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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

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 [1–6].

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) [7–11]. In spite of the 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 still considered a challenge [7,12–17]. In certain DWTPs, the supernatant of the sludge is discharged to the water source or is recycled to the head of the DWTPs [11,18,19]. The quality of stored sludge impacts the load of cyanobacteria and toxins in the sludge supernatant [11].

Several studies have reported that toxic cyanobacterial cells (e.g., *Microcystis aeruginosa*, *Dolichospermum circinale*, *Oscillatoria* sp., and *Cylindrospermopsis raciborskii*) can survive and release cyanotoxins in the stored sludge for up to 12 days [7,14,20–24]. Recent studies hypothesized toxic cyanobacterial cells can even grow in the stored sludge up to 16 days leading to cyanotoxin release up to four times higher than the expected concentration [25]. Recent evidence documenting the growth of cyanotoxin-producing species during controlled extended storage of sludge raises the need for additional treatment controls to be implemented to limit this risk [26].

$KMnO_4$ and H_2O_2 were successfully applied to manage cyanobacteria and cyanotoxins in DWTPs and source waters [27–32]. 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. [18] 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 also decreased 21% of the 2-Methylisoborneol (MIB) concentration. However, 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 [11,33].

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.

2. Materials and Methods

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 was reported in this DWTP in previous studies [8,11,13], and detailed information on the diversity of the blooms and water quality at this site is presented in [11].

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 [11]. The samples for laboratory oxidation were taken from the solids of the sludge holding tank on 31 July, 7 and 17 August 2018. On-site oxidation was performed on sludge holding tank on 17 August and 5 September 2018.

2.2. Oxidation and Stagnation Procedures

2.2.1. Oxidation

The experimental plan for the oxidation assays is presented in Table 1. The maximum dose of $KMnO_4$ (10 mg/L) was selected based on a previous study on the oxidation of sludge collected from the dewatering tank of a DWTP [18]. 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 [29,34].

Table 1. Experimental plant of oxidation assays. DOC: Dissolved organic carbon.

Date	Oxidant	Initial Applied Dose (mg/L)	Contact Time (h)	Oxidant/DOC Ratio
31 July	$KMnO_4$	5	1: Laboratory assay	1.4
		10		2.8
7 August		5		1.6
		10		3.1
	H_2O_2	10	24: Laboratory assay	3.1
		20		6.3
17 August	$KMnO_4$	5	1: Laboratory assay 24, 72: On-site assay (10 mg/L)	1.5
		10		3.1
5 September	$KMnO_4$	10	24, 48: On-site assay (10 mg/L)	1.0

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 3000 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 °C). 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 shock oxidation. Powdered $KMnO_4$ (>97.5%, Carus, LaSalle, IL, USA) was added to 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 discharged into 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 [35]. 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 [35,36].

Oxidant exposure (CT) as residual concentration (mg/L) multiplied by contact time (min) was calculated for each oxidation assay according to Equation (1) [29].

$$CT = \int_0^t \text{Oxidant}\, dt = \frac{C_0}{k}\left(e^{kt} - 1\right) \quad (1)$$

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 7 August sample was 1.0 (Table 2).

Table 2. Apparent decay rate constant in cyanobacteria-laden sludge oxidation. Contact times: $KMnO_4$: 1 h, H_2O_2: 24 h.

Date	**31 July**		**7 August**		**17 August**		**17 August**	
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

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 °C) to simulate sludge holding tank conditions. 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.

2.3. Sample Preparation and Analysis

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 [37–39]. 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.

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) [40].

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, Canada). 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 × 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 carried out 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. [41] and Roy-Lachapelle et al. [42].

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 performed by Génome Québec. Libraries were generated by the NEBNext Ultra II DNA Library Prep Kit for Illumina (New England BioLabs, MA, USA) and according to the manufacturer's instruction. Adapters and PCR primers were provided from IDT (Coralville, IA, USA). SparQ beads (Qiagen, Toronto, ON, Canada) were applied for the size selection of libraries. The Kapa Illumina GA with Revised Primers-SYBR Fast Universal kit (Kapa Biosystems, MA, 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 [11]. DNA extraction was performed in triplicate, while one replicate was sequenced. Reproducibility and variability between replicates of our technique were evaluated by comparing results from triplicates [43].

Statistical analysis was conducted by R (3.6.2) and phyloseq (1.28.0) [44]. Normalization of the taxonomic data was performed using EasyCODA (0.31.1) and centered log-ratio transformation [45]. Redundancy analysis (RDA) was applied for evaluation of the constrained variables. The variance homogeneity was validated prior to implementation of the model [46]. Significant variables (>95%) were selected by permutation test. All details are explained in our previous investigation [11].

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′-TGG GGATGGACTCTCTCACTTC-3′ and mcyD(KS)R1:5′ GGCTTCAACATTCGGAAAACG-3′ [47]. The details of the analysis are presented in Moradinejad et al. [48].

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 [35].

3. Results

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 31 July, 7 and 17 August and increased to 1084 ng/L on 5 September with 88% being cell-bound MCs. DOC remained in the range of 3.19–3.60 mg/L with a peak on 5 September (9.8 mg/L). Similarly, while pH remained in the range of 7.05–7.54 on 31 July, 7 and 17 August (laboratory oxidation dates), it decreased to 6.81 on 5 September (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 2.25×10^6 cells/mL to 2.71×10^6 cells/mL during the studied dates (Table S1). The highest cell counts were reported on 7 August. *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 stagnation (Figure S1).

Shotgun metagenomic sequencing showed that Proteobacteria was the predominant phylum in all studied dates. Cyanobacteria, Bacteroidetes and Actinobacteria were the next predominant phyla. The relative abundance of Cyanobacteria reached its highest level on 17 August, while the lowest relative abundance of Cyanobacteria was recorded on 7 August (Figure S2a). Investigation of Cyanobacteria at the order level revealed that Nostocales was predominant on 31 July and 17 August. In contrast, Chroococcales was the predominant order on 7 August (Figure S2b). Similarly, *Dolichospermum* (Nostocales) was predominant on 31 July and 17 August. *Synechococcus* was the predominant genus on 7 August (Figure S2c).

