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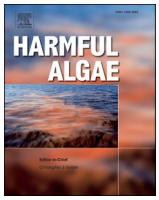

Constructed wetland mesocosms improve the biodegradation of microcystin-LR and cylindrospermopsin by indigenous bacterial consortia

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ABSTRACT

Cyanobacterial blooms releasing harmful cyanotoxins, such as microcystin (MC) and cylindrospermopsin (CYN), are prominent threats to human and animal health. Constructed wetlands (CW) may be a nature-based solution for bioremediation of lake surface water containing cyanotoxins, due to its low-cost requirement of infrastructure and environmentally friendly operation. There is recent evidence that microcystin-LR (MC-LR) can efficiently be removed in CW microcosms where CYN degradation in CW is unknown. Likewise, the mechanistic background regarding cyanotoxins transformation in CW is not yet elucidated. In the present study, the objective was to compare MC-LR and CYN degradation efficiencies by two similar microbial communities obtained from CW mesocosms, by two different experiments setup: 1) in vitro batch experiment in serum bottles with an introduced CW community, and 2) degradation in CW mesocosms. In experiment 1) MC-LR and CYN were spiked at 100 μg L^{-1} and in experiment 2) 200 μg L^{-1} were spiked. Results showed that MC-LR was degraded to ≤1 μg L^{-1} within seven days in both experiments. However, with a markedly higher degradation rate constant in the CW mesocosms (0.18 day^{-1} and 0.75 day^{-1}, respectively). No CYN removal was detected in the in vitro incubations, whereas around 50 % of the spiked CYN was removed in the CW mesocosms. The microbial community responded markedly to the cyanotoxin treatment, with the most prominent increase of bacteria affiliated with *Methylophilaceae* (order: *Methylophilales*, phylum: Proteobacteria). The results strongly indicate that CWs can develop an active microbial community capable of efficient removal of MC-LR and CYN. However, the CW operational conditions need to be optimized to achieve a full CYN degradation. To the best of our knowledge, this study is the first to report the ability of CW mesocosms to degrade CYN.

1. Introduction

Cyanobacteria are proliferating vigorously and form cyanobacterial blooms (Huisman et al., 2018) when nutrients from urban areas, agriculture, and industry and increased emission of greenhouse gases favor their expansion, frequency, duration, and intensity (Paerl and Huisman, 2009; O'Neil et al., 2012; Michalak et al., 2013; Huisman et al., 2018). They also produce hazardous cyanotoxins (Buratti et al., 2017) microcystin (MC) and cylindrospermopsin (CYN) are the most frequently detected and potent cyanotoxins (Svirčev et al., 2019). More than 250 congeners of MC are known (WHO, 2020a), with microcystin-LR (MC-LR) regarded as the most toxic and common congener. CYN is primarily detected in tropical waters but has, in recent decades, been observed to expand into temperate climate zones (WHO, 2020b). The human exposure route to cyanotoxins is primarily through drinking water, however, intake of crops is also a potential exposure route (Chorus and Welker, 2021a). This risk is most prominent in arid and rural areas, where non-treated lake surface water, with a critical content of cyanotoxins, may be used for the irrigation of crops and bioaccumulate (Corbel et al., 2014; Lee et al., 2017). In such locations, solutions with cheap and low-infrastructure requirements may be the only option for purifying water of hazardous agents like cyanotoxins (Norton-Brandão et al., 2013). Cyanobacteria are proliferating vigorously and form cyanobacterial blooms (Huisman et al., 2018) when

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nutrients from urban areas, agriculture, and industry and increased emission of greenhouse gases favor their expansion, frequency, duration, and intensity (Paerl and Huisman, 2009; O'Neil et al., 2012; Michalak et al., 2013; Huisman et al., 2018). They also produce hazardous cyanotoxins (Buratti et al., 2017) microcystin (MC) and cylindrospermopsin (CYN) are the most frequently detected and potent cyanotoxins (Svirčev et al., 2019). More than 245 congeners of MC are known (Meriluoto et al., 2017), with microcystin-LR (MC-LR) regarded as the most toxic and common congener. CYN is primarily detected in tropical waters but has, in recent decades, been observed to expand into temperate climate zones (Kinnear, 2010). The human exposure route to cyanotoxins is primarily through drinking water, however, intake of crops is also a potential exposure route (Chorus and Welker, 2021a). This risk is most prominent in arid and rural areas, where non-treated lake surface water, with a critical content of cyanotoxins, may be used for the irrigation of crops and bioaccumulate (Corbel et al., 2014; Lee et al., 2017). In such locations, solutions with cheap and low-infrastructure requirements may be the only option for purifying water of hazardous agents like cyanotoxins (Norton-Brandão et al., 2013).

