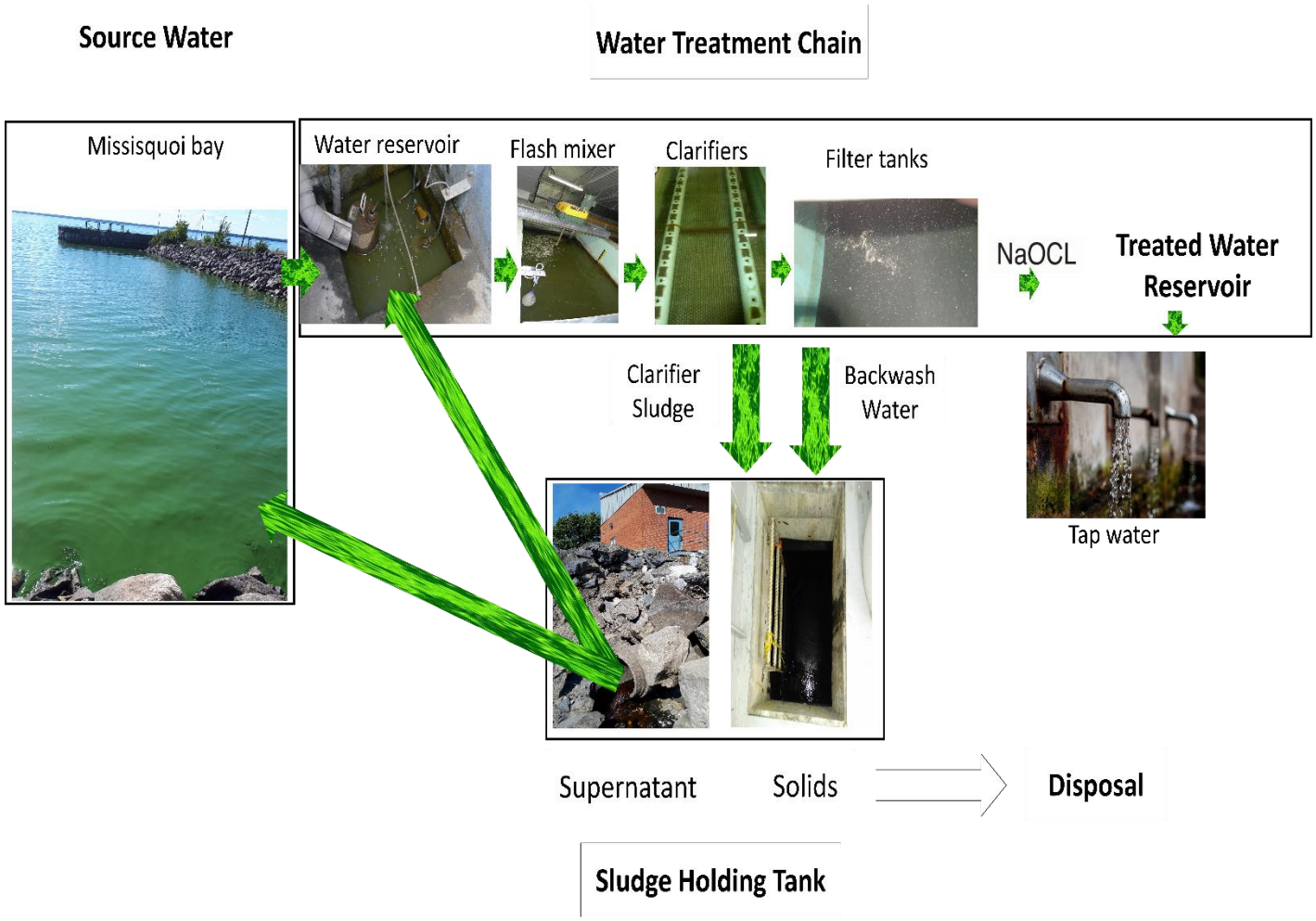

Figure 8-1 Critical accumulation of cyanobacteria and cyanotoxins in the water treatment chain and sludge

Table 8-1 Summary of results and contribution

Chapter	Research topics	Scale	Studied parameters	Achievements
4	What do drive cyanobacteria and cyanotoxin accumulation in the sludge?	Full	• Taxonomic cell counts • MCs • Microbial/ cyanobacterial communities (shotgun metagenomics) • Nutrients (TN, DN, TP, DP, OP, NO_2/NO_3, N-Kjeldahl, TOC)	• Conventional processes removed up to 96% of cyanobacterial cells. However, these processes led to cell accumulation in the sludge up to 31X. • Sludge composition depends on cyanobacterial communities in the intake water and occurred phenomena (cell survival, growth and decay) during sludge storage. • Accumulation of cyanobacteria in the sludge is correlated with nutrient availability in the sludge.
5	Can cyanobacteria grow in the stored sludge (in the dark)?	Full/ Lab	• Taxonomic cell counts • MCs • Microbial/ cyanobacterial communities (shotgun metagenomics)	• Cyanobacterial cells in the stored sludge are subjected to decay, survive and grow. Cell growth was observed in the sludge stored in the dark. • Cell growth was observed in 50% of stagnated sludge samples. • In the worst-case scenario, a 96% cell growth was observed within 16 days of storage. • Cell growth was mostly observed in the resistant potential MC-producer genera.
6	Can oxidation mitigate cyanobacteria and cyanotoxin accumulation in the stored sludge?	Full/ Lab	• Taxonomic cell counts • MCs • Microbial/ cyanobacterial communities (shotgun metagenomics) • *mcyD* (ddPCR)	• $KMnO_4$ and H_2O_2 could decrease 62% and 77% of taxonomic cell counts in the sludge, respectively. Additionally, up to 41% and 98% decreased in total MC level was observed by H_2O_2 and $KMnO_4$, respectively. • Persistence and decline of Cyanobacteria depends on the cyanobacterial composition. When Nostocales is abundant, cyanobacterial abundance is susceptible to decrease. In the presence of resistant genus such as *Microcystis* and *Synechococcus*, cyanobacterial abundance can persist.

Table 8-1 Summary of results and contribution (continue)

Chapter	Research topics	Scale	Studied parameters	Achievements
6	Can oxidation mitigate cyanobacteria and cyanotoxin accumulation in the stored sludge?	Full/ Lab	• Taxonomic cell counts • MCs • Microbial/ cyanobacterial communities (shotgun metagenomics) • *mcyD*	• Cell growth was observed in certain oxidized sludge samples (9 out of 22) during stagnation. The maximum cell growth was 161% using 20 mg/L H_2O_2 and after 17 days of stagnation. • There was not a remarkable cell decrease by oxidation as compared to only stagnation. • Oxidation and stagnation both shift cyanobacterial community towards MC-producer genera as *Microcystis* and *Aphanocapsa*. • Stagnation of oxidized sludge caused up to 13X increase in *mcyD* gene copy numbers.
7	Contribution to management of cyanobacteria and cyanotoxins in DWTPs and their associated sludge.	Review	-	• A strategy including i) Source water assessment, ii) Water treatment breakthrough assessment and iii) Sludge and supernatant assessment and management is suggested to control cyanobacteria in DWTPs and associated sludge. • Intake water, sludge supernatant and sludge (solids) should be monitored to mitigate potential risks related to cyanobacteria and cyanotoxins. Regarding the intake water and supernatant, taxonomic cell counts, cyanotoxins and T&O agents should be monitored. Regarding sludge (solids), the cyanotoxin level should be monitored to determine a safe disposal method. • Improvement (adjustment) of physico-chemical processes along with oxidation and activated carbon processes can be applied in the treatment chain to control cyanobacteria and cyanotoxin breakthrough in filter tanks. • Recycling and discharging of the supernatant containing cyanobacteria and cyanotoxins should regulated. Land application and landfill should be restricted for solids containing cyanotoxins.

8.1 Analytical and Molecular Techniques to Study Cyanobacteria-laden sludge

8.1.1 Selection of Techniques: Requirements and Achievements

In this research microscopic (taxonomic cell counts), NGS (taxonomy and function shotgun metagenomic sequencing), molecular (ddPCR), and analytical (MC, solid and nutrient measurement) techniques were applied for better understanding the fate of cyanobacteria and cyanotoxins in the sludge.

Microscopy taxonomic cell counts and MC concentration measurement are two common techniques widely applied for investigation of cyanobacteria and cyanotoxins (Almuhtaram et al., 2018; Pestana et al., 2016; Water Research Foundation (WRF) and Water Research Australia, 2015; Zamyadi et al., 2013a; Zamyadi et al., 2012a, 2013b; Zamyadi et al., 2010; Zamyadi et al., 2012c). Molecular techniques (NGS and ddPCR) have shown strong performance to predict/correlate cyanobacterial blooms and cyanotoxin concentration in water sources (Berry et al., 2017; Fortin et al., 2010; Tromas et al., 2017). In addition, these techniques have been applied to better understand the fate of cyanobacteria in DWTPs (Zamyadi et al., 2019) and sludge (Pei et al., 2017; Xu et al., 2018). Recently, NGS (16S and shotgun metagenomic) techniques were applied to study the fate of cyanobacterial communities after oxidation of water contained cyanobacteria (Lusty and Gobler, 2020; Moradinejad et al., 2020). These techniques study cyanobacterial communities as part of whole microbial communities. Thus interaction of cyanobacterial communities with other phyla can be investigated. Also, different levels of cyanobacterial (order and genus) persistence may result in different behaviour during oxidative stress such as oxidation and storage. Shotgun metagenomic sequencing studies whole genomes at the phylum, order, genes, and species levels that provide important information. This technique also provides function data (Costa and Weese, 2019; Romanis et al., 2021).

Due to the current challenges and questions on the fate of cyanobacteria during sludge storage (ex. questions about cyanobacterial cell survival, growth, death, or lysis), investigation on i) cyanobacterial diversity, ii) the interaction between cyanobacteria and other phyla, and iii) potentiality of cyanobacterial persistence; can be helpful.

NGS techniques provide relative values that cannot be applied directly in engineering works. Therefore, we combined shotgun metagenomic techniques with microscopy taxonomic cell counts to provide a comprehensive set of data including relative and absolute values. In fact, taxonomic cell counts provide absolute values about the impact of storage and oxidation on cyanobacterial cells, while shotgun metagenomic sequencing provides a relative picture of cyanobacterial and microbial communities, their interactions, persistence and resistance of communities (order and genus) in the sludge. Combination of NGS (shotgun metagenomic sequencing) and taxonomic cell count results can answer the following questions: i) What is the fate of cyanobacteria in the presence of oxidative stress (microscopy taxonomic cell counts), and ii) How and why cyanobacterial communities are changed in the presence of other microbial communities and oxidative stress (shotgun metagenomic sequencing).

As discussed in Chapter 4Chapter 5 and Chapter 6, taxonomic cell counts showed cell grow, survival and death in the cyanobacteria-laden sludge during storage and oxidation. Furthermore, shotgun metagenomic showed that sludge storage and oxidation ($KMnO_4$) selectively shift cyanobacterial community towards resistant orders such as Synechococcales and Chroococcales. Meanwhile, Nostocales is a sensitive order during sludge storage and oxidation. In contrast, no selective shift was observed during oxidation via H_2O_2. Combination of these findings highlighted that even if taxonomic cell counts decreased during sludge storage, potential MC producer species belong to Chroococcales and Synechococcales orders can persist and increase health-related risk during sludge storage and oxidation (Figure 5-4, Figure 5-5, Figure 6-1, Figure 6-2, Figure 6-3, Figure 6-4, Figure 6-7 and Figure 6-8)

The ddPCR technique showed a remarkable increase in *mcyD* gene copy numbers after oxidation followed by storage (Figure 6-9c), although MC concentrations remained quite low (below 92 ng/L) due to biodegradation of dissolved MCs (Figure 6-9a). Thus, a high MC concentration is expected during oxidation/ stagnation if either the initial concentration/ copy numbers of MCs/ *mcyD* is high or biodegradation occurred in a low extent. Therefore, ddPCR is a useful tool for cyanotoxin prediction during sludge handling, however the limitations should be considered (*see 8.1.2*).

8.1.2 Comparison of Microscopy Taxonomic Cell counts and MC Quantification with Shotgun Metagenomic and ddPCR Results

Overall, microscopy taxonomic cell counts were in line with shotgun metagenomic results, in our study. For instance, shotgun metagenomic sequencing confirmed the *Dolichospermum* bloom in the intake water, which was in accordance with microscopy taxonomic cell count results (Figure 4-2 and Figure 4-5). Similarly, shotgun metagenomic results corresponded with microscopy taxonomic cell counts in the sludge (Figure 5-1 and Figure 5-2) and stagnated sludge (Figure 5-5) at the genus and order levels. However, as discussed in section 4.3.3, shotgun metagenomic did not completely match microscopy taxonomic cell counts at the species level. A recent study also reported a good correlation between microscopy taxonomic cell counts and shotgun metagenomic sequencing at the genus level, however, the authors reported a weak correlation at the species level (Moradinejad et al., 2020).

Each of these techniques can yield different communities due to limitations of each technique. Some limitations of microscopy taxonomic cell counts are (America Water Works Association (AWWA), 2010; Casero et al., 2019; Hawkins et al., 2005; Park et al., 2018):

- Cell damage and lysis can affect the morphology of the cells and negatively affect microscopy taxonomic cell count results.
- Misidentification of morphologically similar species can occur during microscopic cell counts.
- The impact of conservation agent on biovolume may affect cell count results.
- Cell enumeration in the aggregated conditions is complex.

Furthermore, the presence of debris and sediments increases the probability of cross interference in the microscopic technique.

In the shotgun metagenomic technique, DNA can be extracted from even lysed and dead cells. However, insufficient recovery rate of DNA, contamination of DNA during extraction and lack of a standard pipeline contained all cyanobacterial species may affect the results (Bag et al., 2016; Gevers et al., 2012; Kuczynski et al., 2011; Teeling and Glockner, 2012).

In our study, a significant correlation between *mcyD* gene copy numbers and total/ cell-bound MC concentrations were detected in the intake water (R > 0.94, $p < 2.4 \times 10^{-5}$) and sludge (R >0.84, $p < 0.004$ in total and cell-bound MCs) samples. In contrast, less correlation was detected between *mcyD* gene copy numbers and dissolved MCs (R = 0.63, $p = 0.07$) in the sludge (Table 8-2). This might be due to either simultaneous cell lysis, cell-bound MC release and dissolved MC (bio) degradation, or complexity of gene expression/ not expression in the sludge. Accordingly, a significant correlation (R = 0.84, $p = 0.008$) was detected between cell-bound MCs and *mcyD* gene copy numbers in the sludge stagnated by up to 38 days, whereas, no correlation was detected in total and dissolved MCs after stagnation (Table 8-2). Similarly, weak correlations (from no correlation to R= 0.55), has been reported after oxidation in water samples (Moradinejad, 2021b). This shows that although ddPCR is an appropriate tool for prediction of MC concentrations, it is not a suitable approach in presence of oxidative stress such as oxidation and storage.

Table 8-2 Correlation of *mcyD* gene copy numbers with MC concentrations using the Pearson correlation (R) in source (intake) water and DWTP's sludge of Missisquoi Bay (Lake Champlain)

	***mcyD* vs. total MCs**	***mcyD* vs. cell-bound MCs**	***mcyD* vs. dissolved MCs**	
Sample	**R (*p*-value)**			**Reference**
Intake water	0.97 (2.4 E-5)	0.94 (1.4 E-5)	0.97 (1.4 E-5)	This study
Sludge	0.86 (0.002)	0.84 (0.004)	0.63 (0.07)	This study
Stagnated sludge (7-38 days)	0.39*	0.84 (0.008)	-0.18*	This study
Source water	0.97 (<0.05)	0.95 (<0.05)	0.95 (<0.05)	(Moradinejad et al., 2021a)
Source water	> 0.91 (<0.05)	-	-	(Fortin et al., 2010)

*Red colors: insignificant data ($p > 0.1$).