3.2. Oxidation of Cyanobacteria-Laden Sludge in Controlled Conditions

The first-order rate constants and half-life of applied oxidants are presented in (Table 2). Rate constants varied from 0.044 min^{-1} to 0.054 min^{-1} using 5 mg/L $KMnO_4$ 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}–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 31 July 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 7 August.

Oxidation with $KMnO_4$ caused different shifts of diversity as revealed by taxonomic cyanobacterial cell counts. On 31 July, 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 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 7 and 17 August, respectively (Figures 2 and 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 2).

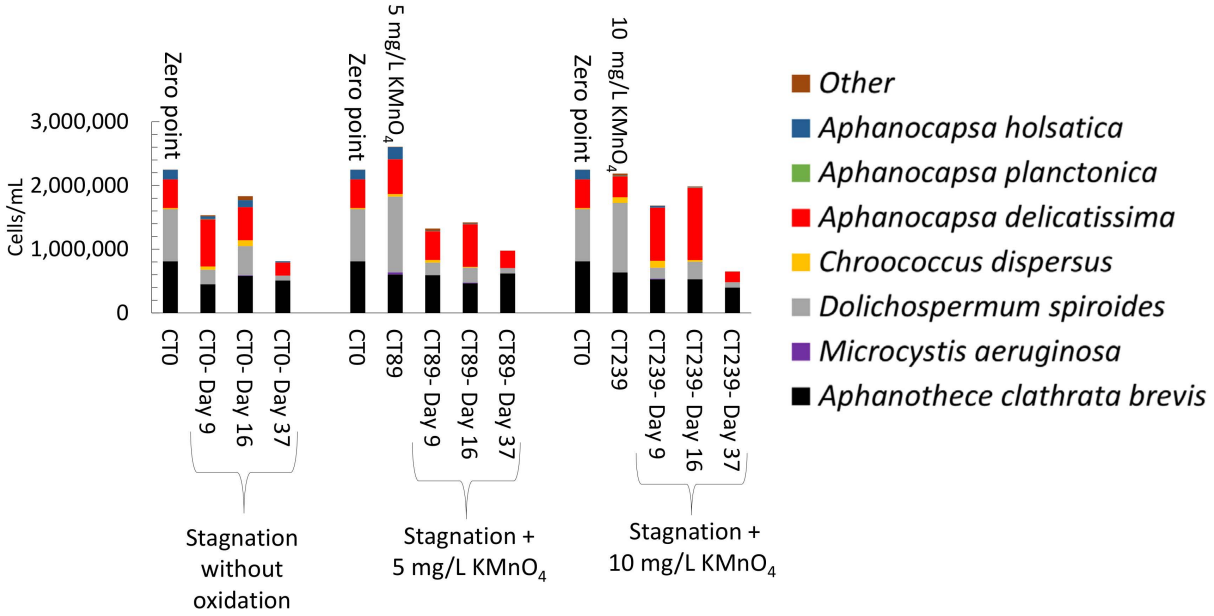

Figure 1. Fate of cyanobacterial cells (taxonomic cell counts) during sludge oxidation and stagnation in the laboratory on 31 July using 5 and 10 mg/L $KMnO_4$. Zero point: Before oxidation, CT: exposure (mg·min/L), Day: stagnation day.

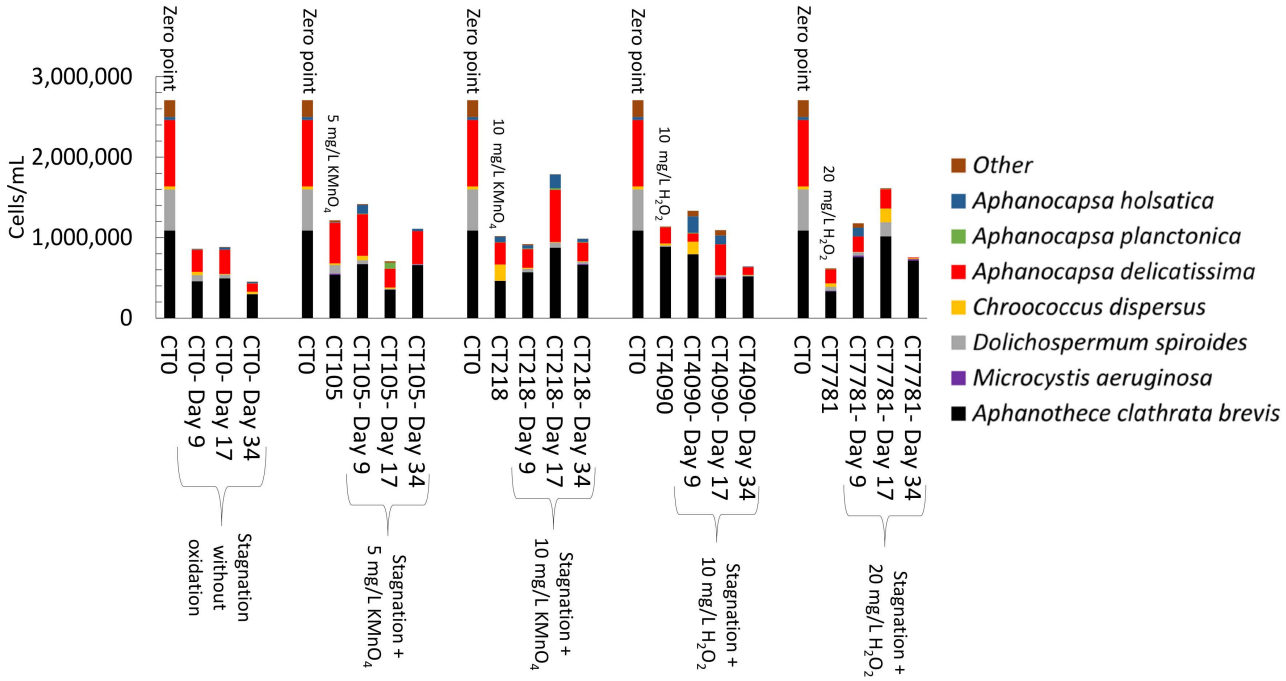

Figure 2. Fate of cyanobacterial cells (taxonomic cell counts) during sludge oxidation and stagnation in the laboratory on 7 August using 5, 10 mg/L $KMnO_4$ and 10, 20 mg/L H_2O_2. Zero point: Before oxidation, CT: exposure (mg·min/L), Day: stagnation day.