Wetland systems may purify water by breaking down organic pollutants and assimilating nutrients by indigenous bacteria and plants. Nature-based solutions, like constructed wetland (CW) systems, have been proposed for treating cyanotoxin contaminated water (Bavithra et al., 2020). In order to apply CWs to treat cyanotoxin-containing waters, the biodegradation potential by microorganisms needs to be fully exploited. More than 70 bacterial isolates have been confirmed to degrade MC-LR and congeners (Massey and Yang, 2020). One enzymatic pathway for MC degradation has been described in *Sphingomonas* sp. ACM-3962 (Bourne et al., 1996), consisting of four genes; *mlrA-D*, where *mlrA-C* are coding for hydrolytic enzymes (Bourne et al., 2001). However, a few reports suggest that unknown alternative biodegradation routes also exist (Lezcano et al., 2016; Mou et al., 2013). The literature on biodegradation of other cyanotoxins is very scarce. Some studies have reported biodegradation of CYN by bacterial consortia in natural water bodies (Smith et al., 2008; Klitzke et al., 2010) and by single-strains (Mohamed and Alamri, 2012; Dziga et al., 2016; Martínez-Ruiz et al., 2020b; Mohamed et al., 2022). Enzymes or biodegradation pathways for CYN are not known.

Bioremediation of cyanotoxin-contaminated water by CWs has focused on MC-LR management at microcosm scale (Wang et al., 2018, 2022; Bavithra et al., 2020; Cheng et al., 2021, 2022b, 2022a) It has been demonstrated that experimental CW systems can efficiently remove MC-LR as well as nutrients. Wang et al. (2018) showed that the initial MC-LR concentration and the addition of six known MC-LR degrading cultures (bioaugmentation), increased the degradation rate in CWs. Following bioaugmentation in CWs, the MC-LR concentration was pushed below the guideline value for drinking water of 1 μg L^{-1}(WHO, 2020a) at a six times faster pace compared to non-bioaugmented biotic controls. Cyanotoxin-degrading bacteria co-exist with the toxic cyanobacteria (Parveen et al., 2013) and can be employed in CWs receiving a constant influx of indigenous degrader bacteria from the inflow water. The operation mechanisms of CWs (continuous influx of nutrients, substratum for establishment of biofilm, plant contribution via exudates) are expected to stimulate the degradation of the cyanotoxins (Cheng et al., 2021).

The main aim of the present study was to evaluate the cyanotoxin biodegradation potential of a microbial community from CW mesocosms. This was approached by combining two experiments: 1) in vitro biodegradation of cyanotoxins in a batch experiment inoculated with the microbial community from experiment 2, and 2) biodegradation of cyanotoxins in a CW mesocosm. As several types of cyanotoxins may be present during a cyanobacterial bloom (Howard et al., 2021) their mixed effect on cyanotoxin biodegradation need to be addressed, thus the present study included both MC-LR and CYN spiked individually and in combination. We hypothesize (1) that the CW mesocosms are more efficient in removing MC-LR and CYN than the in vitro CW community obtained from the CW mesocosms; (2) the bacterial community composition in the CW mesocosms will change its structure when exposed to MC-LR, CYN, and cyanobacterial metabolites. This study contributes to establishing CWs as a suitable and cost-effective strategy for increasing the safety of exploiting surface waters for human and ecosystem services.

2. Materials and methods

2.1. Culturing of cyanobacteria and extraction of cyanotoxins

Two cyanobacterial strains were employed to produce the cyanotoxins used for spiking in the present study; *Microcystis aeruginosa* LEGE 91,094 and *Chrysosporum ovalisporum* LEGE x-001 formerly *Aphanizomenon ovalisporum* (obtained from the Blue Biotechnology and Ecotoxicology Culture Collection (LEGE-CC), Portugal) producing MC-LR and CYN, respectively and maintained in Z8 media optimized for cyanobacterial cultivation (Kotai, 1972). Cyanotoxins were extracted from 15-L photobioreactors (Alpha-Aqua, Denmark) using a three-phase sequential extraction method. Details regarding cyanobacterial cultures and cyanotoxin extraction from the biomass can be found in this article's supplementary material.