Several studies reported that *mcyD* is correlated with MC concentrations in water samples (Davis et al., 2009; Fortin et al., 2010; Moradinejad et al., 2021a; Rinta-Kanto et al., 2009). In contrast, no correlations were reported between these values in some other investigations (Baxa et al., 2010; Beversdorf et al., 2015; Hotto et al., 2008; Li et al., 2014a). The complexity of toxin expression/ not expression by toxic genes (Pacheco et al., 2016), and gene extraction challenges (Kim et al., 2013) are challenges of gene copy number enumeration by ddPCR technique. Therefore, the concept of the evaluation of the MC producer genes to predict MC (cell-bound and dissolved)

concentrations in environmental samples is still complex. However, studies on intake water (Fortin et al., 2010; Moradinejad et al., 2021a) and sludge (this study) on Missisquoi Bay source confirms correlation between *mcyD* gene copy numbers and MC concentrations.

8.1.3 Reproducibility and Variability

Overall, 571 samples were taken in this research study during 2017 and 2018, of which 207 samples were sludge. Due to the high number of the samples, complexity of analysis, time, and cost limitations, one replication was done for microscopy taxonomic cell counts, ddPCR and shotgun metagenomic sequencing.

The reproducibility of taxonomic cell count technique has been already validated (RSD = 4%) by one of the previous study performed by our group (Zamyadi et al., 2012c). The protocol, analysis location, equipment, and technician were the same as that study in our research.

The variability and reproducibility of shotgun metagenomic data were validated by one study of our project (ATRAPP) on the mesocosm samples where triplicate samples were taken (Vatani, 2021). The results confirmed reproducibility at the phylum, order and genus levels of microbial and cyanobacterial communities (Figure 8-2).

Reproducibility of ddPCR was validated by duplication on three samples showing relative standard deviation between 0.2- 7% (Figure 8-3).

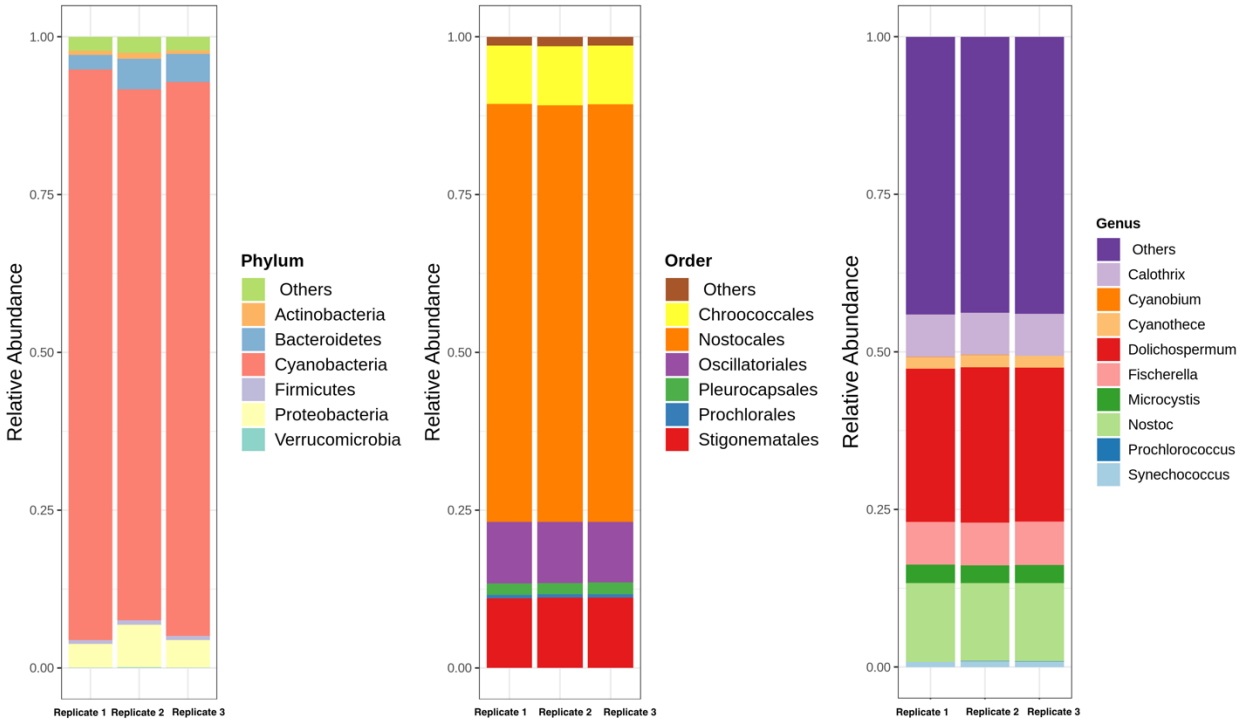

Figure 8-2 Evaluation of reproducibility and variability of shotgun metagenomic sequencing on mesocosm samples at the at phylum, order, and genus levels using three replications, adapted from (Vatani, 2021).

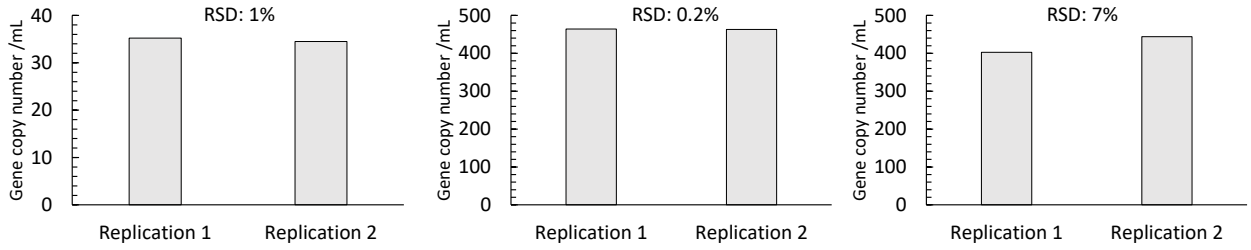

Figure 8-3 Reproducibility of the ddPCR analysis

8.2 Critical Accumulation of Cyanobacteria and Cyanotoxins in DWTPs and sludge

8.2.1 Validation of the Previous Investigations, New hypothesis

Cyanobacterial cells enter from the source to DWTPs. Conventional processes can acceptably trap the cells and decrease cell counts in effluent of filters (Zamyadi et al., 2013a; Zamyadi et al., 2013b). However, remarkable accumulation of cyanobacterial cells occurs in the sludge of DWTPs and its supernatant during conventional processes (Almuhtaram et al., 2018; Ho et al., 2012; Zamyadi et al., 2013b; Zamyadi et al., 2012c). The trapped cells can return to the intake water during either: i) Supernatant recycling to the head of the plant, or ii) Supernatant discharge to the source.

In this thesis (Chapter 4), we validated the previous data about more than 97% cell removal by flocculation, coagulation, and sedimentation (Figure 4-2). The majority of the remained cells were removed by filtration, however taxonomic cell counts in the finished reservoir (after chlorination) increased up to 120% as compared to the effluent of the filters (Figure 4-2).

Total taxonomic cell counts in the sludge holding tank remained 3-31 times higher than the cell counts in the intake water (Figure 4-2). Total taxonomic cell counts in the supernatant of sludge holding tank remained 94-98% lower than those in the sludge and 69-97% lower than those in the intake water. In contrast to previous studies reporting high cyanotoxin accumulation in the sludge and supernatant, we measured low concentration levels of MCs in the sludge (below 281 ng/L) and sludge supernatant (below 128 ng/L) (Figure 4-3). This MC concentration trend was also in contradiction to our cell count results showing high cell accumulation in the sludge. The reason might be either the low level of toxic genes in the influx cells or i) MC biodegradation by MC degrader species belong to Proteobacteria, Actinobacteria and Firmicutes (Kormas and Lymperopoulou, 2013) or, ii) MC degradation on the flocs and sediments (Ma et al., 2016b; Maghsoudi et al., 2015). In addition, we hypothesized that MC degradation (in sludge) on the remained PAC injected into the intake water might occur. These phenomena were also hypothesized during sludge storage (stagnation) in the controlled conditions in Chapter 5 and Chapter 6.

8.2.2 Microbial and Cyanobacterial Diversity in Intake Water

Shotgun metagenomic sequencing showed that microbial communities shifted before and after cyanobacterial blooms. Before the bloom, Proteobacteria (26%-56%) followed by Actinobacteria (12%-26%) were predominant in the intake water (Figure 4-5). During the bloom, Cyanobacteria (19%-38%) and Bacteroidetes (12%-32%) became predominant phyla. This increase in the relative abundance of Cyanobacteria is in accordance with taxonomic cell count results showing an increase in the cell counts of cyanobacterial cell counts in this period. A simultaneous increase in the relative abundance of Cyanobacteria and Bacteroidetes was previously reported by Guedes et al. (2018) and Kim et al. (2020). In fact, nutrient-rich conditions enhance the persistence of Bacteroidetes, which is susceptible for the growth of Cyanobacteria (Cai et al., 2014). Similarly, we demonstrated a significant influence of total (TN) and dissolved nitrogen (DN) on the Bacteroidetes community (Figure A- 1, Table A- 1). After the bloom period (September 5th), the relative abundance of Cyanobacteria and Bacteroidetes decreased, whereas Proteobacteria became the predominant phylum in the intake water (Figure 4-5).

At the cyanobacterial genus level, *Synechococcus* (Synechococcales) and *Microcystis* (Chroococcales) were predominant before the cyanobacterial bloom. In the late August (August 25th, 2017), the relative abundance of *Dolichospermum* and *Nostoc* (Nostocales) increased. The relative abundance of *Dolichospermum* reached its maximum level on September 1st (Figure 4-6). This corresponds with taxonomic cell count results showing a *Dolichospermum* bloom on September 1st (Figure 4-2). An investigation on the Lake Champlain (the source water of the intake water of this study) showed that the relative abundance of *Microcystis* and *Dolichospermum* repeatedly alternated in bloom and non-bloom events, however we showed in Chapter 4 that the relative abundance of *Synechococcus* also shifted from highly abundant before the bloom to low abundance during *Dolichospermum* bloom (Figure 4-6).

Overall, bacterial and cyanobacterial compositions dynamically changed during non-bloom and bloom events. Cyanobacterial diversity (Shannon index) changed between 4.51 and 4.18 in the intake water before the bloom but reached its lowest level on September 1st (4.06), showing there is a predominant genus in this date (*Dolichospermum*) (Figure A- 4). This in in agreement with taxonomic cell count results showing that *Dolichospermum* was predominant in the bloom date (Figure 4-2 and Figure 4-6).

8.2.3 Impact of Intake Water Diversity on Sludge and Supernatant

Similar to the intake water, Proteobacteria and Actinobacteria were the predominant phyla in the sludge holding tank (ST) and supernatant (SST). Cyanobacteria, Bacteroidetes, Verrucomicrobia and Firmicutes were also detected in low relative abundance in the sludge holding tank and supernatant (Figure 4-5). Shannon index shows that similar microbial diversity was observed in the intake water and sludge holding tank during sludge storage, however microbial diversity in the supernatant remarkably decreased showing that Proteobacteria was predominant in the supernatant (Figure A- 5, Figure 4-5).

The cyanobacterial composition of sludge holding tank and its supernatant did not match to the intake water. Precisely, for the sludge samples taken on August 25th and September 5th, sludge storage time were 13 and 1 day, respectively. For instance, higher relative abundance of *Synechococcus* and lower relative abundance of *Dolichospermum* were observed in the sludge holding tank and its supernatant as compared to the intake water (Figure 4-6). The relative abundance of *Microcystis* in the sludge holding tank was higher than in the intake water on September 5th (1 day storage time). We showed in Chapter 5 that cyanobacterial genera as *Synechococcus* (representative of Synechococcales) and *Microcystis* (representative of Chroococcales) are more resistant during sludge storage as compared to *Dolichospermum* (representative of Nostocales). This resistance is attributed to the ability to form glycoprotein S-layer (Rachel et al., 1997), protecting the cells against ecological stress (Callieri, 2017; Sleytr et al., 1999; Šmarda et al., 2002). Additionally, cell survival and lysis during sludge storage occur simultaneously in the sludge (Ho et al., 2012; Water Research Foundation (WRF) and Water Research Australia, 2015).

The interaction of sludge holding tank and its supernatant is more complex. On August 30th, the relative abundance of *Microcystis*, *Synechococcus*, and *Dolichospermum* was almost equal in the sludge and supernatant. In contrast, On September 5th, the relative abundance of *Microcystis* and *Synechococcus* was higher in the sludge holding tank than the supernatant, while an opposite trend was observed for *Dolichospermum* (Figure 4-6). Chao index (richness) in the intake water and the sludge holding tank were quite similar, while this index was lower in the supernatant. This suggests that cyanobacterial communities in the sludge and intake water were quite similar; however cyanobacterial communities were different in these two points with the supernatant (Figure 4-8a).

8.3 Important Parameters on Sludge and Supernatant Communities

As previously mentioned, the fate of cyanobacteria and cyanotoxins in the stored sludge and its supernatant is complex due to simultaneous phenomena as cell lysis, cyanotoxin release and dissolved cyanotoxin degradation (Ho et al., 2012; Water Research Foundation (WRF) and Water Research Australia, 2015). Meanwhile, various environmental parameters impact sludge, and sludge supernatant communities during storage. The roles of these parameters have not been well understood (Dreyfus et al., 2016; Pestana et al., 2016). In this study, we investigated some important parameters as: i) Initial composition in the intake water and sludge before storage, ii) The degree of cyanobacterial orders and genus resistance, and iii) Nutrient parameters.

According to our results presented in Chapter 4, sludge storage time is one of the important parameters affecting cyanobacterial compositions in the sludge and supernatant. As discussed in Section 8.1, sludge communities are influenced by incoming cyanobacterial diversity (intake water) during storage time. However, cyanobacterial composition of the sludge is not exactly reflects the intake water communities. We showed in Chapter 5 that cyanobacterial cells are not only survived or lysed in the stored sludge, but also they can grow (Figure 5-4). The dynamic and reasons of cell growth was not the objective of this research study but for the first time, we justified heterotrophic growth of cyanobacterial cells in the sludge stored in the dark.

Initial cyanobacterial composition (intake water or sludge before storage) is another important parameter that shapes sludge communities during storage. Some cyanobacterial orders such as Synechococcales and Chroococcales are resistant against stress (eg. storage, oxidation), while filamentous orders such as Nostocales are more sensitive. Among our 2017 field monitoring, we showed that although the intake water mostly contained Nostocales, Chroococcales remained the predominant order in the sludge (Figure 4-9). Furthermore, in Chapter 5 and Chapter 6, we demonstrated that cyanobacterial communities shifted from the more sensitive order (as Nostocales) towards more resistant orders (Synechococcales and Chroococcales) in the presence of oxidative stress such as sludge storage and oxidation (Figure 5-5, Figure 6-4, Figure 6-6, Figure 6-8 and Figure C- 5). As previously discussed in Chapter 5 and Section 8.1, glycoprotein S-layer in these orders can protect cells against stress (Callieri, 2017; Rachel et al., 1997; Sleytr et al., 1999; Šmarda et al., 2002). One reason for the sensitivity of Nostocales might be trichomes breakage (Pestana et al., 2019) due to stress attributed to the sludge storage.

Higher concentrations of nutrients including nitrogen, phosphorus and carbon favours microbial growth (Jankowiak et al., 2019; Pei et al., 2017; Xu et al., 2018). We showed a significant correlation (p <0.05) between availability of OP and the presence of Nostocales in the intake water. The relative abundance of genes related to phosphorus metabolisms in Nostocales is 4.5 times higher than Chroococcales (Lu et al., 2019). In contrast, TN, N-Kjeldahl, TP, PP and TOC had a strong impact on cyanobacterial composition in the sludge mostly contained Chroococcales (Figure 4-9). However, no correlations between sludge supernatant and studied nutrient parameters were found.