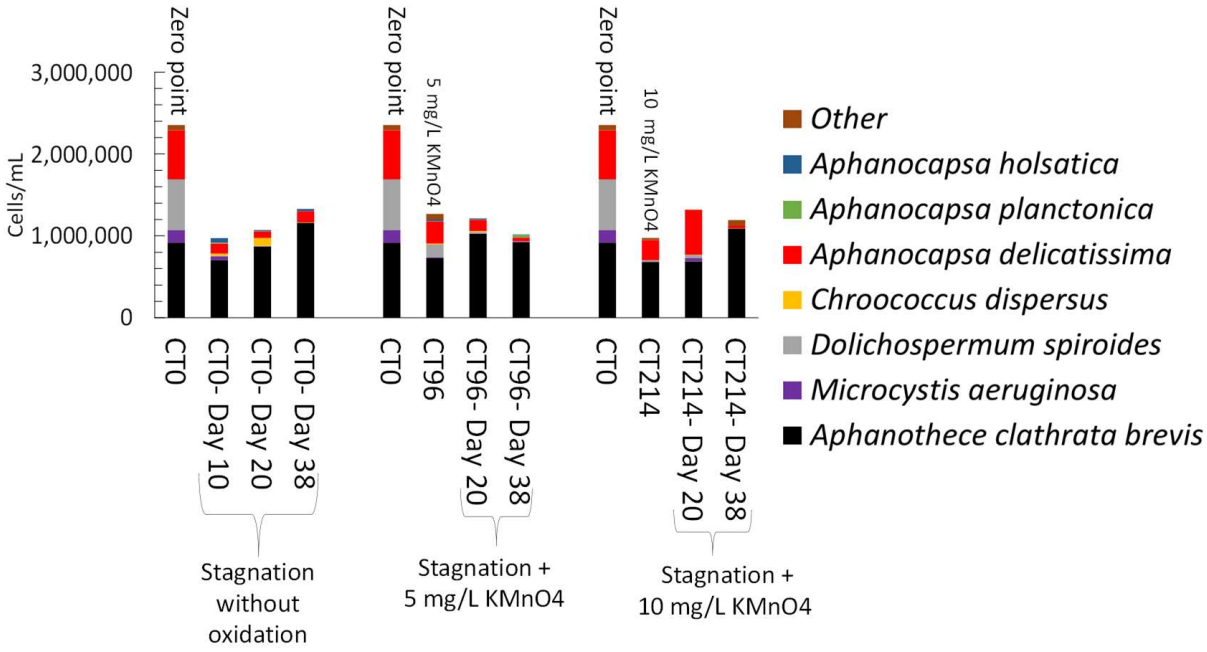

Figure 3. Fate of cyanobacterial cells (taxonomic cell counts) during sludge oxidation and stagnation in the laboratory on 17 August using 5 and 10 mg/L $KMnO_4$. Zero point: Before oxidation, CT: exposure (mg·min/L), Day: stagnation day.

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 (7 and 17 August) and oxidants ($KMnO_4$ and H_2O_2) (Figure 4a). The relative abundance trends are coherent with absolute abundance observations based on taxonomic cell counts (Figures 2, 3 and 4a).

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

Overall, two different trends were observed in the microbial communities after oxidation: (i) on 7 August, the diversity (Shannon index) remained almost constant after oxidation in all scenarios (Figure 5a), (ii) on 17 August, the Shannon index increased after oxidation. This could be caused by the higher relative abundance of Cyanobacteria on 17 August (<70%) as compared to 7 August (<33%) (Figure 4a). Indeed, at the order level, Chroococcales was predominant on 7 August and was not impacted by oxidation (Figure 4b). On 17 August, 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 6a,b).

At the cyanobacterial level and on 7 August, 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 17 August, both doses of $KMnO_4$ decreased cyanobacterial diversity (Figure 5b). The relative abundance of *Synechococcus*, *Microcystis*, *Cyanobium* and *Prochlorococcus* persisted during oxidation, while that of *Dolichospermum* decreased (Figure 4b,c). Precisely, on 7 August, 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 17 August, 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 6c,d). The initial microbial community before oxidation and the CT determines the extent of changes caused by oxidation within the microbial composition as reported by Moradinejad et al. [29].