2.2. CW mesocosm construction and pre-conditioning

Experiments were conducted in mesocosms simulating sub-surface vertical flow CWs. Four mesocosms were created in black 12 L plastic plant containers with a 3 cm bottom layer of granite gravel (10 mm) (Champost, Denmark) for drainage. The remaining space was filled with filter sand (2 mm) (Aquaproof, Denmark). Two of the containers were planted with *Juncus effusus,* as replicates of planted mesocosms, M_P1 and M_P2. Two other mesocosms were used as unplanted replicates (M_U1 and M_U2) (Fig. S1). Data is further presented in the paper per replicate. The substratum (sand/gravel) used in the mesocosms was prior to the experiment tested for sorption of cyanotoxins, finding no significant sorption of MC-LR or CYN (Fig. S2) (Larsson, 2022). The mesocosms were kept in a temperature-controlled room at 15 °C and a light intensity of 22 μmol photons m^{-2} s^{-1} with a light/dark cycle of 16/8 h. The systems were fed with a hydraulic loading rate of 3.5 cm day^{-1} using a multi-channel peristaltic pump (Ole Dich Instrumentmakers, Denmark) and operated in batch mode recirculating the inflow and the outflow from the same feeding recipient.

To establish a natural microbial community, the systems were fed water from eutrophic Lake Arresø (55°58′27.6″N 12°07′08.5″E) for four months (July to November 2022), switching to synthetic eutrophic lake media (Table S1) for two more months until experimentation. Lake Arresø has a historical record of cyanotoxins (Podduturi et al., 2021) and some authors even successfully isolated potential cyanotoxin degraders (Jørgensen et al., 2022). Therefore, Lake Arresø waster was used in order to increase the potential for genetic biodegradation capacity of the bacterial community in the CW mesocosms.

2.3. In vitro CW community degradation of MC-LR and CYN

The in vitro CW community experiment was performed in 120 mL serum bottles. Seventy grams of sand from each of the four preconditioned CW mesocosms containing the microbial community were pooled in a 5 L blue cap bottle containing 2.5 L of sterile mineral salt medium (MSM) (Table S2) and shaken vigorously for 10 min. Sand, roots, and debris were removed by filtration (6-μm pore size). Seventy mL aliquots of the medium with the CW community were transferred to 120 mL serum bottles. The remaining medium was filter sterilized (0.22 μm pore size). An MC degrader, *Sphingosinicella microcystinivorans* Y2 (Park et al., 2001) (DSMZ culture collection, DSM 19,791), was used as a positive control and compared to the efficiency of the in vitro CW

community. Before the experiment, *S. microcystinivorans* Y2 was grown in R2A medium (Reimer et al., 2022) for three days, centrifuged, and washed twice with MSM. Two mL (~2.1 × 10^6 cells mL^{-1}) of *S. microcystinivorans* Y2 was inoculated into 68 mL filter sterilized MSM medium. Abiotic controls were created from the filter sterilized MSM medium. Before spiking, the cyanotoxin extracts were lyophilized to remove methanol and resuspended in H_2O, and quantified (Section 2.5). To study the single and combined effect of MC-LR and CYN on the biodegradation efficiency, the in vitro CW community and *S. microcystinivorans* Y2 were incubated in triplicates and with three cyanotoxin-spiked treatments: 1) MC-LR, 2) CYN, and 3) MC-LR and CYN. Non-spiked controls without cyanotoxin were created for each treatment (in vitro CW community, *S. microcystinivorans* Y2, and abiotic). The resulting concentration of MC-LR and CYN in the spiked treatments was approximately 100 μg L^{-1}, an environmental relevant concentration range (Chorus and Welker, 2021b). The cultures were incubated at 15 °C on a shaker at 150 RPM. Samples (100 μl) for cyanotoxin quantification and samples (2 mL) for DNA extraction were taken at aseptic conditions after 0, 2, 4, 6, 8, 24, 28, 32, 52, 77, 125, 173 h. The content of total organic carbon (TOC) and total nitrogen (TN) was measured at time 0 in the filter sterilized MSM bottle (without cyanotoxin extracts) and at the end of the experiment in each of the serum bottles. All samples were stored at −20 °C until analysis.

2.4. CW mesocosm degradation of MC-LR and CYN

Each of the four mesocosms had a designated flask containing 2 L of synthetic eutrophic water (Table S1) and spiked with approximately 200 μg L^{-1} of MC-LR and CYN, as the only spiking treatment. Similarly, to the in-vitro experiment, the goal was to use an environmental relevant concentration. The range here was twice as before, as the starting lyophilized biomass used for mesocosm experiment was more enriched. The flasks were wrapped with aluminum foil to prevent eventual photodegradation and growth of phototrophs. The inlet and outlet tubes were placed in the same flask, so the water was recirculated to monitor the degradation of the cyanotoxins over time. The systems were fed by pulses (HRL 3.5 cm day^{-1}). One mL samples were taken after 0, 1, 3, 5, 7, 24, 30, 48, 72, 144, 168 h and analyzed for the concentration of MC-LR and CYN. Samples were taken directly from the flask containing the medium with the cyanotoxins after agitation. Forty mL of medium was sampled for TOC and TN at time 0 (with cyanotoxins) and at the end of the experiment, from all flasks. Approximately 30 mL of sand was taken just before spiking on day 0 and each day during the experiment. Samples were taken directly from each mesocosm for extraction of DNA. Sand samples were collected at three different places in each mesocosm and pooled, to get representative samples from the mesocosms. Samples were taken at a depth of 5 cm and stored in 50 mL Falcon tubes. Chemical samples were taken as technical duplicates. All samples were stored at −20 °C until analysis.