Finally, microbial diversity was not followed a trend from the intake water and sludge towards the supernatant (Figure A- 5). In fact, the supernatant composition reflects incoming sludge and the subsequent buoyancy of the community in the sludge. Sludge storage may cause cell breakage lead to vesicle damage and interruption of buoyancy regulations (Arii et al., 2015; Reynolds et al., 1987).

8.4 Oxidation of Cyanobacteria-laden Sludge

According to our achievements (Chapter 4) about challenges of cyanobacteria-laden sludge and its supernatant in DWTPs, an appropriate treatment approach is essential.

$KMnO_4$ and H_2O_2 have been widely applied to control cyanobacteria and cyanotoxins in water samples (Fan et al., 2013a; Fan et al., 2013b; Li et al., 2014b; Matthijs et al., 2012; Piezer et al., 2020b), and to enhance coagulation process throughout the water treatment chain (Chen and Yeh, 2005; Xie et al., 2013). However, there is only one study on treatment of cyanobacteria-laden sludge using $KMnO_4$ (Zamyadi et al., 2016b), and no study about the impact of H_2O_2 on sludge containing cyanobacteria and cyanotoxins.

The half reaction of $KMnO_4$ at pH range from 3.5 to 12 is presented in Equation 8-1. During oxidation via $KMnO_4$, three electrons are exchanged lead to reduction of Mn^{+7} to Mn^{+4} (Laksono and Kim, 2017).

$$MnO_4^- + 2H_2O + 3e^- => MnO_2(\mathrm{s}) + 4OH^- \qquad \text{(Equation 8-1)}$$

H_2O_2 is decomposed to hydroxyl radicals (OH•) through the Fenton reaction presented in Equation 8-2 (Latifi et al., 2009) then, hydroxyl radicals lead to i) decrease of cyanobacterial cell counts and

cyanotoxin concentrations (Fan et al., 2013b; Fan et al., 2014b; Zamyadi et al., 2020), and ii) cyanobacterial DNA damage (Latifi et al., 2009).

$$Fe^{2+} + H_2O_2 => OH^- + FeO^{2+} + H^+ => Fe^{3+} + OH^- + OH\bullet \quad \text{(Equation 8-2)}$$

According to our laboratory results presented in Chapter 6, H_2O_2 (10 and 20 mg/L) and $KMnO_4$ (5 and 10 mg/L) decreased cyanobacterial taxonomic cell counts up to 77% and 62%, respectively (Figure 6-1-Figure 6-3). One exception was the sample taken on July 31st where only slight cell count decrease (3%) was observed after oxidation by 10 mg/L $KMnO_4$, whereas cell counts slightly increased (16%) after 5 mg/L $KMnO_4$. This unexpected trend after $KMnO_4$ oxidation might be due to either i) the cells were presented in the colony form in the July 31st sample and oxidation burst the cells from the colony forms, or ii) uncertainty of microscopy taxonomic cell counts as discussed in section 8.1.1.

In two on-site shock oxidation treatments with continuous incoming of fresh sludge from the clarifier, lower efficacy (Max. 43%) was observed as compared to the laboratory oxidation results (Figure 6-7). The contact times in the on-site shock experiments (24 h to 72 h) were longer than that in the (controlled) laboratory experiments (1 h). Thus, very low $KMnO_4$ (almost zero) residual was expected at the end of the mentioned contact times. Also, fresh sludge (solids) was pumped from the clarifier to the holding tank during shock on-site oxidation. Additionally, cell settling from the supernatant to the sludge could affect cell counts. Finally, cell growth might occur in the sludge after depletion of the $KMnO_4$ residual. All these reasons resulted in lower efficiency of on-site shock oxidation as compared to the controlled oxidation.

MC concentrations in the intake water and sludge were low during our experiments (< 161 ng/L). Although similar $KMnO_4$ doses (5 and 10 mg/L) was applied and CTs were close (for 5 mg/L $KMnO_4$, CT: 89-105 mg.min/L, and for 10 mg/L $KMnO_4$, CT: 214-239 mg.min/L) in three oxidation days, the percentage of MC concentration decrease remarkably different in three experiments, from 20% to ~100% in cell-bound MCs, and from ~0 to 98% in total MCs (Figure C-4). Interestingly, taxonomic cell count results showed that potential MC producer species (ex. *Microcystis aeruginosa, Dolichospermum spiroides, Dolichospermum circinale, Chroococcus dispersus, Aphanocapsa delicatissima* and *Aphanocapsa holsatica*) had different decreasing trends in three laboratory oxidation days by $KMnO_4$ (Figure 6-1-Figure 6-3).

Shotgun metagenomic sequencing is helpful to study the impact of oxidation: As we demonstrated in Chapter 4, sludge communities dynamically changed due to changes in incoming cyanobacterial communities (intake water), and the sludge storage time. Indeed, the difference in microbial and cyanobacterial communities in three experiment days could impact the oxidation results. Precisely, on August 7th, Chroococcales was the predominant cyanobacterial order and resisted against oxidation (Figure 6-4b). Thus, the relative abundance of Cyanobacteria slightly decreased (16% - 50%) by both oxidants ($KMnO_4$ and H_2O_2) and applied doses. Consequently, the microbial diversity (Shannon index) remained constant in this oxidation day (Figure 6-5a). In contrast, on August 17th, Nostocales was predominant and was sensitive against oxidation. Consequently, cyanobacterial diversity (Shannon index) decreased as cyanobacterial compositions shifted from Nostocales towards Chroococcales (Figure 6-4b). At the genus level and after oxidation, the relative abundance of *Dolichospermum* decreased while that of *Microcystis* remained almost constant (Figure 6-4c). Meanwhile, the relative abundance of *Synechococcus* increased. Thus, regardless of the initial composition of the sludge, oxidation shifts cyanobacterial communities towards resistant genera such as *Microcystis* (Chroococcales) and *Synechococcus* (Synechococcales) (Figure 6-1-Figure 6-3). Similar trend was observed in the on-site shock oxidation scenario (Figure 6-8b). This is important because the resistant genera (ex. *Microcystis* and *Synechococcus*) are mostly potential cyanotoxin producers (Carmichael and Li, 2006; Gin et al., 2021; Lone et al., 2015). At the phylum level, the relative abundance of Proteobacteria increased after oxidation, while the relative abundance of Cyanobacteria decreased (Figure 6-4a and Figure 6-8a). This trend is in accordance with the impact of stagnation on microbial community shift presented in Chapter 4 and Chapter 5.

Furthermore, our results showed that the relative abundance of cyanobacteria is influenced by the relative CT (Figure 6-6). Therefore, similar to cyanobacterial oxidation in water samples (Fan et al., 2013a; Greenstein et al., 2020b; Zhou et al., 2013), CT can be used to evaluate cyanobacteria and cyanotoxin oxidation in the sludge.

Regarding oxidation of cultured toxic cyanobacteria via $KMnO_4$, it has been documented that dissolved MCs are degraded initially then, $KMnO_4$ compromises the cell integrity. If the concentration of the residual $KMnO_4$ is sufficient, released cell-bound MCs will be degraded (Li et al., 2014b; Piezer et al., 2020b). However, in our study this trend was not respected neither in the laboratory nor in on-site shock oxidation (Figure 6-9 and Figure C- 4). This might be due to the

differences of natural cyanobacteria and cultured cyanobacterial cells such as multicellularity, presence of organic matter in the sample, different cell integrity in a bloom and the presence of the cells in colony forms (Coral et al., 2013a; Greenstein et al., 2020b; He and Wert, 2016; Zamyadi et al., 2015a; Zamyadi et al., 2012b). Laboratory and on-site shock oxidations could not degrade MCs concentration completely (Figure 6-9 and Figure C- 4). The maximum MC level decrease in laboratory scale was around 98% observed on July 31st using 10 mg/L $KMnO_4$ (Figure C- 4a). However, in other scenarios, the maximum MC decrease was 41% (Figure C- 4b,c). During on-site shock oxidation on August 17th, total MC concentration increased up to 37% and 589% after 24 h and 72 h, respectively (Figure 6-9a). In contrast, on-site shock oxidation on September 5th could decrease total MC level up to 25% and 3% after 24 h and 48 h, as compared to before oxidation (Figure 6-9b). Furthermore, short-term (24 h) reduction on taxonomic cell count was observed in both on-site shock oxidation scenarios (Figure 6-7a,b). Increase of the contact time led to raising the cell counts after 24 h. However, on August 17th, cell counts remained below the initial value after 72 h (24%), while on September 5th, cell counts surpassed the initial level after 48 h (13%) (Figure 6-7a,b).

Total taxonomic cell counts and potential MC producer species increased in the sludge supernatant by up to 144X and 317X, respectively after on-site shock oxidation (24 h to 72 h) (Supernatant: Figure C- 3a, b). Accordingly, MC concentrations increased by up to 140% in 3 out of 4 samples of the sludge supernatant (Figure C- 3c, d). This highlights the importance of CT (dose and contact time) optimization. Also, this achievement is in accordance with our observation in Chapter 4 and (Dreyfus et al., 2016; Pestana et al., 2016; Zamyadi et al., 2016b), about the challenge of supernatant recycling to the head of the DWTPs during toxic cyanobacterial blooms.

Indeed, microbial and cyanobacterial communities affect the result of sludge oxidation. Additionally, the difference of cultured and natural cyanobacteria (blooms) should be considered during oxidation. Also, as we demonstrated that the oxidant dose and contact time should be selected in a way to provide sufficient CT during oxidation (Jalili et al., 2022a).

Conducting a mass balance of the cyanobacterial cell counts in the sludge holding tank would be desirable. Unfortunately, in this study, establishing a mass balance cannot be conducted for the following reasons: i) Water and sludge were sampled periodically; ii) Input to the sludge is discontinuous and sludge concentration during extraction varies significantly and, iii) Supernatant

is returned to the source and was not monitored continuously. Additionally, cell decay and growth can occur as shown in the controlled laboratory studies.

In this study, the maximum cyanobacterial cell counts observed in the supernatant was 2.0 x 10^6 cells/mL. Considering the discharged/recycling flow rate is around 10% of the flow rate of the intake water (Zamyadi et al., 2016b), recycling of the supernatant to the head of the studied DWTP, could increase cell counts in the intake water by up to 2.0 x 10^5 cells/mL.

8.5 Impact of Sludge Storage on Cyanobacteria and Cyanotoxins

8.5.1 Sludge Storage without Oxidation

Proliferation of cyanobacterial cells in the stored sludge has been suggested by recent studies (Dreyfus et al., 2016; Pestana et al., 2016; Water Research Foundation (WRF) and Water Research Australia, 2015). These studies applied cell quota (if all cell-bound metabolite were released), metabolite measurement, or microscopy taxonomic cell counts. However, according to the methodology of those studies other hypotheses such as i) cell quota underestimation, ii) increase in production of metabolite per cells during sludge storage, and iii) additional cell settlement from the supernatant to the sludge were also suggested. In Chapter 4, we hypothesized that prolonged sludge storage (by up to 13 days), increase the probability of cell growth (Jalili et al., 2021).

In Chapter 5, for the first time we observed the cell growth of natural cyanobacterial cells in the sludge in controlled conditions stored from 7 to 38 days in the dark. Cell growth was observed in both total taxonomic cell counts and species.

Cell growth in the stored sludge was observed in different trends (Figure 5-4):

- On July 27th and November 1st, around 50% of total cell counts progressively decreased within 31 days.
- On July 31st, August 7th and 17th, 3%- 20% cell increase (growth) was observed within 17 days as compared to 9 days of stagnation. Then, 44%- 83% cell decreased occurred within 31- 37th stagnation days. However, taxonomic cell counts remained below the initial values during stagnation.

- On August 10th, 16 and 21% cell growth was observed after 9 and 17 days, respectively. However, a 48% decrease in total cell counts was observed after 34 days, and total cell counts remained below the initial value.
- On September 5th and October 16th, progressive cell growth was observed during stagnation and total cell counts remained higher than the initial values after 30 days (on October 16th) and 35 days (on September 5th). The highest cell growth occurred in the sample taken on October 16th where taxonomic cell counts increased from 2.7 x 10^6 cells/mL (before storage) to 3.1 x 10^6 cells/mL (16%) after 8 days, 5.3 x 10^6 cells/mL (94%) and 4.2 x 10^6 cells/mL (56%) after 30 days (Figure 5-4).

Additionally, even in scenarios that total cell counts decreased during stagnation, cell growth was observed in certain species. For instance, in the July 27th sample, *Aphanocapsa delicatissima* increased from 7.5 x 10^4 cells/mL at day 7th to 2.0 x 10^5 cells/mL at day 14th (164%). In this date, *Aphanocapsa holsatica* increased from 6.9 x 10^3 cells/mL to 1.4 x 10^5 cells/mL (1892%) (Figure 5-4). However, since the other species decreased in that period, total cell counts decreased consequently. This is in accordance with our results in Chapter 4 about dynamic changes of cyanobacterial communities in the stored sludge.

Meanwhile, cyanobacterial functions increased or persisted in certain dates during sludge storage (Figure 5-6):

- "Pentose phosphate pathway (*OpcA*)"

This marker is the most related function that justifies cell growth of cyanobacterial cells in the dark. In fact, "Pentose phosphate pathway (*OpcA*) is specific to heterotrophic growth of cyanobacteria in the dark (Min and Golden, 2000), and under nitrogen starvation conditions (Shimmori et al., 2018; Summers et al., 1995).The relative abundance of this biomarker increased in the October 16th sample and persisted in some other dates.

- "Cyanobacterial circadian clock"

This is one of the most important cyanobacterial markers responsible for gene expression regulation, cell metabolism, chromosome compaction and synchronizing cyanobacterial activities (Diamond et al., 2015; Dong et al., 2010; Liu et al., 1995; Mori et al., 1996; Smith and Williams,

2006; Taton et al., 2020). The relative abundance of this biomarker increased in the October 16th sample and persisted in some other stagnated samples.

- "Transcription factors cyanobacterial *RpoD*-like sigma"

This function is attributed to the cell viability and cell survival under stress in cyanobacteria (Imamura and Asayama, 2009). The relative abundance of this biomarker increased in the October 16th sample and persisted in some other stagnated samples.

- "Heterocyst formation in Cyanobacteria"

This biomarker is related to filamentous genera with the heterocyst such as Nostocales order (ex. *Dolichospermum* and *Nostoc*) attributed to nitrogen fixation (Herrero et al., 2016; Kumar et al., 2010). This biomarker decreased in almost all stagnated samples which is in line with decrease of taxonomic cell counts of *Dolichospermum* during stagnation (Figure 5-4). The exception was the increase of this biomarker in the October 16th sample where the *Dolichospermum spiroides* cell count increased during stagnation (Figure 5-4).

Overall, cell death, survival, and growth were observed in different stored (stagnated) sludge samples. One suggested pathway of cell growth that we observed by function metagenomics was switching from photoautotrophic growth to heterotroph growth through "Pentose phosphate pathway (*OpcA*)" cyanobacterial biomarker.

Both taxonomic cell counts and shotgun metagenomic sequencing showed that Synechococcales and Chroococcales were susceptible to grow, whereas Nostocales was the most sensitive order during stagnation (Figure 5-5). This is in accordance with our function metagenomic results as well as our results in Chapter 4 about the resistance of Chroococcales and sensitivity of Nostocales during sludge storage (Figure 4-9). As mentioned previously, the importance of this finding is that the majority of MC producer genera as *Microcystis*, *Chroococcus* (Chroococcales) and *Aphanocapsa* (Synechococcales) can grow during sludge storage.