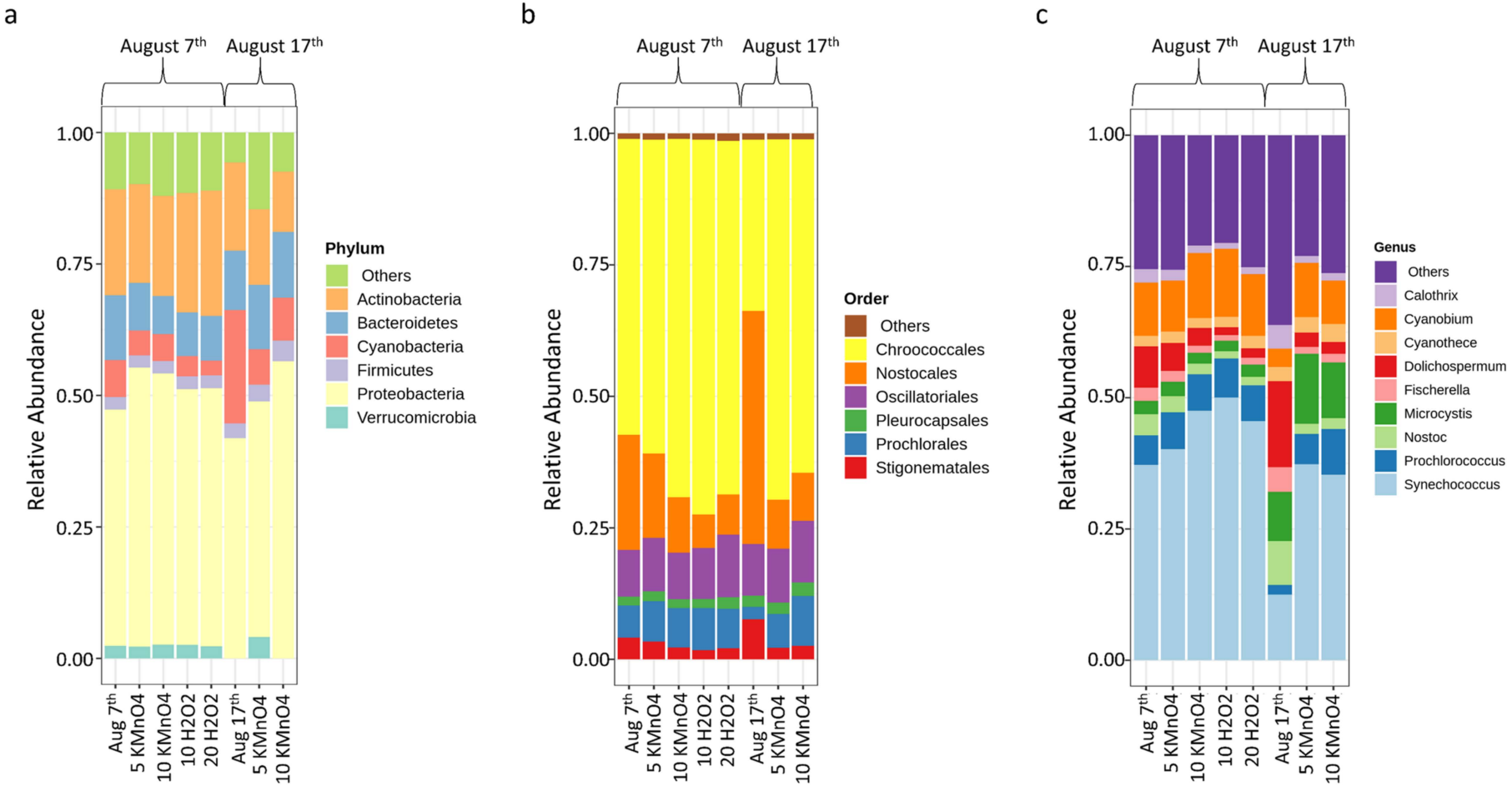

Figure 4. Impact of laboratory oxidation on (**a**) Microbial relative abundance at the phylum level, (**b**) Cyanobacterial relative abundance at the orders and (**c**) Cyanobacterial relative abundance at the genus level on 7 and 17 August.

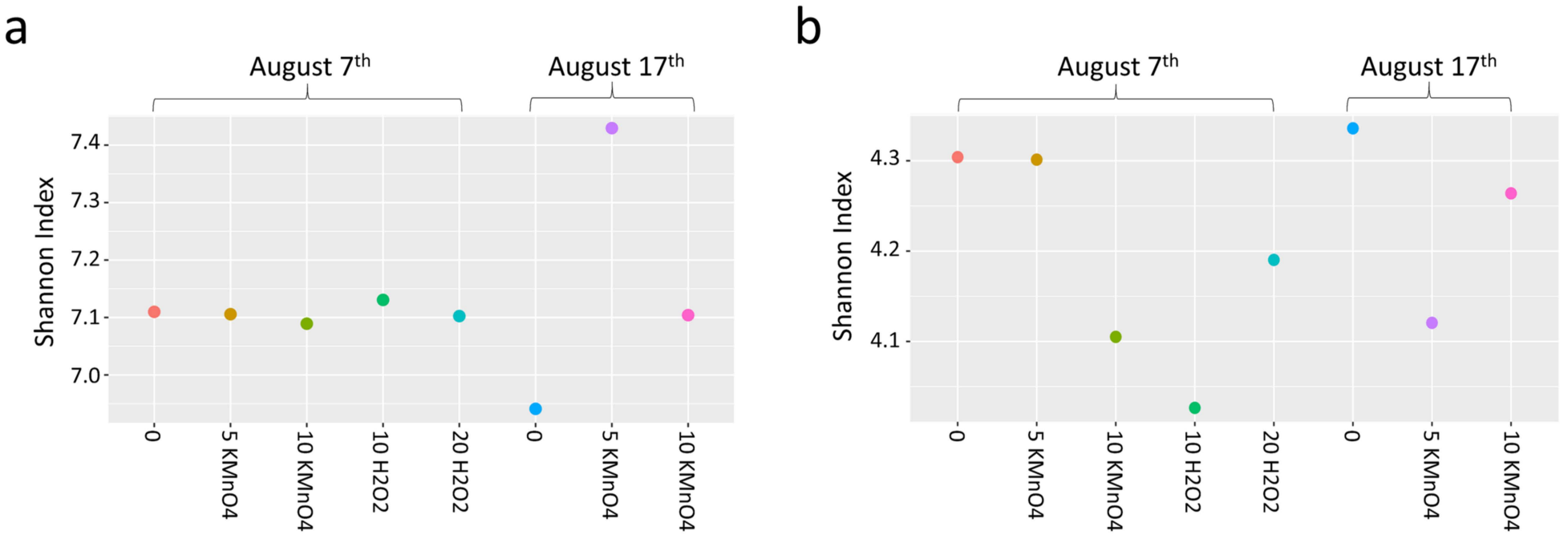

Figure 5. Impact of laboratory oxidation on (**a**) Microbial and (**b**) Cyanobacterial diversity (Shannon index) on 7 August and 17 August, 0: Sludge samples before oxidation.