2.5. MC-LR and CYN quantification by LC-MS/MS

Quantification of MC-LR and CYN was performed by high-performance liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) using a 1200 Series binary LC gradient system (Agilent Technologies, United States) coupled to a 5500 QTRAP-MS (Sciex, United States). Separation was performed at 20 °C and a flow of 350 μl min^{-1} using a Synergy Polar-RP column (150 mm x 2 mm, particles size 4 μm) (Phenomenex, United States). Two acidic mobile phases were used, A) consisted of water with 0.2 % formic acid and B) contained methanol with 0.2 % formic acid, following an optimized gradient (Table S3). The MS was operated with electrospray ionization in positive mode at 400 °C and capillary voltage of 4500 V. Compound specific MS/MS-parameters (Table S4) were set using scheduled MRM mode with a target scan time of 1 s and MRM detection window of 60 s. Sample (aqueous samples or biomass extracts) were directly injected in the instrument; injection volume was 10 μl and the needle was washed with acetonitrile between injections. All samples (aqueous samples or biomass extracts) were spiked with 20 μl of internal standard (Novakits, France; MC-LR d7, CYN $^{15}N_5$, purity: 98.7 % and 99 %, respectively) to correct the matrix effect's influence on the quantification. The six-points calibration curve (from 0.1 to 200 μg L^{-1}) was prepared in water, using analytical standards from CIFGA (Spain; purity MC-LR 96 % and CYN 99 %). Method performance is detailed in Table S5. The limits of quantification were 0.50 μg L^{-1} and 0.1 μg L^{-1} for MC-LR and CYN respectively. Characteristic chromatograms of the LC-MS/MS method are also provided (Figure S3-S6).

2.6. Rate constant and half life

The degradation rate constant, k, for MC-LR and CYN degradation was calculated by using a 1st order kinetics model, as:

$$\ln(C) = \ln(C_0) - kt$$

Where C is the cyanotoxin concentration at time t, C_0 is the initial spiking concentration, k is the rate constant, and t is time.

The half-life was calculated based on the formula:

$$T_{1/2} = \frac{\ln(2)}{k}$$

2.7. TOC and TN quantification

TOC and TN were measured on a Shimadzu TOC-L_{CPH} total organic carbon analyzer equipped with a TNM-L total nitrogen unit (Shimadzu, Japan). TOC was quantified by measuring the non-purgeable organic carbon which excludes the inorganic carbon fraction. A standard series for quantification of TOC and TN were used and known concentrations of sucrose were used as a control standard.

2.8. DNA extraction

For experiment 1, 500 μl of culture from the 2-ml sample taken for DNA extraction was used in the FastDNA™ SPIN Kit for Soil (MP biomedicals, United States). Most of the samples taken in the vitro experiment (experiment 1), had too low DNA concentrations to be measured by Qubit. Thus, molecular analysis of the in vitro CW community was not possible. Sand samples from experiment 2, were thoroughly homogenized and aliquots (~0.6 g) were used for DNA extraction, in duplicates. Three hundred μl of liquid *S. microcystinivorans* Y2 culture, grown in R2A medium, was used for DNA extraction to acquire DNA for the qPCR standard. FastDNA™ SPIN Kit for Soil (MP biomedicals, United States) was used and performed following the manufacture's protocol. followed by measurement of DNA concentrations by Qubit (Thermo Fischer Scientific, United States) using the Qubit dsDNA HS assay kit.

2.9. Real-time qPCR of 16S rRNA and mlrA

Quantifications of bacterial 16S rRNA and *mlrA* genes were achieved by qPCR on a CFX Connect Real-Time Detection System (Bio-Rad, United States). The qPCR reaction mixture and cycling conditions for 16S rRNA qPCR were performed as described in (Gobbi et al., 2019). Each sample was run in triplicate. The standard curve was linear over the range of 1.83 × 10^2–1.83 × 10^7 gene copies. The run parameters for each qPCR run were slope=−4.079 and −4.141, R^2 = 0,996 and 0,999 with efficiency of 75.9 % and 74.4 %.