8.5.2 Sludge Storage after Oxidation

Overall and as compared to only oxidized samples, stagnation followed by $KMnO_4$ oxidation caused cell growth by up to 70% in 4 out of 16 samples (Figure 6-1-Figure 6-3). Similarly, up to 161% cell growth was observed in 4 out of 6 samples oxidized by H_2O_2 and then stagnated (Figure 6-2). Interestingly, although H_2O_2 had a better performance in decreasing of taxonomic cell counts, oxidized cells by H_2O_2, had higher cell growth as compared to $KMnO_4$ during stagnation (Figure 6-1-Figure 6-3). Cell growth was also observed in potential MC producer genera such as *Microcystis aeruginosa*, *Aphanocapsa delicatissima*, and *Dolichospermum spiroides*. Our results also reveal that the combination of oxidation and stagnation reduces taxonomic cell counts by 12-76% depending on the duration of stagnation, falling short of global removal observed without oxidation that range from 19-83% .

Stagnation time significantly affected cyanobacterial communities in the oxidized samples. Shotgun metagenomic results revealed that stagnation shifted sludge samples oxidized by $KMnO_4$ (both laboratory and shock on-site) towards resistant orders such as Chroococcales and Pleurocapsales (Figure 6-6 and Figure C- 5). In contrast, H_2O_2 oxidation followed by stagnation selectively decreased the relative abundance of Chroococcales (Figure C- 5).

Our research also shows that oxidation can stimulate the increase of *mcyD* gene copy numbers leading to increase of MC production genera during stagnation. After on-site oxidation (72 h) followed by controlled stagnation (closed system), *mcyD* genes copy numbers increased by up to 13X (Figure 6-9c). Similarly, the ratio of potential MC producer species after oxidation (72 h) followed by stagnation increased in 2 out of 3 samples (days 10 and 38) as compared to oxidized sludge before stagnation (Figure 6-7a). The increase in *mcyD* gene copy numbers suggests either cell growth or gene expression regulation in the presence of oxidation and stagnation.

8.5.3 Summary

According to Chapter 4, Chapter 5 and Chapter 6, sludge storage time is an important term that influences cyanobacterial communities during storage (with or without oxidation). This should be considered during sludge handling and management.

Additionally, the combination of oxidation and stagnation does not increase taxonomic cell count removal or MC level decrease. In contrast, oxidation can play a role as a stimulator to increase cell growth probability. Overall, combined oxidation/stagnation increased taxonomic cell counts in 11 out of 18 samples as compared to samples with only stagnation.

Both storage and oxidation are considered as oxidative stresses that affect cyanobacterial communities almost in the same way. In general, resistant genera such as Chroococcales are more susceptible to survive and grow in the presence of these conditions, while Nostocales is more sensitive.

In spite of potential MC producer species growth as well as a remarkable increase in *mcyD* genes in the oxidized/ stagnated samples as compared to only stagnated samples, MC concentrations remained low during stagnation and oxidation/stagnation scenarios (Figure 6-1-Figure 6-3,Figure 6-7,Figure 6-9 and Figure C- 4). This can be due to simultaneous phenomena as i) biodegradation

by MC degrader species, and ii) adsorption on flocs and remained PAC (Jalili et al., 2021; Kormas and Lymperopoulou, 2013; Ma et al., 2016b).

8.6 Implementation of Sludge Management in DWTPs

Solid residuals (including clarifier's sludge and backwash water) vary from 0.1% to 4% of the influent water volume of DWTPs depends on treatment processes (Crittenden et al., 2012).

In general, the sludge supernatant is recycled to the head of the DWTP or is discharged into the source (Chow et al., 1999; Drikas et al., 2001; Jalili et al., 2021). The solid phase can be either transferred to the WWTP or is applied for land application, or disposal by landfill (Qrenawi and Rabah, 2021; Turner et al., 2019; United States Environmental Protection Agency (USEPA), 2011). Less environmental friendly approaches such as untreated residual discharge into lakes or ponds can be also applied in some undeveloped and developing areas (Ahmad et al., 2016). Re-use of DWTP's residuals is growing (Jung et al., 2016; Suman et al., 2017; Turner et al., 2019; Xu et al., 2015). In Canada, the sludge supernatant is often discharged into the source and solids are transferred to WWTPs (Jalili et al., 2021).

The presence of cyanobacteria and cyanotoxins affects sludge handling. An investigation demonstrated that the half-life of microcystin analogues vary from 8 to 18 days in the soil (Chen et al., 2006). Since, there is the risk of soil and groundwater contamination, landfill and field application of cyanobacteria-laden sludge should be avoided. Overall, cyanobacteria-laden sludge should be treated before disposal either in situ or via sending to wastewater treatment plants.

This research (*See section 8.2.1*) validates previous investigations about very large accumulation of cyanobacterial cells in the sludge (Almuhtaram et al., 2018; Drikas et al., 2001; Zamyadi et al., 2013a; Zamyadi et al., 2013b; Zamyadi et al., 2012c). Additionally, we showed dynamic changes of cyanobacterial communities in the stored sludge Chapter 4) leading to cell growth, lysis, and cyanotoxin release (Chapter 5). Furthermore, we showed in Chapter 5 that oxidation could not remove cyanobacterial cells completely from the sludge. Additionally, our results reveal that oxidation followed by storage does not bring additional benefit as compared to only sludge storage in cyanobacteria and cyanotoxin degradation (Chapter 5). Also, storage time is an important parameter that affects cyanobacterial cell counts and communities in the sludge.

Therefore, for development of an efficient strategy to manage cyanobacteria-laden sludge, following subjects should be considered:

- Cyanobacterial cells can grow even after 7 days of storage.
- MC producer genera belong to Synechococcales (*Aphanocapsa*) and Chroococcales (*Microcystis* and *Chroococcus*) are more susceptible to survive and grow during stressful conditions such as sludge storage. This increases the risk of MC release in the sludge and in the supernatant consequently.
- Probability of cyanotoxin degradation (and biodegradation) in the stored sludge can mitigate cyanotoxin accumulation in the stored sludge and its supernatant.

One study reported that pre-ozonation in the water treatment chain can decrease cell accumulation in the clarifier's sludge by up to 630% (Zamyadi et al., 2013b). Several studies demonstrated that pre-oxidation: i) Enhances coagulation/ sedimentation efficiency to remove cyanobacterial cells, and ii) Decrease required coagulant dose; due to the DOM release that can integrate cells together (Ma et al., 2012b; Ma et al., 2012c; Piezer et al., 2020b; Qi et al., 2016).

Therefore, regarding cyanobacteria-laden sludge management, one strategy can be minimizing cell accumulation in the sludge. In fact, cyanobacterial management should be simultaneously applied in both water treatment chain and sludge.

In parallel, we showed that cyanobacterial communities dynamically change in the water (intake or source) and consequently in the sludge Chapter 4). Therefore, frequent monitoring of cyanobacteria in the treatment chain, sludge and supernatant is required to minimize related risks to cyanobacteria and cyanotoxins. Several studies highlighted the importance of water monitoring for the management of cyanobacteria and cyanotoxins in DWTP (Chorus and Welker, 2021; Ibelings et al., 2014; McQuaid et al., 2011; Zamyadi et al., 2016a; Zamyadi et al., 2013a; Zamyadi et al., 2012c).

Overall, sludge management needs three simultaneous actions as i) Water and sludge monitoring, ii) Water treatment process optimization, and iii) Sludge/ supernatant handling.

In Chapter 6, achievements of our experiments were merged with a recent study on water oxidation (Moradinejad, 2021b) to provide a management strategy to control cyanobacteria and metabolites

in DWTPs and their associated sludge. In the suggested framework (Figure 7-8), taxonomic cell counts and metabolites (MCs and T&O agents) should be monitored.

Cell count monitoring can be performed by either on-line probe (RFU) or biovolume values (Chorus and Welker, 2021; McQuaid et al., 2011). A study on cyanobacterial and cyanotoxin management suggested 0.3 mm^3/L biovolume as the vigilance level (Chorus and Welker, 2021). The advantage of on-line monitoring is to provide time-series data. This facilitates further action during increase of the cell counts in the treatment chain (Zamyadi et al., 2016a). However, correlation of biovolume and RFU values is a challenge. In fact, correlation of RFU and biovolume is site-specific (Bowling et al., 2016; Cotterill et al., 2019; Thomson-Laing et al., 2020). Therefore, calibration of RFU values using biovolume for each water and before source monitoring is recommended. Cell counts should be monitored in the treatment chain, sludge (solids) and sludge supernatant. Monitoring of MC level has been widely recommended in several guidelines such as WHO (1.0 μg/L MC-LR) ((WHO), 2020c; World Health Organization (WHO), 1998) and Health Canada (1.5 μg/L) (Health Canada and Federal-Provincial-Territorial Commitee on Drinking Water, 2016). The T&O agents (eg. geosmin and MIB) negatively affect the water quality, raise client's complains and decrease the public's confidence however, they are not health-related concerns (Hobson et al., 2010; Kim and Park, 2021; Ridal et al., 2001; Smith, 2011; Zamyadi et al., 2015b). Therefore, their thresholds can be set as detection level by human's nose. MCs and T&O agents should be monitored in the treatment chain as well as the sludge supernatant. MCs should be monitored in the sludge (solids). Additionally, cell and metabolite breakthrough should be evaluated in the filter tanks.

Cyanobacterial breakthrough can be controlled by optimization of coagulation/clarification and filtration processes. Metabolite breakthrough should be controlled i) CT adjustment in post-oxidation and then, ii) Supplementary treatment as pre-oxidation, PAC or GAC.

Sludge supernatant is subjected to be monitored (cell counts, MCs and T&O) in case it is recycled to the head of the DWTP or is discharged to the source. If sludge (solids) is contained elevated level of MCs, land application and landfill should be restricted. Instead, the sludge should be subjected to treatment either on-site or by transferring to WWTPs.

8.6.1 Cost Implication of Monitoring of DWTPs and associated sludge

Cost and processing time for measurement of cyanobacteria and cyanotoxins of this study are summarized in Table 8-3.

Table 8-3 Cost and processing time of cyanobacteria and cyanotoxin measurement techniques [1]

Analysis	Output type	Cost CAD [2] per sample	Processing time (day per sample)
Microscopy taxonomic cell counts	Absolute	120	1
Total cyanotoxins [3]	Absolute	284	5
Dissolved cyanotoxins	Absolute	284	5
Cell-bound cyanotoxins	Absolute	564	5
Shotgun metagenomics	Relative	200	30 [4]
Gene copy numbers (ddPCR)	Absolute	20	7

[1] Processing times are estimated based on the net required working time. Uncontrolled conditions such as repetition and technician busyness may cause a significant delay; costs were calculated based on the current project (ATRAPP) and previous projects of the contributors.
[2] CAD: Canadian dollar.
[3] Cyanotoxins: i.e. common cyanotoxins as MCs, ATXs, STXs, CYNs
[4] Processing time includes DNA/ RNA extraction, library preparation, sequencing and bioinformatics.

According to our decision framework (Figure 7-8), two absolute-value provider techniques as microscopy taxonomic cell counts and cyanotoxin measurement should be applied for cyanobacteria/cyanotoxin management.

Indeed, cost and time are two important criteria to select measurement techniques in the engineering world. However, firstly, the objective of the measurement should be identified. As discussed in 8.1.1 and 8.6, an appropriate technique for the measurement of cyanobacteria and cyanotoxins should provide absolute data to be interpretable for the process designers, engineers, and operators. Shotgun metagenomic data cannot be used directly in water and sludge management; however, it provides valuable information about persistence of potential toxic genera during treatment and sludge storage. Microscopy taxonomic cell counts provides important information about cell numbers and biovolume in a sample, however it cannot differentiate toxic and non-toxic cells. Therefore, cyanotoxin measurement should be performed to provide information about the presence and the level of cyanotoxin in the sludge. The presence of both cell-bound and dissolved cyanotoxins in the sludge and its supernatant increases the health risk; hence both should be considered during sludge handling. Therefore, to optimize time and cost, only total cyanotoxins

can be measured. Finally, as discussed previously in 8.1.2, although measurement of gene copy numbers by ddPCR provides helpful data for prediction of cyanotoxin production in DWTPs and sludge, it still cannot be replaced with direct measurement of cyanotoxin. However, gene copy quantification can be applied for estimation/ prediction of toxicity in the stored sludge for further actions.

CAPEX and OPEX costs of DWTP and sludge monitoring are presented in Table 8-4. The monitoring costs are amortized in a 5-year period in Table 8-5.

Table 8-4 Capital and operational cost of cyanobacteria and cyanotoxin monitoring in DWTP and sludge according to the suggested decision framework (for the 1st year)

Analysis	**CAPEX CAD**	**OPEX [1] CAD**
On-line probe	30,000 [1]	200
Microscopy taxonomic cell counts (biovolume)	-	300 [2]
Total cyanotoxins (MCs)	-	4500 [3]

[1] Including, the probe, measurement chamber, telemetry components and required mechanical/instrumental components.
[2] Taxonomic cell count (biovolume) cost is calculated based on two calibrations per year, transportation fee included.
[3] MC measurement cost is calculated based on ELISA kit for MC measurement in the intake water, filtered water, sludge and sludge supernatant for 100 days per year, transportation fee included.

Table 8-5 A 5-year analysis cost of a DWTP monitoring for cyanobacteria and cyanotoxin management

Period	**Probe**		**Taxonomic cell counts cost [4]**	**Total cyanotoxins cost [4]**	**Total annual cost [4]**
	Amortized cost [2]	**Annual cost [3,4]**			
1st year	5625	200	300	4500	10,625
2nd year	5625	204	306	4590	10,725
3rd year	5625	208	312	4682	10,827
4th year	5625	212	318	4775	10,931
5th year	5625	217	325	4871	11,037
Total	28,125	1041	1561	23,418	**54,145**

[1] Cost in Canadian dollar
[2] Amortized cost (Ross et al., 2013) is calculated based on the present value: 30,000, salvage value: 5000, interest rate: 2%.
[3] The probe annual cost includes battery and calibration solution fee.
[4] A 2% cost increase is considered per year.

CHAPTER 9 CONCLUSIONS AND RECOMMENDATIONS

9.1 Conclusions

This study enhanced our understanding about cyanobacteria-laden sludge including i) Cyanobacterial accumulation in the sludge during conventional processes in DWTPs, ii) Impact of oxidation ($KMnO_4$ and H_2O_2) on cyanobacterial communities of sludge, iii) Impact of sludge storage (stagnation) on cyanobacterial cell growth, and iv) Cyanobacteria/cyanotoxin management/handling in DWTPs and their sludge/ sludge supernatant. For the first time, combination of microscopy taxonomic cell counts and shotgun metagenomic sequencing as the main techniques of cyanobacterial composition monitoring; and ddPCR as well as physico-chemical parameters as supplementary analyses were applied for investigation of natural cyanobacterial blooms in sludge.

Conclusions of our findings are presented as follows:

(A) Cyanobacterial accumulation in the sludge during conventional processes in DWTPs:

- Conventional treatment eliminated up to 97% of the cyanobacterial cells, as revealed by cell counts. Overall, 96% of *Microcystis* and *Dolichospermum* were eliminated by these processes. At the first glance, this is an effective approach to control cyanobacterial cells. However, coagulation, and sedimentation lead to accumulation of cyanobacterial cells in the sludge. Even a low cell number in the intake water (3.9×10^4 cells/mL) led to 31 times as much cell accumulation in the sludge.
- Selective removal of cyanobacteria at the genus and species levels by coagulation, sedimentation has been highlighted by both shotgun metagenomic sequencing and taxonomic cell counts. Sludge cyanobacterial composition differs from the intake water; even if only samples from the same day are compared. Sludge diversity reflects both selective removal by conventional processes and the accumulation of captured cells over a period of time as determined by sludge age.
- Cyanobacterial communities in the intake water were influenced by OP, while the sludge communities were correlated with TN, N- Kjeldahl, TP, PP, and TOC. None of these nutrient parameters seemed to affect the SST.

- Bacterial and cyanobacterial communities of sludge in the holding tank markedly differed from those measured in sludge supernatant. The prediction of cyanobacterial communities in the supernatant is a challenge, as it is often recycled, possibly adding cyanobacteria, and cyanotoxins to the intake water.
- Understanding the community structure and dynamics in the sludge and sludge supernatant is important for a quantitative cyanobacterial risk assessment. Water operators need to be able to predict the exchanges between the sludge and the supernatant.