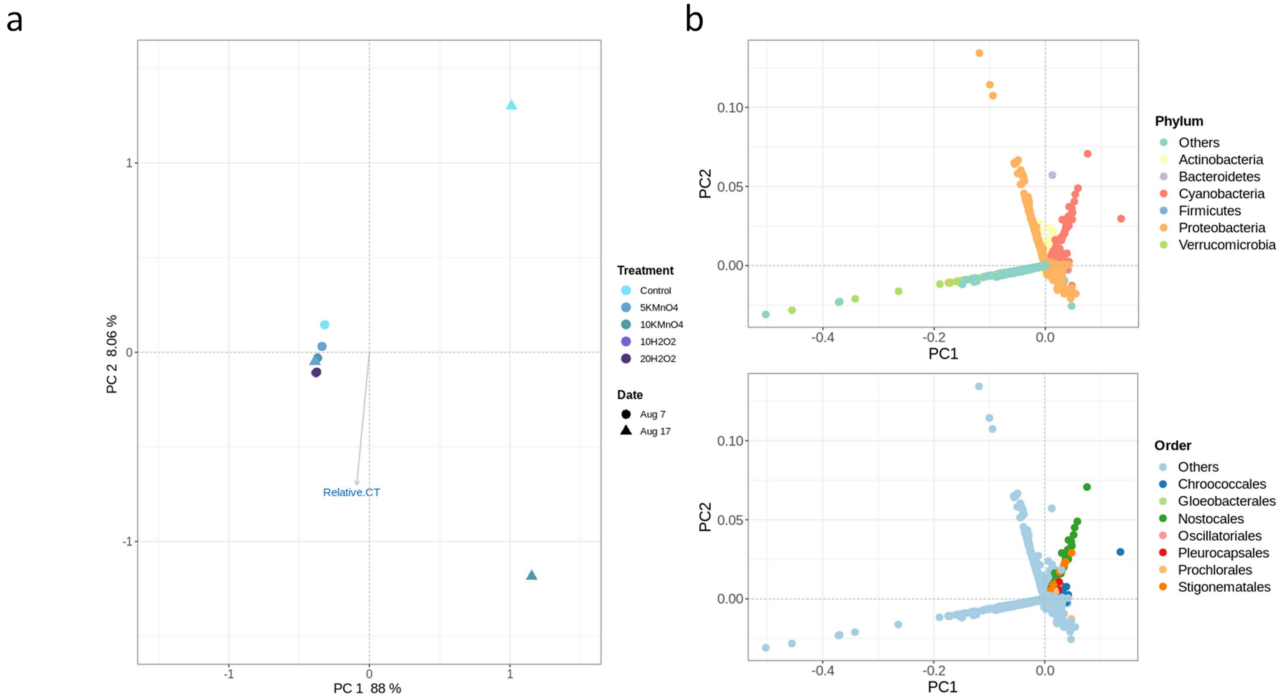

Figure 6. *Cont.*

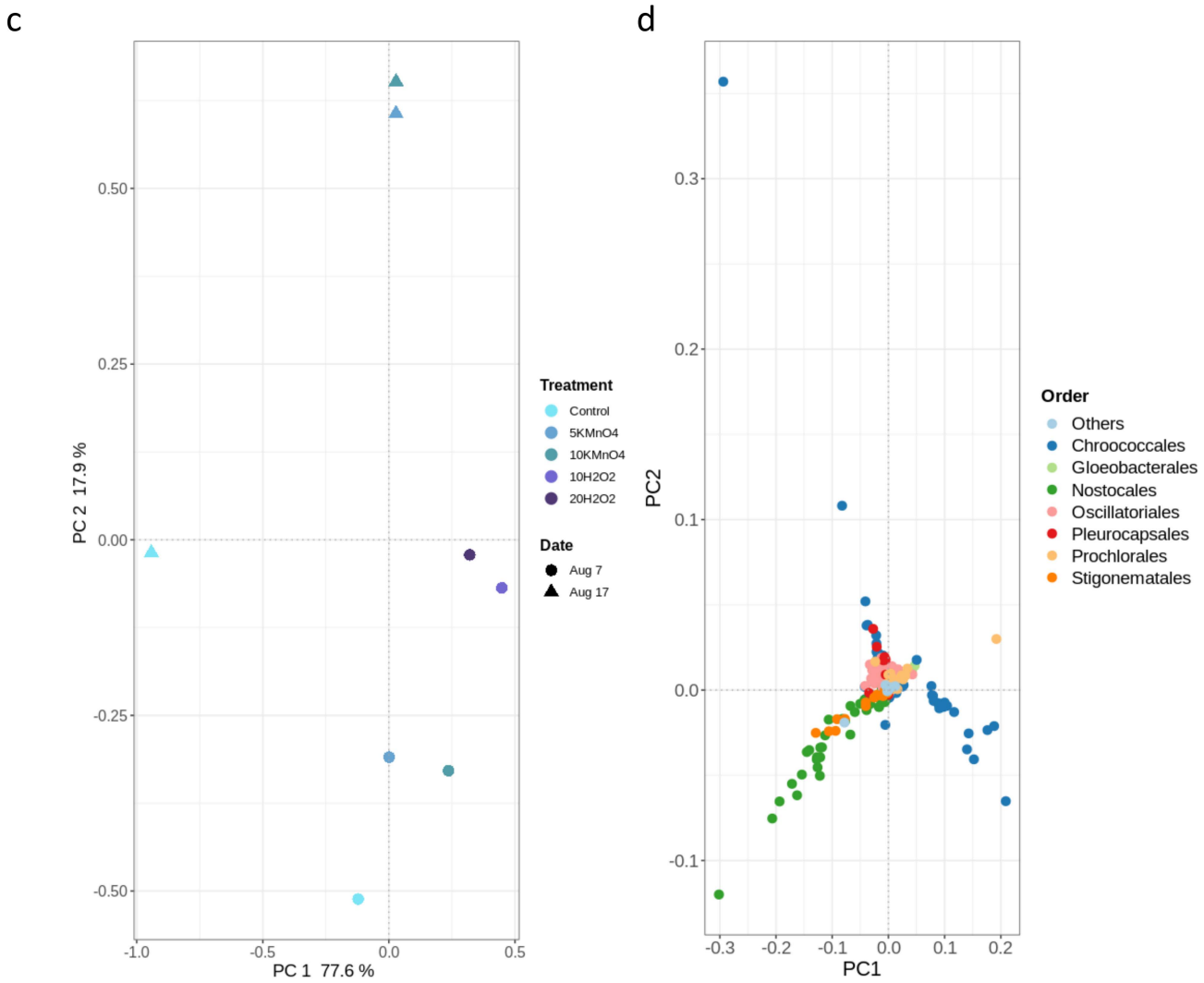

Figure 6. Principal component analysis (PCA) of (**a**) Microbial communities, (**b**) Microbial/cyanobacterial communities grouped at the phylum and order levels, (**c**) Cyanobacterial communities, (**d**) Cyanobacterial species grouped at the order level on 7 and 17 August in the laboratory oxidation.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

3.3. Oxidation of Cyanobacteria-Laden Sludge in a Full-Scale Plant

On-site sludge 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. 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 Section 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 3.3×10^4 cells/mL after 72 h. Additionally, *Dolichospermum spiroides* depletion continued (99% removal) (Figure 7a). On 5 September and after 24 h, total taxonomic cell counts decreased by 34%. Precisely, an 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 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 applications of 10 mg/L $KMnO_4$ in a sludge thickener reported a similar range of total taxonomic cell count reduction ranging from 13–98% [18].