Quantification of the *mlrA* gene was conducted as described by Hoefel et al. (2009). The standard curve was linear over the range of 1.53 × 10^1–1.53×10^6 gene copies. Each sample was run in triplicate. The run parameters for each qPCR run were slope=−3.267 and −3.277, R^2 = 0.998 and 0.999, with efficiency of 102.3 % and 101.9 %. DNA

extracts from *S. microcystinivorans* Y2 were used as a positive control. The standard curve was made as a dilution series in nuclease free dH_2O using a 641 bp *mlrA* gene fragment created with primer pair mlrAF2 (5′-GATTTCGTGAGCTGCTAAGCC-3′) and mlrAR2 (5′-CCTCCCACAAATCAGGACGAG-3′). Primers were made using Primer-Blast (Ye et al., 2012). The *mlrA* amplicon was constructed by performing end-point PCR on DNA extracts from *S. microcystinivorans* Y2. Amplicon molecular weight (MW) was determined based on the *mlrA* gene sequence obtained from *S. microcystinivorans* Y2 (accession number AB114203.1) (Saito et al., 2003).

2.10. 16S rRNA amplicon sequencing and bioinformatic analysis

Samples for 16S rRNA amplicon sequencing were taken at time 0 and on the final day of the experiment to investigate the effect the cyanotoxin extracts have on the bacterial community structure in the CW mesocosms. The amplicon libraries were built as described by (Gobbi et al., 2019) with slight modifications. PCRBIO ultramix polymerase (PCR Biosystems, United Kingdom) was used for both amplification and indexing PCRs on a T100 Thermal Cycler (Bio-Rad, United States). The library was sequenced using the V2 500 cycle reagent kit on the Illumina MiSeq instrument (Illumina, United States) at the Aarhus University (Roskilde, Denmark) Sequencing Center.

The 16S rRNA amplicon bioinformatic analysis of the microbial community found in the CW mesocosms was performed through the QIIME 2 pipeline, version 2020.8.0. (Bolyen et al., 2019). The bioinformatics pipeline was followed as described by (Gobbi et al., 2019) with slight modifications. Following inspection of rarefaction curves to check for saturation, the output was rarefied to 1696 reads to include the sample with the lowest read number. Taxonomy was assigned to the ASVs with q2-feature-classifier classify-consensus-vsearch (Bokulich et al., 2018) using trained classifiers for V3-V4 region sequences using the Silva 138 99 % database (Pruesse et al., 2007). Duplicate samples for each mesocosm were merged and the taxonomy generated was filtered to only allow taxa represented in at least two mesocosms and with a score of more than 100 features (removed taxa with a relative abundance below 0.5 %). Plots for ASVs observed, Shannon diversity index, taxa bars, and Bray-Curtis Emperor PCoA were generated with QIIME 2.

2.11. Statistical analysis

Significant community structure changes between start and end were evaluated with PERMANOVA on the unweighted Unifrac metric with 999 permutations. A significant difference is determined at a p-value <0.05.

3. Results

3.1. MC-LR and CYN degradation

The in vitro CW community degraded MC-LR completely within seven days of incubation, but with a three-day lag phase (Fig. 1A), where *S. microcystinivorans* Y2 degraded all the MC-LR within 24 h and with no lag phase. The MC-LR degradation in any of the treatments was the same either MC-LR was spiked alone or in combination with CYN. (Fig. 1A). CYN degradation was not detected in any of the treatments (Fig. 1B).

In contrast to the in vitro CW community, the CW mesocosms initiated the degradation of MC-LR with no lag phase, and after seven days, ~1 μg L^{-1} MC-LR remained (Fig. 2A) corresponding to a degradation efficiency >99 %. The planted CW mesocosm, M_P2, degraded MC-LR faster than the unplanted CWs. In contrast to the in vitro CW community, the CW mesocosms degraded approximately half of the spiked CYN within 24 h (Fig. 2B), whereafter the degradation did not proceed further despite the degradation initiated instantaneously (Fig. 2B).

Calculating the degradation kinetics, it appeared that *S. microcystinivorans* Y2 had the highest degradation rate constant and shortest half-life for MC-LR degradation observed in the present study (Table 1). The CW mesocosms had higher rate constants during MC-LR degradation compared to the in vitro CW community. During degradation of CYN (from time 0–24 h), the rate constants were in proximity to the rate constants for the MC-LR degradation, and the half-life had a mean between mesocosms at around one day for both MC-LR and CYN.