(B) Impact of oxidative stress (oxidation and storage) on cyanobacterial communities of sludge:

- Cyanobacterial decay and survival were quantified during sludge stagnation under controlled conditions. Cyanobacterial cell growth was observed by total taxonomic cell counts in 50% of the sludge samples during extended stagnation in the dark within 8-38 days. Growth of cells was dominated by potential MC producers such as *Microcystis*, *Aphanocapsa*, *Chroococcus,* and *Dolichospermum*. Cyanobacterial biomarkers (level 4 subsystems) related to "Circadian clock", "*RpoD*-like sigma" and "Pentose phosphate pathway" increased during stagnation, confirming cyanobacterial growth even in the dark.
- In general, controlled laboratory oxidation with $KMnO_4$ and H_2O_2 decreased total taxonomic counts and potential MC producers in 6 out of 8 samples. However, stagnation after oxidation led to an increase in 9 out of 22 samples.
- Both laboratory oxidation and stagnation affected cyanobacterial diversity. Chroococcales (*Microcystis*) and Synechococcales (*Synechococcus*) were the most persistent orders, whereas Nostocales (*Dolichospermum*) was less resistant. Opposite to $KMnO_4$ oxidation, in which no selective removal within the cyanobacterial community was observed; H_2O_2 selectively reduced the relative abundance of Chroococcales. Under the conditions tested, persistence and/or growth of potential MC producers during storage raise the need to monitor cell counts and cyanotoxins of the sludge and supernatant.
- Total MCs after stagnation preceded by shock on-site oxidation (10 mg/L $KMnO_4$) remained below initial MCs concentrations. However, *mcyD* gene copies surprisingly increased during stagnation, supporting the growth of potential MC producer species. Short-term (24 h) reduction on total taxonomic cell counts was observed.

- When evaluating strategies to manage stored sludge, oxidation and subsequent storage should be considered. As compared to storage only, sludge oxidation with $KMnO_4$ and H_2O_2 did not bring significant additional benefits for the mitigation of cyanobacterial cells and cyanotoxins during storage. In some cases, oxidation prior to storage led to enhanced growth of potential MC producers in the sludge.

(C) Sludge monitoring, management and handling:

Since various phenomena such as cell accumulation, survival, lysis and growth occur in the sludge, monitoring strategies focusing on sporadic measurement of the diversity in the intake water cannot capture the risk associated with the storage and disposal of the sludge. Instead, sludge (solids) and supernatant should be monitor during cyanobacterial seasons.

- Challenges in controlling cyanobacteria in sludge demonstrate the need to consider strategies that minimize cyanobacteria and cyanotoxin accumulation in the stored sludge, such as water oxidation, activated carbon application, and source mitigation.
- Cyanobacteria/ cyanotoxin management/ handling should be simultaneously applied in DWTPs and their sludge. Cyanobacteria and cyanotoxins management in DWTPs is a triangular activity including monitoring, treatment and sludge handling and interrelation. A decision framework including three following steps can support operators to control cyanobacteria and cyanotoxin in DWTPs and sludge: i) Source water risk assessment, ii) Treatment breakthrough assessment and management, and iii) Sludge and supernatant risk assessment and management.
    - *Source water risk assessment*: Source water should be periodically monitored during cyanobacterial bloom seasons. This helps to take an appropriate decision during elevated cell counts or cyanotoxin concentrations. On-line probes provide time-series data that increase flexibility of operators to control cyanobacterial cells. However, correlation between RFU and biovolume is required.
    - *Treatment breakthrough assessment and management:* The efficiency of coagulation should be evaluated during cyanobacterial presence inside DWTPs. Cell breakthrough into the filtration tanks reflects inefficiency of coagulation. In this condition, adjustment of coagulation process such as coagulant dose optimization or decrease the filtration rate are required. In case of cyanotoxin or

T&O presence in the filtration tank, further treatment such as post- / pre- oxidation or activated carbon (PAC and GAC) should be considered.

- *Sludge and supernatant risk assessment and management:* If sludge supernatant is recycled to the head of the DWTP or is discharged into the source, cell counts (or RFU), cyanotoxin and T&O levels should be monitored. If the values are not in the recommended ranges, the risk assessment should be performed. If supernatant has a negative impact on the intake water/source quality recycling/ discharge should be stopped. Sludge (solids) containing MCs cannot be used for land application purpose or landfill due to long half-life in the sol. Instead, a treatment is required. Sludge treatment can be performed either on-site or by transferring to a WWTP.

9.2 Recommendations

According to findings of this research study, following recommendations are suggested for further studies:

- To study the impact of continuous oxidation (instead of shock and batch oxidation) on cyanobacterial communities, taxonomic cell counts and cyanotoxins during cyanobacterial blooms.
- To investigate the impact of pre-oxidation of intake water on the cyanobacterial composition of sludge in a full scale DWTP.
- To apply metatranscriptomic technique to evaluate the expression and activation of active genes in cell growth during sludge storage and oxidation.
- To study the impact of oxidative stress (oxidation and sludge storage) on sludge contained various filamentous cyanobacterial orders such as Nostocales, Oscillatoriales and in the absence (or a low presence) of resistant orders such as Chroococcales and Synechococcales.
- To develop a time-series monitoring technique for analysis of cyanobacteria in the sludge.
- To investigate the fate of ATX-a, STXs and CYN in the stored sludge and its supernatant.

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APPENDICES

Appendix A Supplementary data, Article 1

Journal: *Toxins*

Title: Can Cyanobacterial Diversity in the Source Predict the Diversity in Sludge and the Risk of Toxin Release in a Drinking Water Treatment Plant?

Authors: Farhad Jalili, Hana Trigui, Juan Francisco Guerra Maldonado, Sarah Dorner, Arash Zamyadi, B. Jesse Shapiro, Yves Terrat, Nathalie Fortin, Sébastien Sauvé and Michèle Prévost

Table A- 1 Water characteristics of the studied plant in Missisquoi Bay during the sampling campaign from July to October 2017.

Treatment Step	Parameters	July	August	September	October	Specifications
Raw water (RW)	Turbidity (NTU)	2.4–93.3	0.14–102.5	2.7–79.8	1.5–153	-
	pH	6.0–8.2	5.6–8.2	6.4–9.1	5.9–8.7	-
Clarifier (CW)	Turbidity (NTU)	0.29–20	0.01–10.23	0.01–20.1	0.24–21.1	Effective clarifier depth: 4.90 m, Max. sludge bed: 2.95 m, Hydraulic retention time: 1 h, Solid retention time: 48 h
	pH	6.1–7.3	6.66–7.11	6.20–7.00	6.54–7.05	
Dual sand-antrachite filter (FW)	Turbidity (NTU)	0.16–0.4	0.15–0.4	0.12–0.4	0.1–0.5	Retention time: 2 h
Treated water (TW)	Turbidity (NTU)	0.24–0.59	0.22–0.50	0.20–0.45	0.15–0.41	-
	pH	6.51–8.02	7.04–8.00	6.95–8.74	7.10–8.14	-

Table A-2 Concentrations of cell-bound and dissolved microcystins (MCs) in the RW (raw water), (ST) sludge holding tank and (SST) sludge holding tank. DL: below detection limit. NS: sample mot taken

Date	RW		ST		SST	
	Cell-bound MCs (ng/L)	Dissolved MCs (ng/L)	Cell-bound MCs (ng/L)	Dissolved MCs (ng/L)	Cell-bound MCs (ng/L)	Dissolved MCs (ng/L)
July 27th 2017	2.9	65.7	5.1	54.8	NS	
August 15th 2017	5.8	81.5	11.6	77.6	3.3	75.6
August 25th 2017	6.0	89.6	36.1	74.2	13.0	86.7
August 30th 2017	9.0	64.0	38.9	52.6	44.7	71.3
September 1st 2017	68.2	191.9	158.9	121.7	NS	
September 5th 2017	DL	DL	12.7	69.2	36.9	91.2
October 27th 2017	20.7	121.9	52.4	116.7	NS	

Table A-3 Concentration of nutrients in raw water (RW), sludge holding tank (ST) and sludge tank supernatant (SST) on July 27th, August 15th, 25th and 30th, September 1st and 5th and October 27th 2017. NS: Sample not taken.

Date	TN (mg N/L)			DN (mg N/L)			N-Kjeldahl (mg N/L)			NH_4 (mg N/L)			DON (mg N/L)			NO2- NO3 (mg N/L)		
	RW	**ST**	**SST**	**RW**	**ST**	**SST**	**RW**	**ST**	**SST**	**RW**	**ST**	**SST**	**RW**	**ST**	**SST**	**RW**	**ST**	**SST**
July 27th 2017	0.87	2.19	NS	0.78	0.76	NS	0.60	1.80	NS	0.17	0.13	NS	0.40	0.30	NS	0.25	0.37	NS
August 15th 2017	0.96	1.98	0.94	0.84	0.83	0.80	0.60	1.40	0.40	0.07	0.06	0.05	0.40	0.20	0.20	0.41	0.56	0.56
August 25th 2017	0.64	3.09	0.47	0.58	0.54	0.43	0.60	3.00	0.30	0.09	0.11	0.06	0.40	0.30	0.20	0.08	0.10	0.13
August 30th 2017	0.97	29.21	0.53	0.68	1.15	0.46	0.80	29.00	0.40	0.12	0.44	0.09	0.40	0.50	0.20	0.13	0.18	0.17
September 1st 2017	1.61	32.77	NS	1.25	1.86	NS	0.01	32.60	NS	0.27	0.46	NS	0.80	1.20	NS	0.23	0.16	NS
September 5th 2017	0.84	5.44	0.52	0.68	0.56	0.51	0.70	5.20	0.30	0.08	0.06	0.08	0.40	0.30	0.20	0.19	0.21	0.21
October 27th 2017	1.12	2.27	NS	0.82	0.72	NS	0.80	1.90	NS	0.07	0.05	NS	0.40	0.30	NS	0.32	0.37	NS

Table A-3 Concentration of nutrients in raw water (RW), sludge holding tank (ST) and sludge tank supernatant (SST) on July 27th, August 15th, 25th and 30th, September 1st and 5th and October 27th 2017. NS: Sample not taken (continue)

Date	TP (µg P/L)			DP (µg P/L)			OP (µg P/L)			PP (µg P/L)			DOP (µg P/L)			TOC mg/L		
	RW	ST	SST	RW	ST	SST	RW	ST	SST	RW	ST	SST	RW	ST	SST	RW	ST	SST
July 27th 2017	54.51	323.41	NS	31.54	2.34	NS	18.00	4.00	NS	23.00	321.00	NS	14.00	2.00	NS	5.30	13.85	NS
August 15th 2017	93.32	476.00	18.00	47.70	3.00	4.00	35.00	3.00	2.00	46.00	473.00	14.00	13.00	2.00	4.00	5.30	220.00	2.70
August 25th 2017	60.64	948.00	36.00	27.50	8.00	11.00	21.00	4.00	6.00	27.00	940.00	25.00	12.00	4.00	4.00	5.30	290.00	2.90
August 30th 2017	78.35	5277.00	21.82	27.49	29.00	4.30	14.00	4.00	3.00	51.00	5248.00	18.00	13.00	25.00	2.00	6.10	1390.00	2.60
September 1st 2017	50.72	5857.00	NS	23.73	60.00	NS	8.00	25.00	NS	27.00	5797.00	NS	16.00	60.00	NS	8.60	1240.00	NS
September 5th 2017	85.89	1176.00	17.65	42.56	7.00	7.12	24.00	3.00	3.00	43.00	1169.00	11.00	19.00	4.00	4.00	5.80	430.00	2.70
October 27th 2017	119.01	554.00	NS	42.29	3.00	NS	29.00	2.00	NS	77.00	551.00	NS	14.00	3.00	NS	5.60	2.40	NS

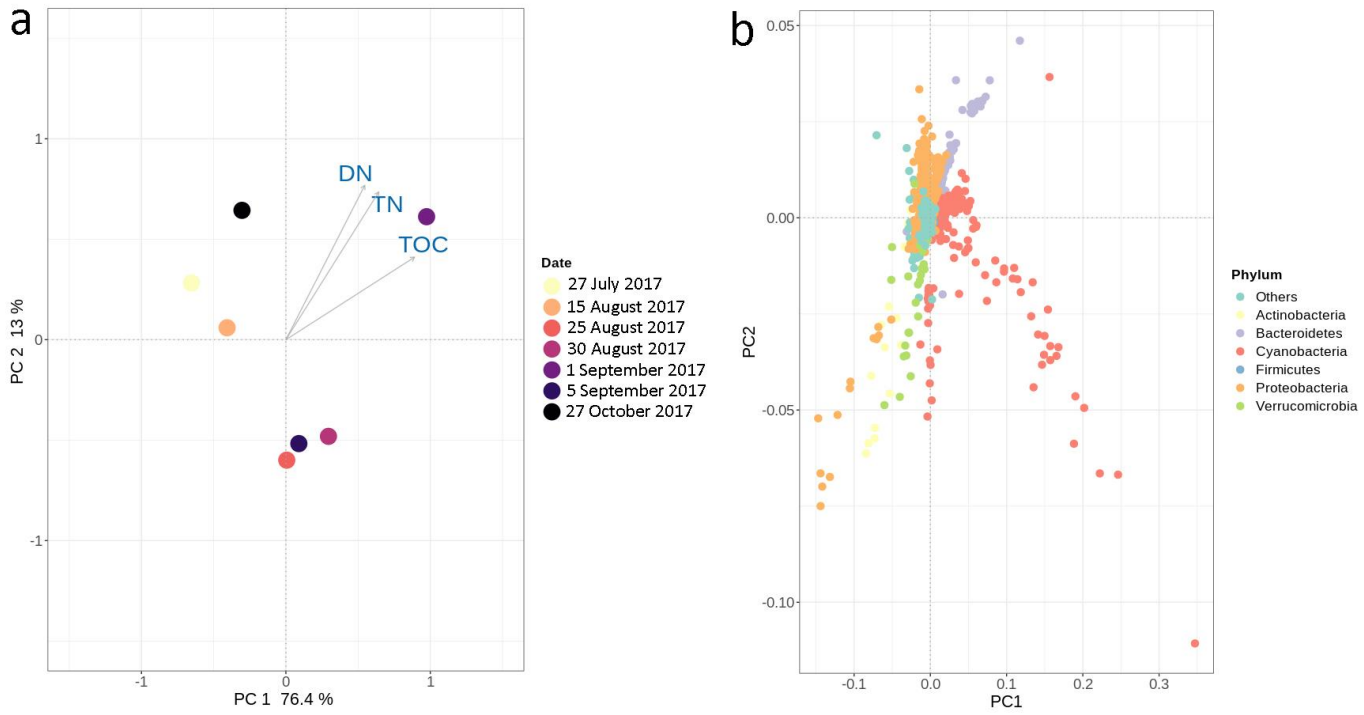

Figure A- 1 Principle components analysis (PCA) of nutrient parameters' impact on a) sampling dates, b) bacterial diversity (phylum level) in raw water (RW) on July 27th, August 15th, 25th and 30th, September 1st and 5th and October 27th 2017. Only the significant parameters were shown ($p < 0.05$).