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 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 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 9a). This is in line with the increase of potential MC producer species such as *Microcystis aeruginosa* (3.3×10^4 cells/mL), *Chroococcus dispersus* (1.1×10^4 cells/mL) and *Aphanocapsa delicatissima* (3.8×10^5 cells/mL) (Figure 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 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.

$KMnO_4$ was 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 analogs of MCs [34,49,50]. In fact, dissolved MCs are easily degraded and $KMnO_4$ compromises cell integrity. If a residual persists, it degrades released cell-bound MCs [31,51]. However, in our work, this trend was not observed on 17 August, as a sharp increase in cell-bound MCs after 72 h was detected due to either the impact of fresh MCs producing species, an increased expression of MC synthesis genes (*mcy*) or growth of surviving MC producing species.

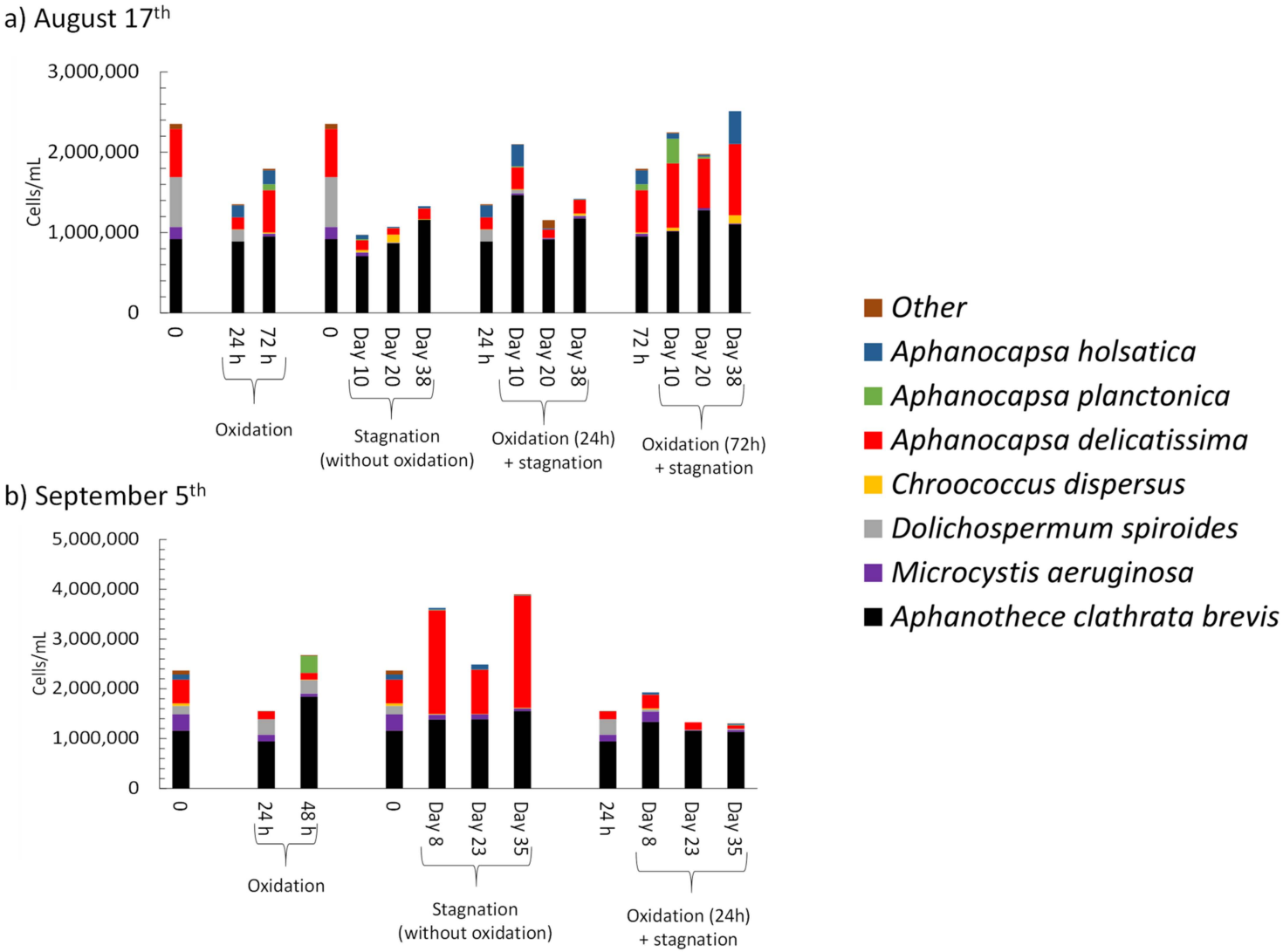

Figure 7. Taxonomic cell counts on (**a**) 17 August, and (**b**) 5 September after on-site oxidation using 10 mg/L $KMnO_4$ and stagnation. 0: Before oxidation, Day: Stagnation day.