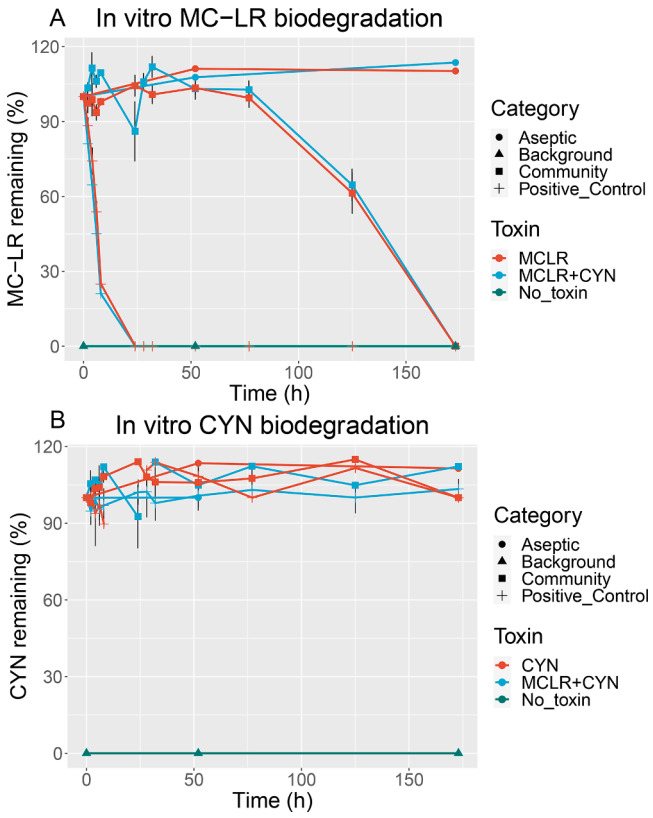

Fig. 1. In vitro degradation of MC-LR (A) and CYN (B) by i) An aseptic spiked control, ii) Background as aseptic non-spiked control, iii) the microbial community derived from the constructed wetland mesocosms, and iv) positive control (*S. microcystinivorans* Y2 - MC-LR degrading strain), ($n = 3$; bars indicate standard deviation). The medium was spiked with either MC-LR or CYN, or a mix of MC-LR and CYN (MCLR+CYN). The background was not spiked (No_toxin).

3.2. TOC and TN removal

The TOC concentration decreased when the in vitro CW community was present in all treatments compared to *S. microcystinivorans* Y2 and the abiotic controls. TN was low (<0.2 mg L^{-1}) at the start of the experiment (time 0) and slightly increased to 0.3–1.5 mg L^{-1} at the end of the experiment when spiked with cyanotoxins, as well as in the abiotic non-spiked treatment (Fig. S7). In general, TN was low in treatments with the only addition of CYN, except in the *S. microcystinivorans* Y2 treatment.

Regarding the CW mesocosms, they were individually fed from flasks that contained the same type of synthetic eutrophic medium, spiked with a mix of MC-LR and CYN. The amount of each cyanotoxin extract spiked into each flask was based on the concentration of MC-LR and CYN in the concentrated extracts, which resulted in starting spike concentrations of 176.25 ± 22.75 μg/L for MC-LR and 183.31 ± 42.75 μg/L for CYN. This summed variability of 36 % in the content of cyanobacterial compounds, and corresponding growth media, might explain the difference in TOC content between mesocosms (Fig. S8A). However, the TN values were similar in all four flasks (Fig. S8B). All four mesocosms had the same pattern of removing the majority of the TOC and TN. After the