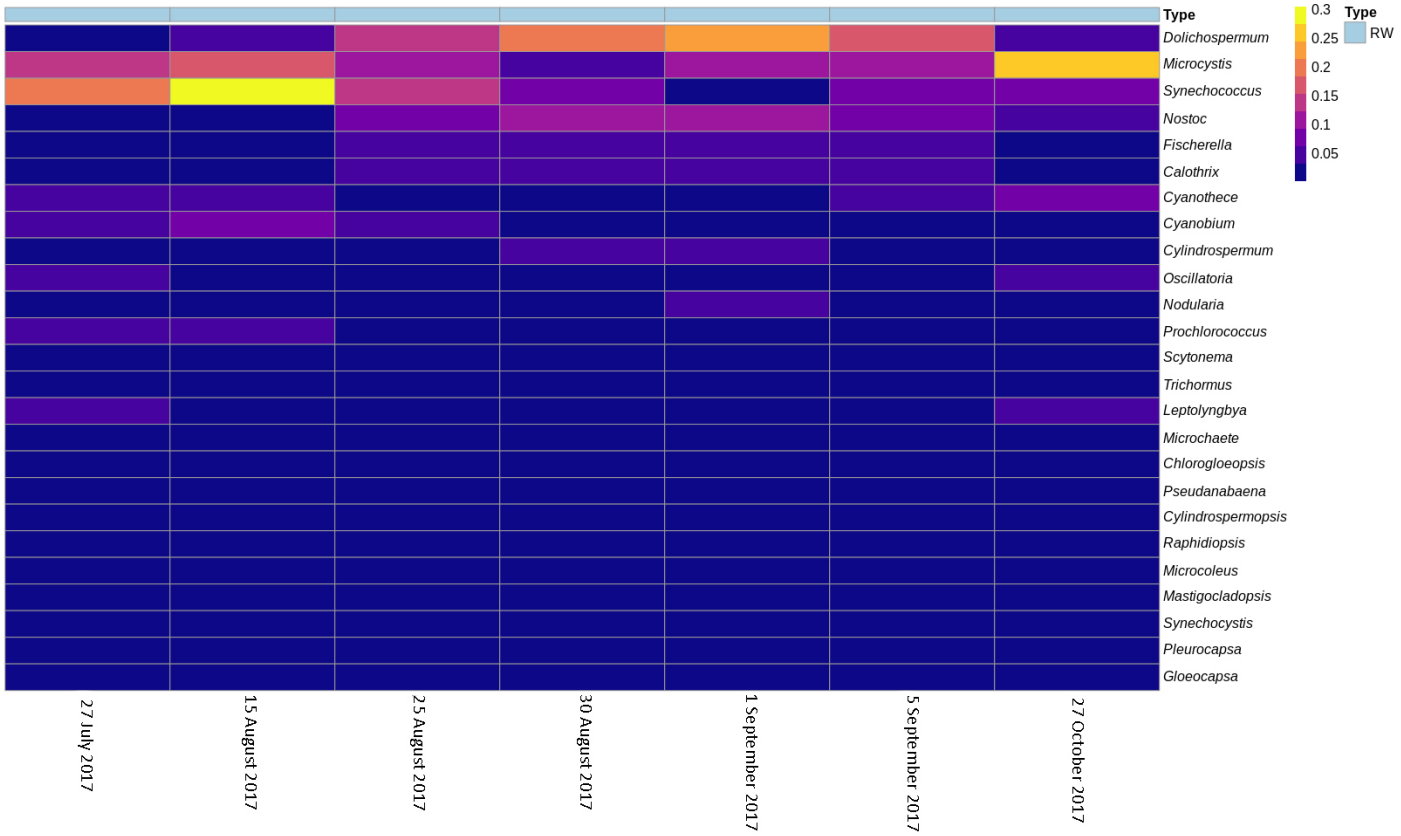

Figure A- 2 Relative abundance of the top 25 major abundant genera in the raw water (RW). Samples taken on July 27th, August 15th, 25th, and 30th, September 1st and 5th, and October 27th 2017.

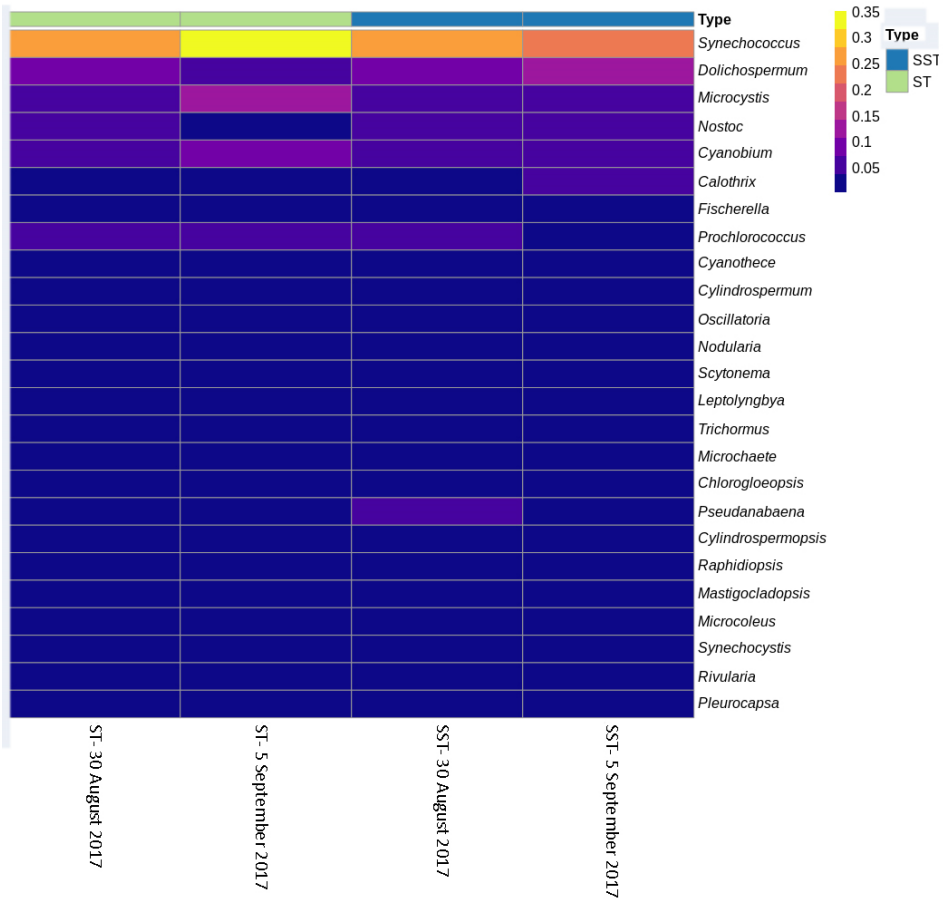

Figure A- 3 Relative abundance of the top 25 major abundant genera in sludge holding tank (ST) and sludge holding tank supernatant (SST). Samples taken on August 30th, and September 5th 2017.

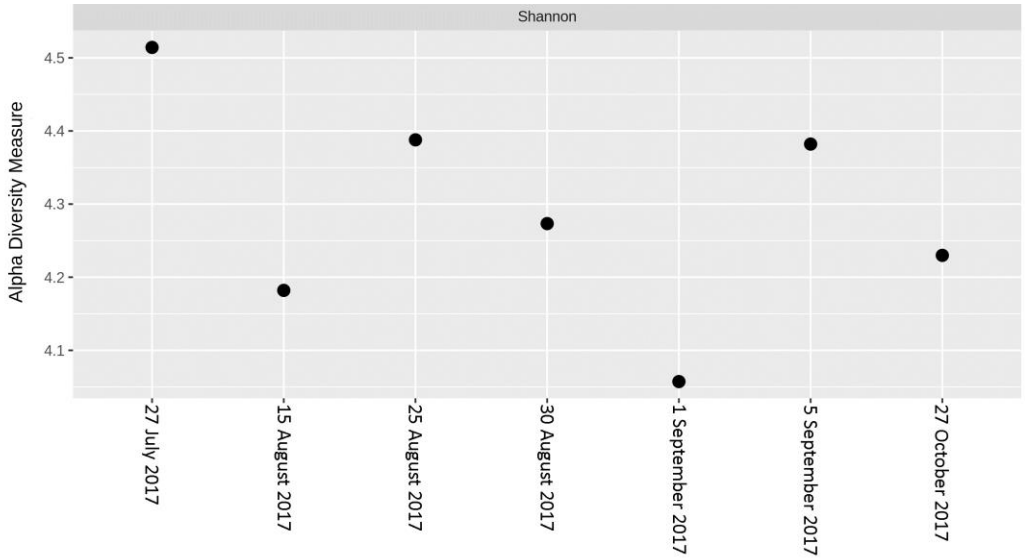

Figure A- 4 Evaluation of the cyanobacterial diversity in raw water (RW) on July 27th, August 15th, 25th and 30th, September 1st, 5th, and October 27th 2017 using Shannon index.

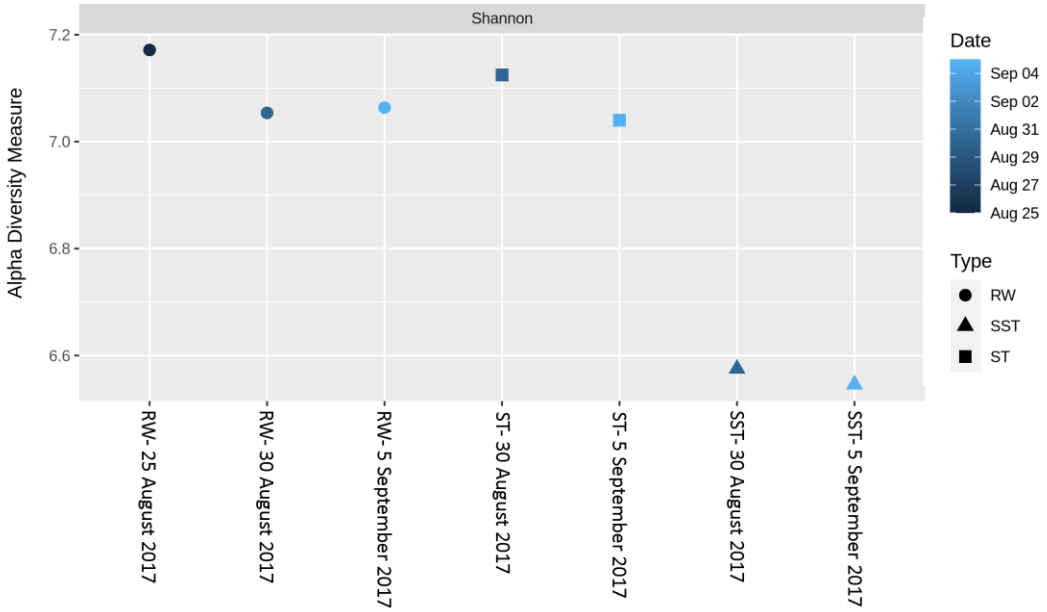

Figure A- 5 Bacterial diversity in the raw water (RW) on August 25th and 30th and September 5th 2017, sludge holding tank (ST) and sludge holding tank supernatant (SST) on August 30th and September 5th 2017.

Appendix B Supplementary data, Article 2

Journal: *Toxins (submitted)*

Title: Impact of stagnation on the diversity of cyanobacteria in drinking water treatment plant sludge

Authors: Farhad Jalili, Hana Trigui, Juan Francisco Guerra Maldonado, Sarah Dorner, Arash Zamyadi, B. Jesse Shapiro, Yves Terrat, Nathalie Fortin, Sébastien Sauvé and Michèle Prévos

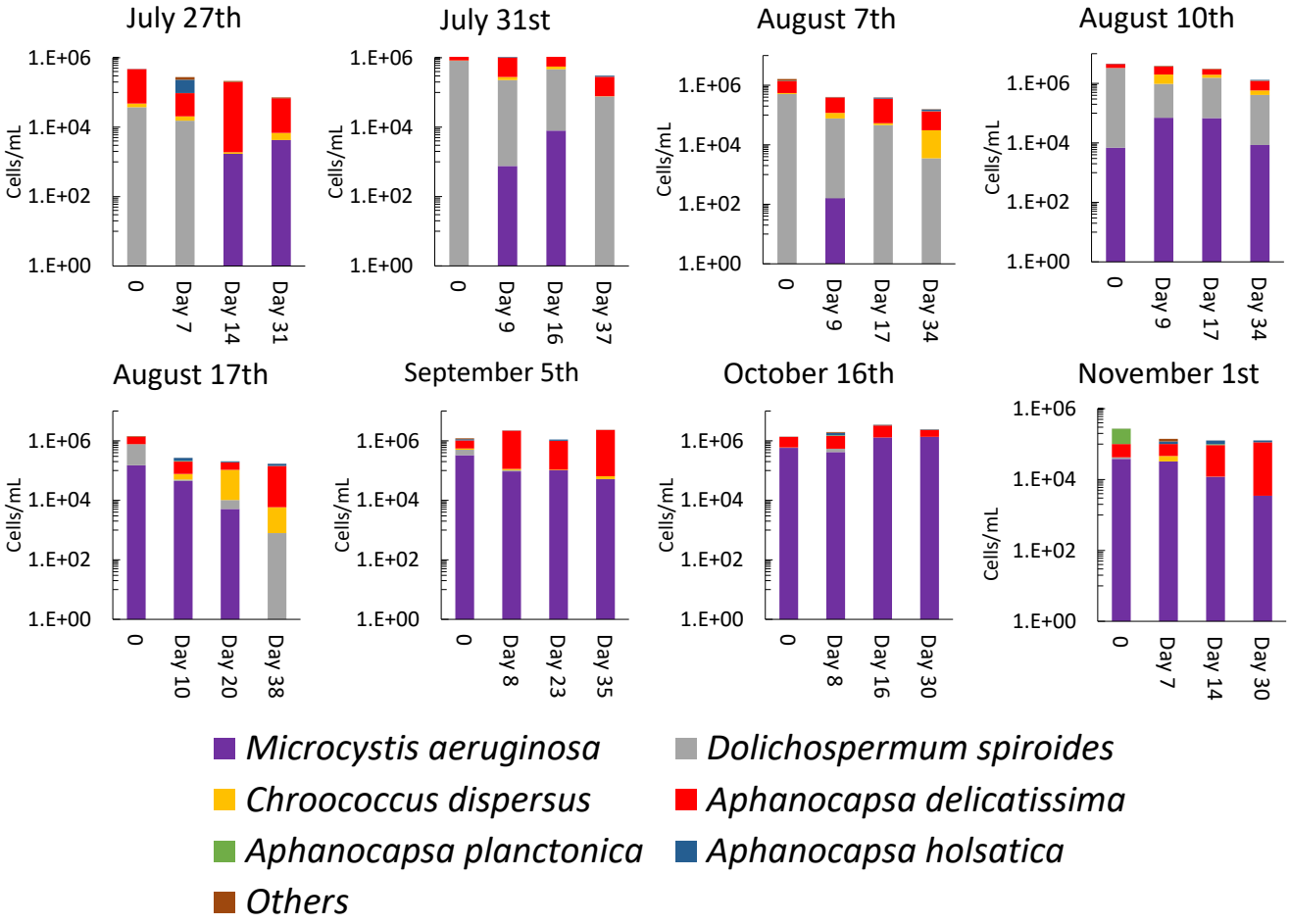

Figure B- 1 Taxonomic cell count speciation (other than *Aphanothece clathrata brevis*) after sludge stagnation, 0: before stagnation. Other: *see Figure 5-1*.