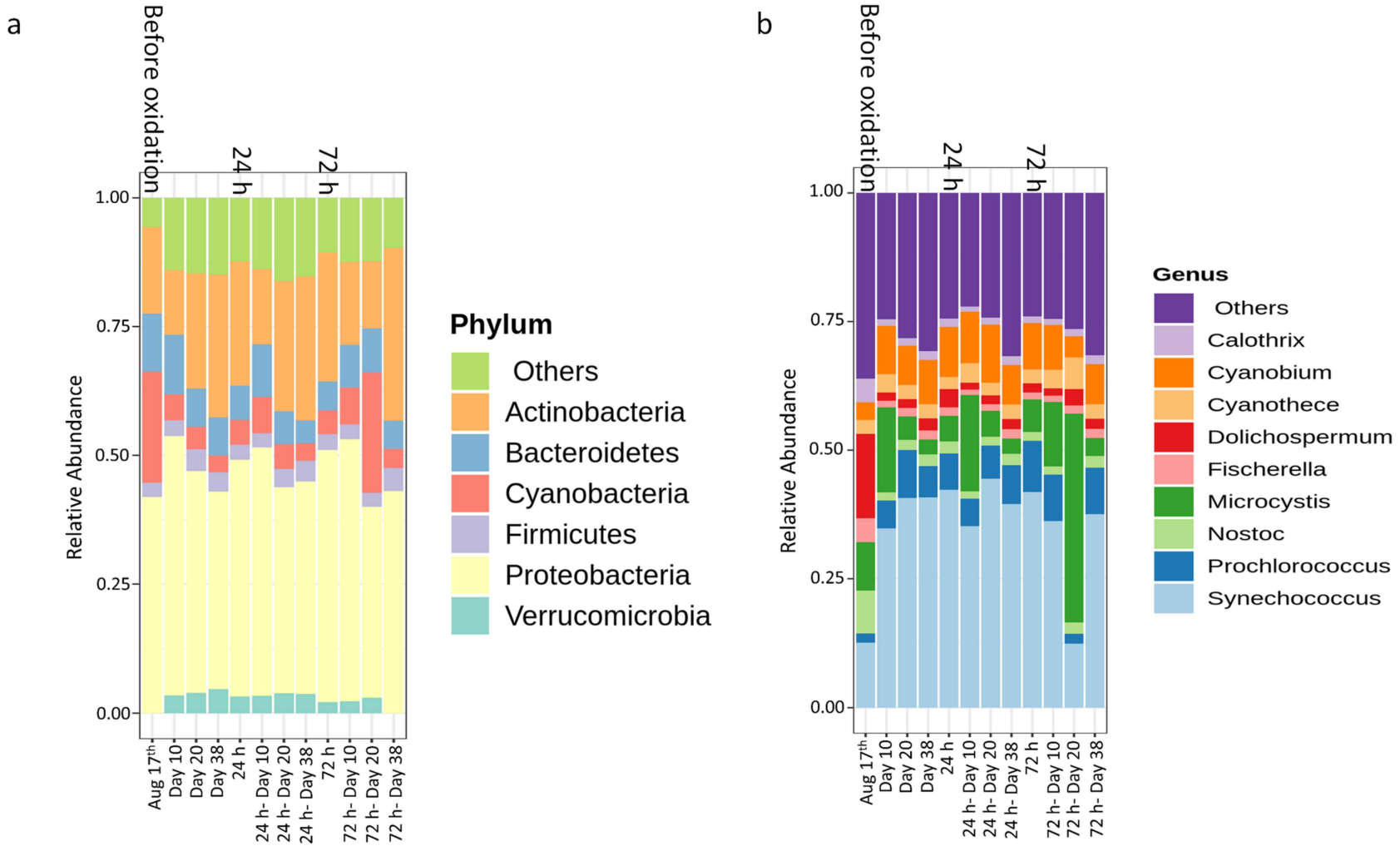

Figure 8. Taxonomic composition before/after on-site oxidation by 10 mg/L KMnO4 and stagnation on 17 August: (**a**) Microbial communities at the phylum level, (**b**) Cyanobacterial communities at the genus level. 24 h and 72 h: Oxidation contact time, Day: Stagnation day.