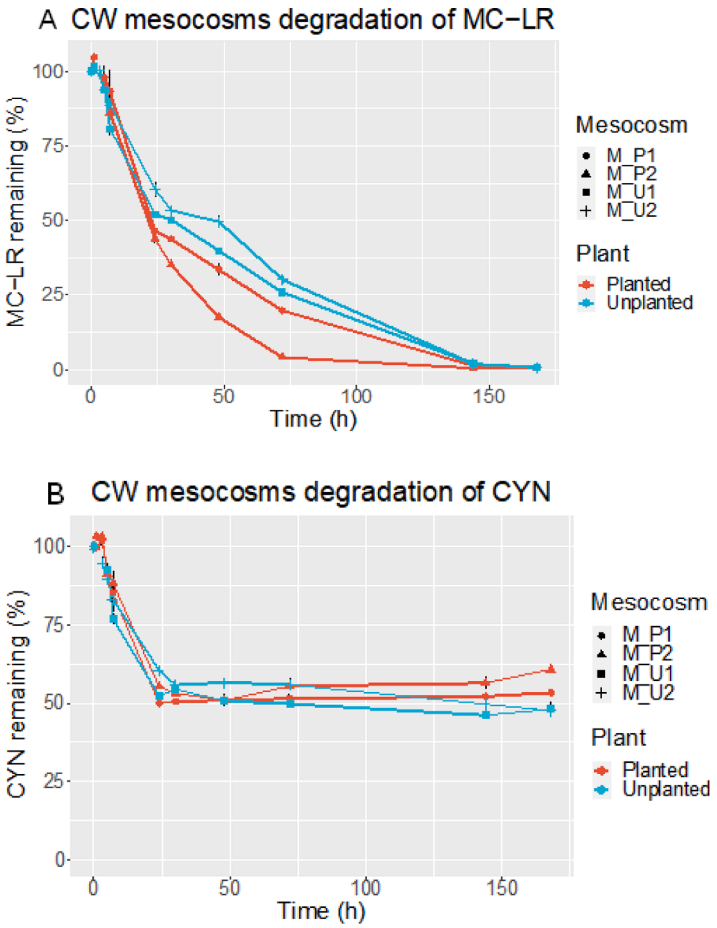

Fig. 2. Degradation of cyanotoxins (A: MC-LR; B: CYN) from CW mesocosms either unplanted (M_U) or planted (M_P). Results for each replicate are shown. The inflow medium was spiked with a mix of MC-LR and CYN.

Table 1
Degradation rate constants and half-life of MC-LR from all incubations and CYN from the CW mesocosms. CW mesocosms were either unplanted (M_U) or planted (M_P). The values are based on 1st order kinetics.

Treatment	Rate constant [a] (day^{-1})	Half-life (days)
In vitro MC-LR		
In vitro CW community MC-LR	0.18	3.85
In vitro CW community MC-LR + CYN	0.17	4.19
S. microcystinivorans Y2 MC-LR	3.94	0.18
S. microcystinivorans Y2 MC-LR + CYN	4.44	0.16
CW Mesocosms MC-LR		
M_U1	0.67	1.03
M_U2	0.71	0.98
M_P1	0.73	0.95
M_P2	0.87	0.80
CW Mesocosms CYN		
M_U1	0.69	1.01
M_U2	0.54	1.28
M_P1	0.73	0.95
M_P2	0.64	1.09

[a] The in vitro CW community rate constant for MC-LR is based on when the degradation was initiated and with the best linearity (52h-125 h). The CYN rate constant is based on the first 24 h of incubation in the CW mesocosms.

incubation period, between 82 and 93 % of TOC and 95–97 % of TN were removed from the mesocosms.

3.3. Bacterial 16S rRNA and mlrA abundance in the CW mesocosms

The introduction of MC-LR and CYN into the CW mesocosms induced a shift in bacterial abundance. In the four mesocosms, the bacterial abundance started at 2.6×10^6–1.3×10^7 16S rRNA gene copies and steadily increased during the treatment period to reach 6.71×0^7–1.5×10^8 gene copies (Fig. 3).

Few to no *mlrA* gene copies were detected in the CW mesocosms, except for M_U2 where ~16,000 gene copies of *mlrA* were detected at 144 h, although with a high variation between duplicate DNA extractions (Fig. S9). However, most DNA samples did not contain any *mlrA* gene copies.

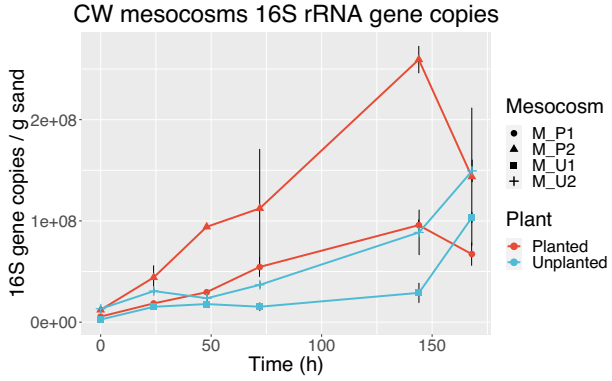

Fig. 3. The abundance of 16S rRNA gene copies in the CW mesocosms either unplanted (M_U) or planted (M_P) during the treatment period. $n = 2$; bars indicate standard deviation.

3.4. Bacterial community structure in the CW mesocosms

A similar trend between the number of ASVs and the calculated Shannon diversity index can be observed in the CW mesocosm samples. All end samples, but M_P1, had higher numbers of ASVs and higher Shannon index' thus revealing that a more diverse community had developed as compared to the start of the experiment (Fig. S10). M_P2 had the highest number of ASVs and Shannon index. A total of eight unique phyla, 25 unique orders, and 34 unique families were present in the CW mesocosms.