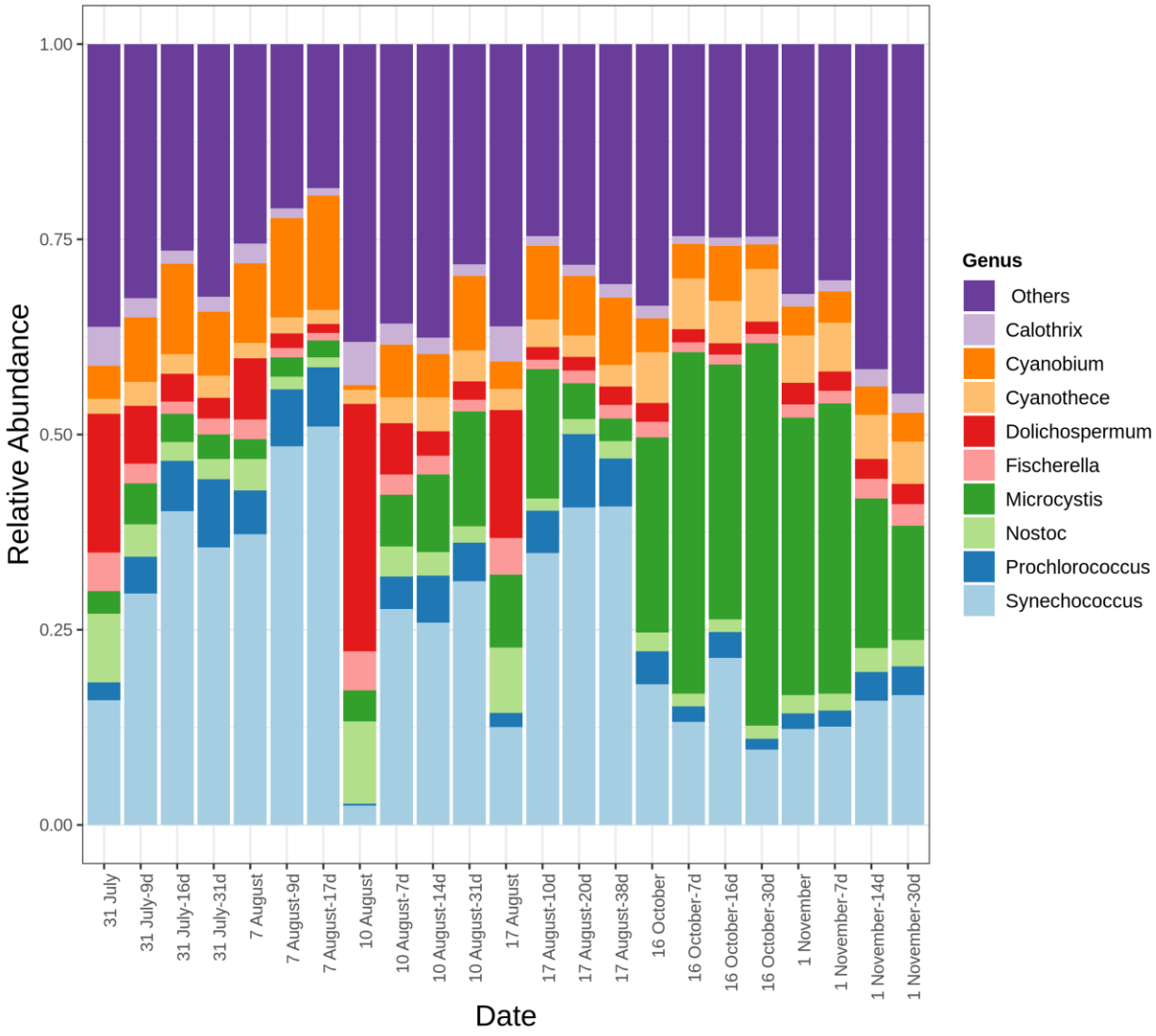

Figure B- 2 Cyanobacterial community at the genus level during stagnation. d:stagnation day

Appendix C Supplementary data, Article 3

Journal: *Journal of Water (Published)*

Title: Oxidation to control cyanobacteria and cyanotoxins in drinking water treatment plants: challenges at the laboratory and full-scale plants

Authors: Farhad Jalili, Hana Trigui, Juan Francisco Guerra Maldonado, Sarah Dorner, Arash Zamyadi, B. Jesse Shapiro, Yves Terrat, Nathalie Fortin, Sébastien Sauvé and Michèle Prévost

Table C- 1 Characteristics of untreated sludge

-: shotgun metagenomic sample not taken, *: shotgun metagenomic sample taken.

Sampling date	Shotgun metagenomic sequencing	Taxonomic cell counts		MCs (ng/L)		DOC (mg/L)	pH	Turbidity (NTU)	TSS (mg/L)	TVS (mg/L)	Sludge storage time (d)
		Cells/mL x10^6	mm3/L	Cell-bound	Dissolved						
July 31st 2018	*	2.25	147.4	24.9	37.9	3.60	7.05	201	716	367	7
August 7th 2018	*	2.71	96.20	22.0	138.5	3.19	7.54	171	728	456	5
August 17th 2018	*	2.35	138.3	41.6	46.6	3.35	7.12	327	1092	434	3
September 5th 2018	-	2.37	52.76	951.8	131.2	9.80	6.81	701	1957	1230	6

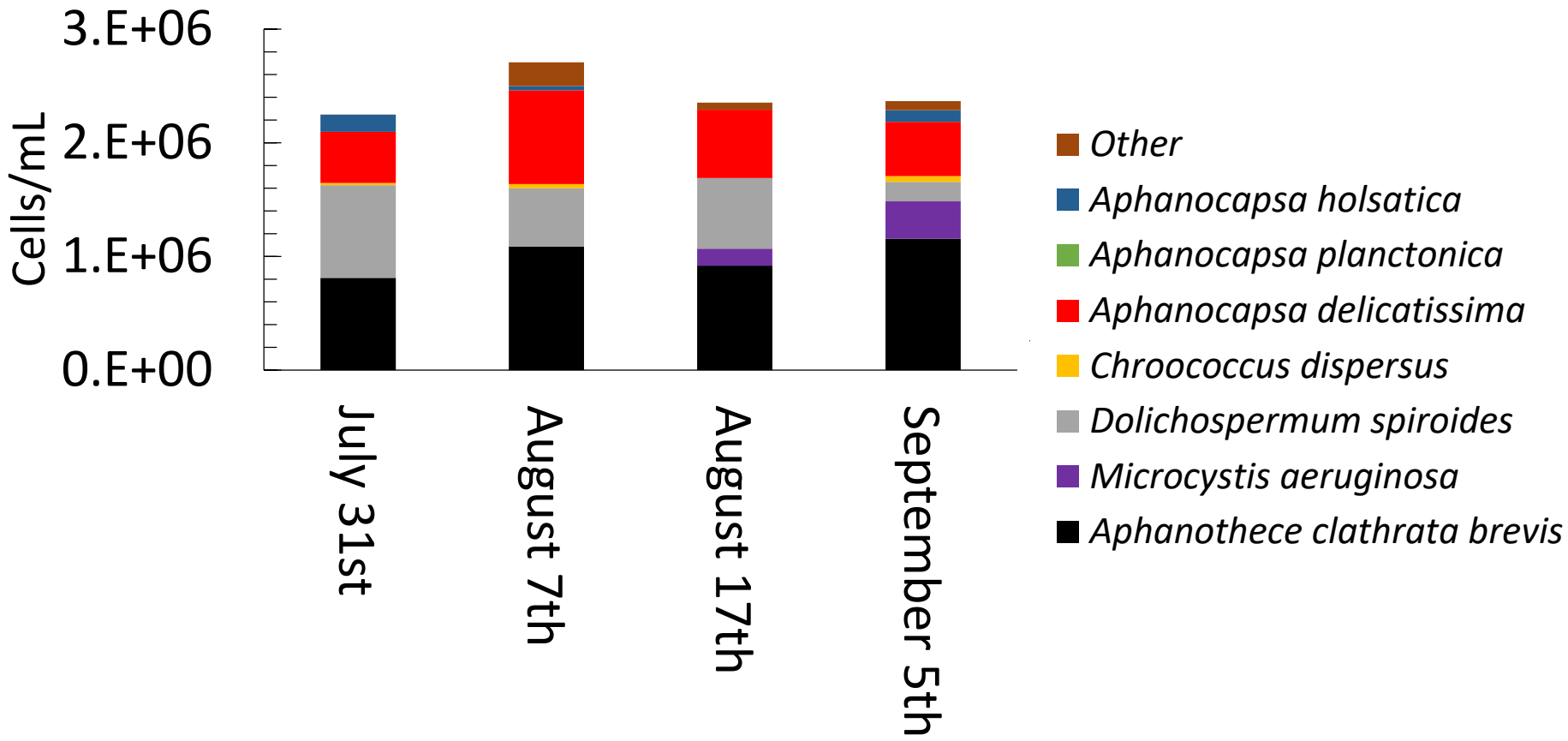

Figure C- 1 Taxonomic cell counts in the untreated sludge before oxidation or stagnation, Other: species with less than 5% of total cell counts.

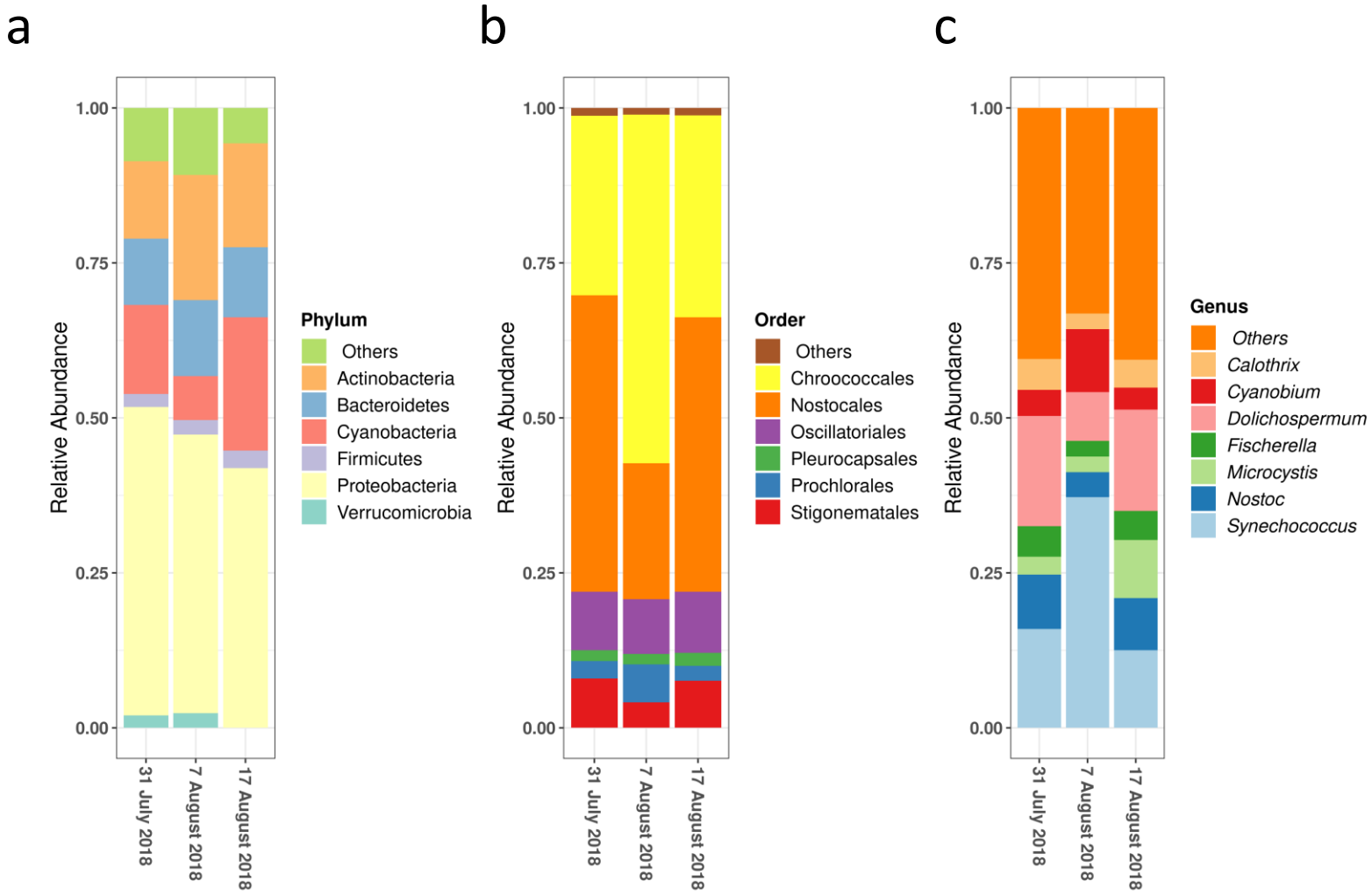

Figure C- 2 a) microbial communities at the phylum level, b) cyanobacterial communities at the order level, and c) cyanobacterial communities at the genus level in the sludge samples.

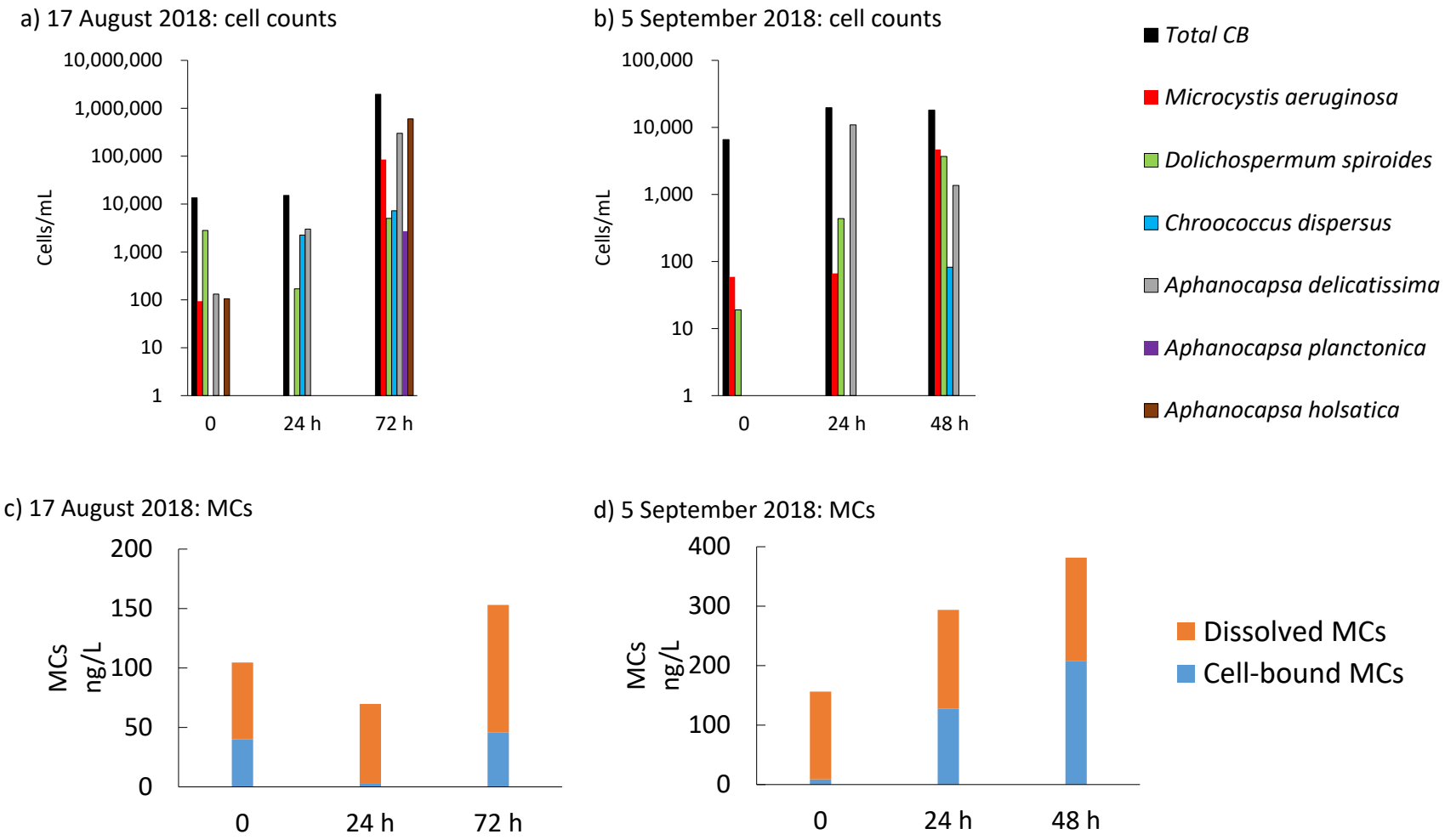

Figure C- 3 Taxonomic cell counts on a) August 17th and b) September 5th; MC concentrations on c) August 17th and d) September 5th in the sludge supernatant after on-site oxidation.