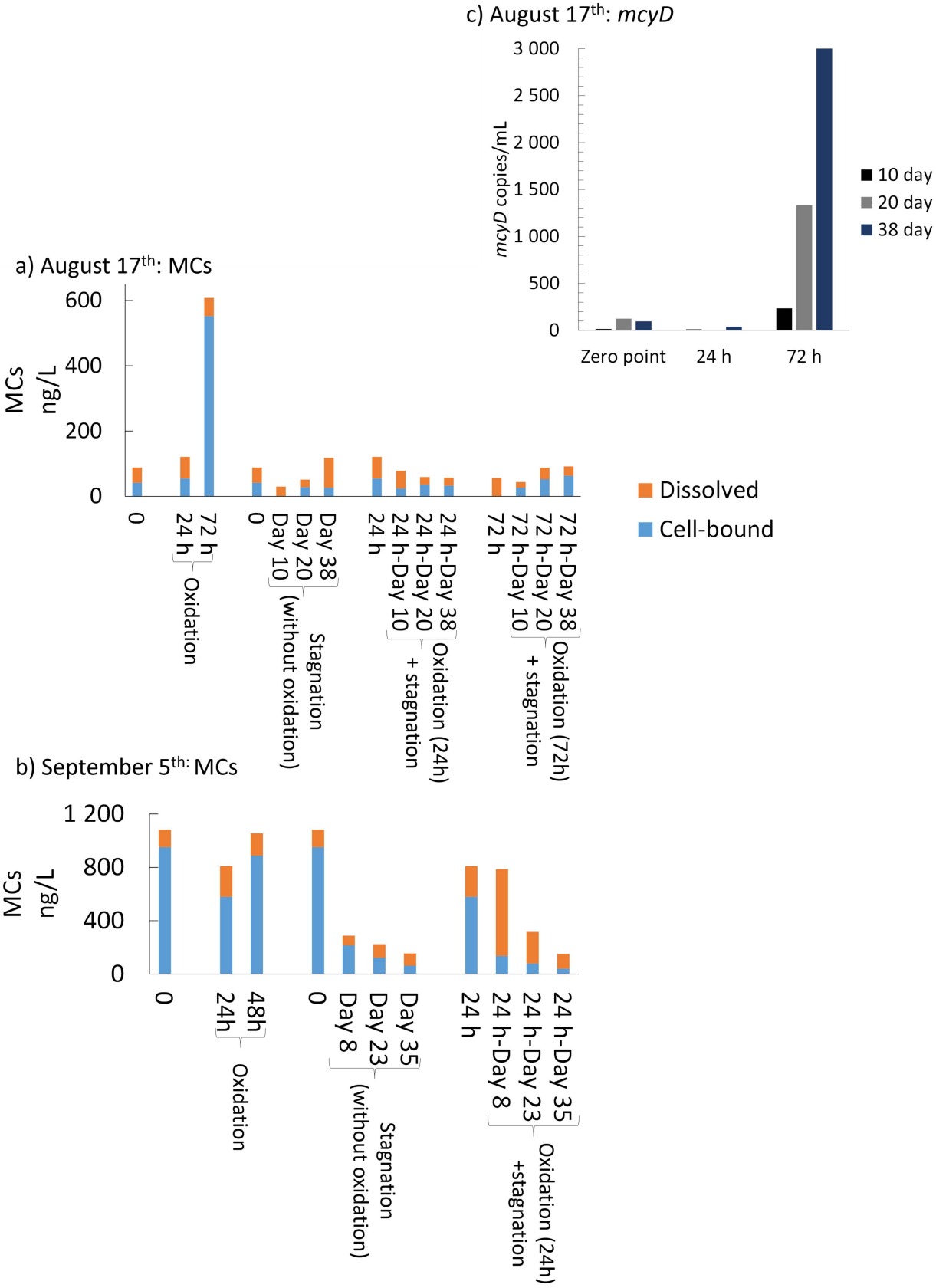

Figure 9. MC concentrations in the sludge after on-site oxidation and stagnation on (**a**) 17 August, (**b**) 5 September, (**c**) *mcyD* gene copy numbers during stagnation on 17 August.

Taxonomic cell counts in the sludge supernatant increased by up to two-fold after on-site oxidation, and by even more than 144X on 17 August after 72 h contact time (Figure S3a,b). Before oxidation, potential MC producing species accounted for 23% and 1% of the total cell counts on 17 August and 5 September, respectively. After oxidation, this percentage increased on 17 August to 36% and 51% after 24 and 72 h oxidation, respectively. On 5 September, 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 three out of four cases after oxidation (Figure S3c,d). In contrast to observations in the sludge (Figure 7), the proportion of dissolved to cell-bound MCs was greater in the supernatant. A dynamic exchange of cells and toxins can occur between sludge and supernatant in the holding tank [11], posing an operational challenge during sludge management. These findings support other reports [18,33,52], outlining the challenge of supernatant recycling to the head of the DWTPs during toxic cyanobacterial blooms.

Small facilities (e.g., the studied DWTP) may not be able to install quickly and operate safely online dosage equipment for continuous on-site oxidation of sludge. Additionally, 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 not for an extended period. Documenting the impact of on-site shock oxidation in these dynamic operating conditions provide valuable information to operators.

3.4. Impact of Stagnation on Oxidized Cyanobacteria-Laden Sludge

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 (Figures 1–3). However, a 17–75% increase in total counts was also observed in some samples (4 out of 16) during stagnation (Figures 1–3). 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 four out of six 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 2). Results presented in Section 3.3 and plotted in Figures 1–3 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 ranges 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 (Figures 1–3). 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 the 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 in 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 as *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 S4).

The impact of $KMnO_4$ and H_2O_2 on cyanobacterial communities is quite different (Figure S5). 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. [29] findings of H_2O_2 oxidation of cyanobacterial bloom water, H_2O_2 oxidation of sludge in this study reveals selective reduction of Chroococcales. The distinct shift is obtained following stagnation only.

3.4.2. Stagnation after On-Site Oxidation

On 17 August 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 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 7a).

The relative abundance of Cyanobacteria increased during oxidation/stagnation as compared to the non-oxidized/stagnated samples (Figure 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 8b). In accordance with our laboratory oxidation/stagnation results, depletion of Nostocales and persistence of Chroococcales order were observed (Figure S6).

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 S7).

Cell-bound MCs in the oxidized sludge (24 and 72 h) remained 1.2–27 times higher than that of non-oxidized sludge during stagnation (Figure 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 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 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 7a). This marked increase corresponds to 7.2×10^5 cells/mL out of which 82% were potential MC producers mainly *Aphanocapsa delicatissima*, *Aphanocapsa holsatica*, and *Chroococcus dispersus*.

On 5 September 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 7b). Then, taxonomic cell counts slightly decreased (15%) after 23 and 35 days of stagnation (Figure 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 7b). On this date, slightly higher MC concentrations were detected (Figure 9b). On-site oxidation did not markedly decrease these MCs (3–25%) as shown by the 24 h and 48 h results (Figure 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 1.2–9.2× higher than non-oxidized/stagnated sludge within 8–35 days (Figure 9b).

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 [27,28] and as revealed by this study, after stagnation. Released dissolved MCs can be oxidized [31], adsorbed onto PAC or flocs [11,53] or can be biodegraded by species belonging to Proteobacteria, Actinobacteria, and Firmicutes [54,55].
- Oxidation causes significant changes in microbial diversity leading to the selective persistence of some cyanobacterial species over other communities [29,30] and nutrient availability [11] 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 [56,57]. 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 in oxidative stress.

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 2.7×10^6 cells/mL to 6.2×10^5 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 6.2×10^5 cells/mL to 1.6×10^6 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 a 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 six out of nine samples as compared to on-site oxidation alone. Partial cell growth was also observed in MC producer genera such 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 copy numbers 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.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/w14040537/s1, Table S1. Characteristics of untreated sludge, Figure S1. Taxonomic cell counts in the untreated sludge before oxidation or stagnation, Figure S2. (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 S3. Taxonomic cell counts on (a) 17 August and (b) 5 September 5; MC concentrations on (c) 17 August and (d) 5 September in the sludge supernatant after on-site oxidation, Figure S4. MC concentrations during laboratory oxidation followed by stagnation on (a) 31 July using 5 and 10 mg $KMnO_4$/L, (b) 7 August using 5, 10 mg/L $KMnO_4$ and 10, 20 mg/L H_2O_2, and (c) 17 August using 5 and 10 mg/L $KMnO_4$, Figure S5. Principal component analysis (PCA) of cyanobacterial communities and cyanobacterial species grouped at the order level on oxidized/stagnated samples in the laboratory scale on (a) 7 August, PC1: 68.6, PC2: 21.5% and (b) 17 August, PC1: 64.8, PC2: 28.1%. Sig-nificant parameters (p <0.05): stagnation time after oxidation by $KMnO_4$, and oxidation by H_2O_2, Figure S6. (a) Principal component analysis (PCA) on sludge samples after on-site oxidation followed by stagnation on 17 August, PC1: 66.5%, PC2: 26.6%, (b) Cyanobacterial species grouped at the order level, Figure S7. Differential heat tree demonstrating changes in microbial and cyanobacterial taxonomic profiles of $KMnO_4$ oxidized sludge for 72 h at T0/T38.

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