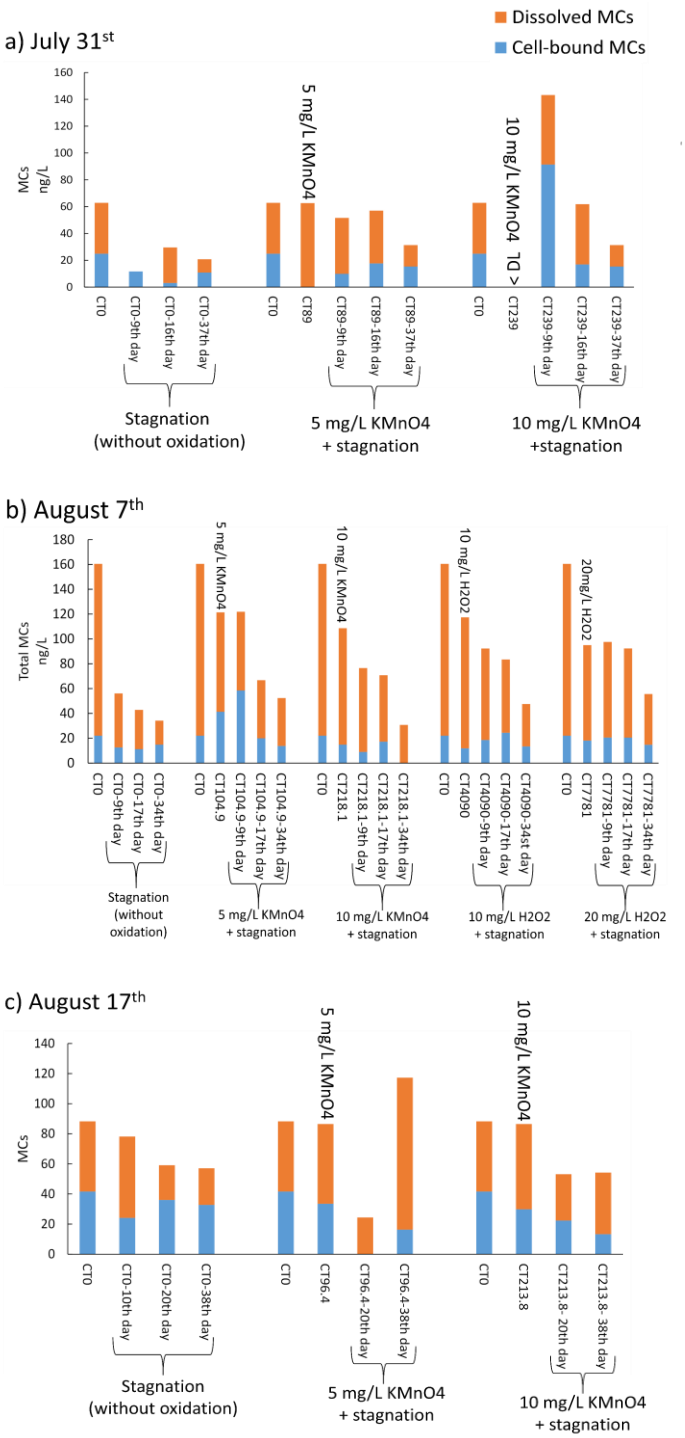

Figure C- 4 MC concentrations during laboratory oxidation followed by stagnation on a) July 31st using 5 and 10 mg $KMnO_4$/L, b) August 7th using 5, 10 mg/L $KMnO_4$ and 10, 20 mg/L H_2O_2, and c) August 17th using 5 and 10 mg/L $KMnO_4$, CT: exposure (mg.min/L), CT0: Before oxidation, day: stagnation day.

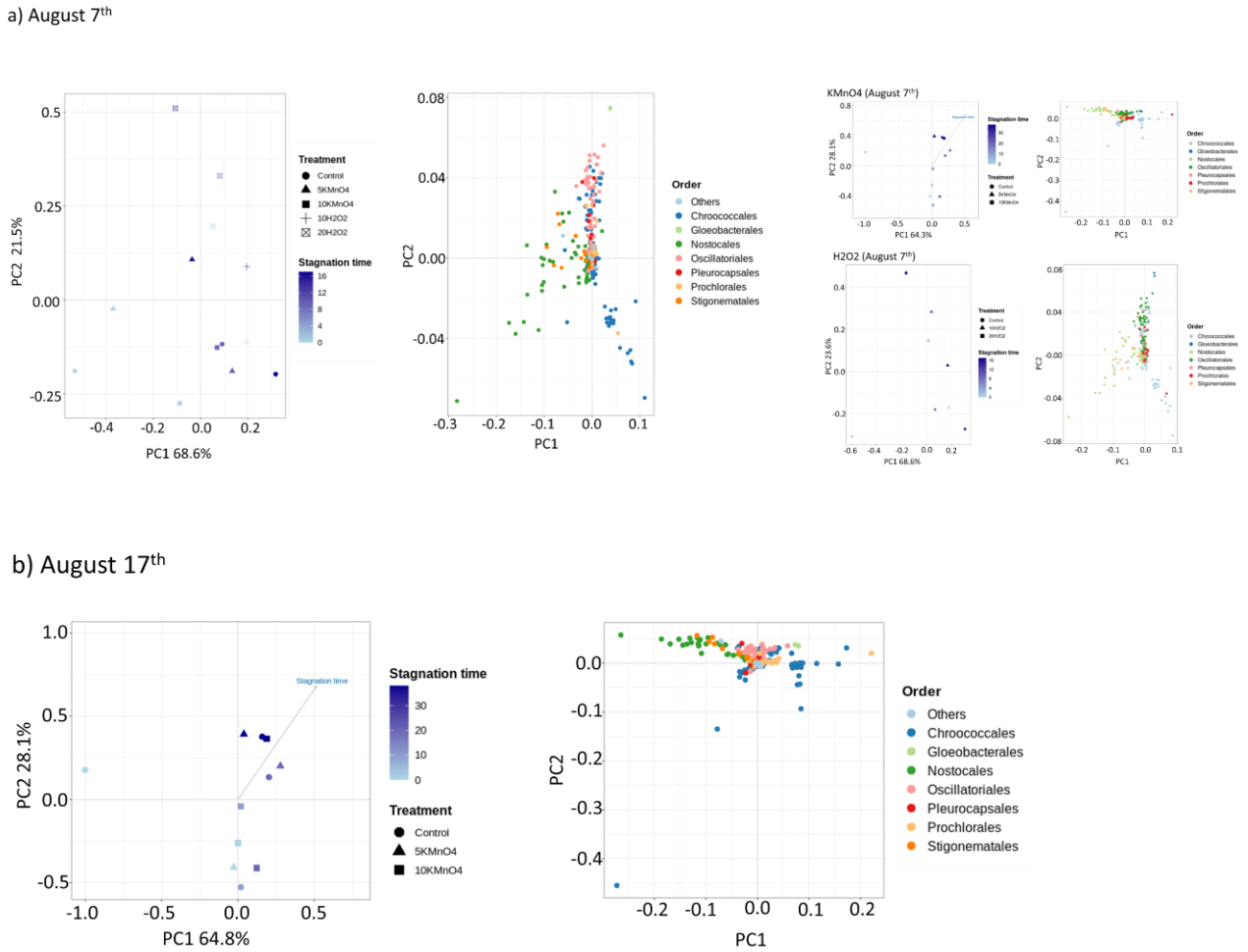

Figure C- 5 PCA analysis of cyanobacterial communities and cyanobacterial species grouped at the order level on oxidized/ stagnated samples in the laboratory scale on a) August 7th, PC1: 68.6, PC2: 21.5% and b) August 17th, PC1: 64.8, PC2: 28.1%. Significant parameters (p <0.05): stagnation time after oxidation by $KMnO_4$, and oxidation by H_2O_2.

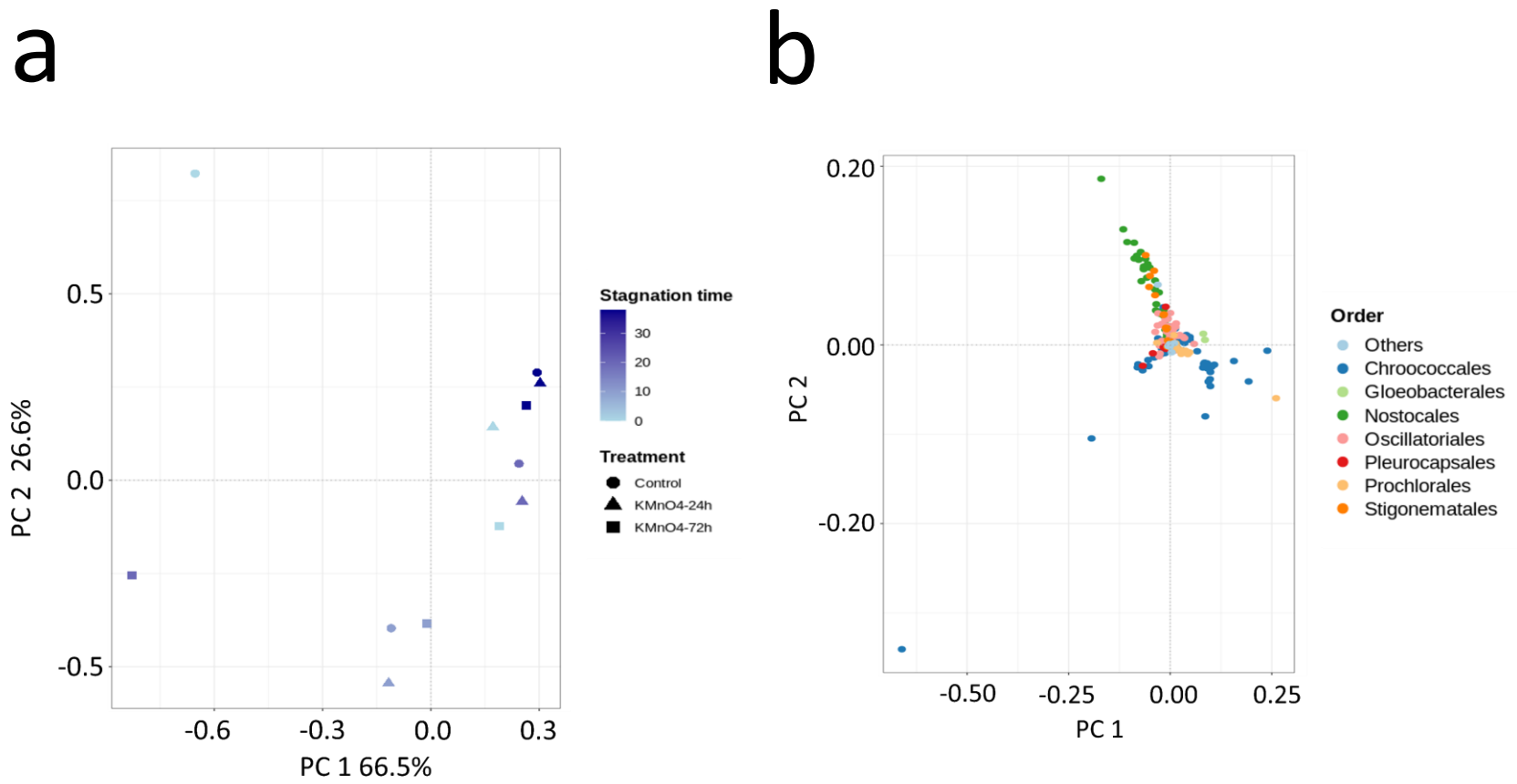

Figure C- 6 a) principal component analysis (PCA) on sludge samples after on-site oxidation followed by stagnation on August 17th, PC1: 66.5%, PC2: 26.6%, b) Cyanobacterial species grouped at the order level.

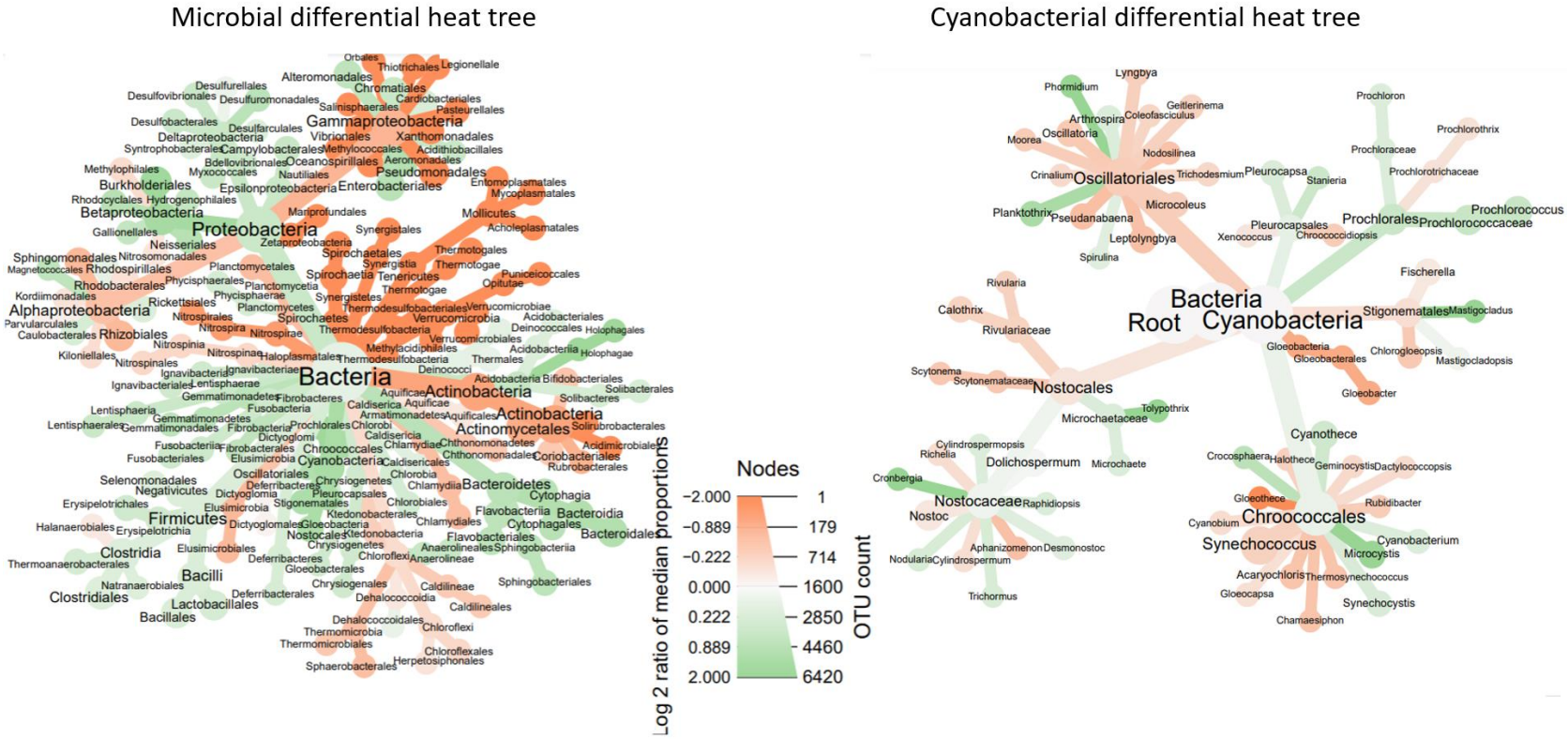

Figure C- 7 Differential heat tree demonstrating changes in microbial and cyanobacterial taxonomic profiles of KMnO4 oxidized sludge for 72 h at T0/T38. T0: not stagnated after oxidation, T38: stagnated for 38 days after oxidation. The intensity color of nodes and edges are correlated with the abundance of taxa in each community. The green color indicates that the taxa is more abundant in the sludge at T0 than the sludge at T38, while the orange color indicates the opposite.