According to the 16S rRNA amplicon analysis, the microbial community from the CW mesocosms showed that a few phyla were highly abundant, with Proteobacteria as the most prominent; at the start of the experiment (40–57 %) and at the end (76–86 %) (Fig. 4A). The phylum Actinobacteria was likewise highly abundant at the start of the experiment (31–48 %) but decreased in relative abundance during the incubation (9–18 %). Bacteroidota increased in abundance during incubation from 0 to 3 % to 3–6 % abundance, while Acidobacteriota all decreased in relative abundance from 2 to 7 % to 0–3 %. Other phyla than the four were only represented at low relative abundance.

A clear time-dependent tendency between mesocosms was observed, with an increase in the relative abundance of bacteria affiliated to the order *Methylophilales* (start 4–11 %, end 33–68 %; Fig. 5B). *Methylophilales* is the primary reason the phylum Proteobacteria increases so heavily in abundance, as other abundant orders within the Proteobacteria have a constant relative abundance (*Rhodobacterales* from 3 to 16 % to 4–19 % and *Sphingomonadales* from 5 to 10 % to 4–8 %) or decreases (*Pseudomonadales* from 12 to 25 % to 2–9 %) from start to end (Fig. 4B). The drop in relative abundance of Actinobacteria is attributed to the decrease of bacteria from the order *Corynebacteriales* (from 21 to 35 % to 5–17 %).

At the family level, *Methylophilaceae* increased heavily in relative abundance and was the most abundant (start 0–2 %, end 22–63 %; Fig. 4C) at the end of the experiment. M_P2 had the lowest increase of *Methylophilaceae* (start 0.5 % to 22 % end) compared to the other three mesocosms which had a very similar increase (0–2 % start to 53–62 % end). *Flavobacteriaceae* was another family which increased in relative abundance from 0 to 1 % to 2–5 %, following incubation. Some of the other families that were abundant at the start dropped in relative abundance throughout the incubation period, such as *Nocardiaceae* (from 21 to 35 % to 5–17 %), *Moraxellaceae* (from 9 to 18 % to 0–5 %), and *Micrococcaceae* (from 6 to 21 % to 1–3 %). Families remaining relatively constant during the treatment were *Sphingomonadaceae* (from 5 to 10 % to 4–8 %), *Comamonadaceae* (from 1 to 5 % to 2–9%), and *Pseudomonadaceae* (from 3 to 7 % to 1–4 %) (Fig. 4C).

The Bray-Curtis PCoA dissimilarity plot of the microbial community formed two distinct groups according to the start and end of the incubation period (Fig. S11). This dissimilarity in the microbial community observed between the start and end shows that the cyanotoxin extracts induce a community shift upon exposure, which is highly dissimilar to the start community in the mesocosms. The observed dissimilarity between start and end communities is significantly different based on the unweighted Unifrac dissimilarity metric (PERMANOVA, $p < 0.01$).

4. Discussion

The outcome of the present study indicates that CWs have a great potential for removing MC-LR and CYN (Table 1) from lake surface water. However, pilot studies with full-scale systems are needed to verify our results obtained at mesocosm scale. To the best of our knowledge, this is the first study to demonstrate CYN biodegradation in CWs. The operation parameters need to be tuned to obtain a better degradation of this cyanotoxin as only half of the CYN was removed (Fig. 2B). The degradation mechanisms of MC-LR and CYN in CWs are largely unknown, but the data presented in our study reveal the importance of having a suitable microbial community and conditions for the degradation of MC-LR and CYN. The superiority of CW systems is shown by the increased degradation capacity of CW mesocosms compared to a similar microbial community outside a CW system (Table 1). This achieved hypothesis (1) that CW mesocosms more efficiently degrade MC-LR and CYN than the respective in vitro CW community. The effect that the plant (*J. effusus*) had on the biodegradation and microbial community was ambiguous. M_P1 had similar degradation efficiency and microbial community structure to the unplanted mesocosms (M_U1 and M_U2), while M_P2 had a faster degradation rate and a dissimilar microbial community to the other CW mesocosms (Fig. 4 and Fig. S11). This suggests that the microbial community was the factor that determined the best degradation performance amongst our systems, rather than the plant presence. However, further studies are required to obtain information regarding the role plants play in cyanotoxin degradation in CWs.

4.1. MC-LR degradation

Initially, the heterogenous microbiota in the in vitro CW communities may have depleted the labile substrates before switching to MC-LR degradation by competent cells (Eleuterio and Batista, 2010; Li et al., 2011). Hence, fast-proliferating microbes may have outcompeted MC-LR-degrading bacteria, initially, until labile compounds were catabolized and MC-LR degradation started (Li et al., 2011). Indeed, a lag phase of three days was observed before MC-LR degradation initiated (Fig. 1A). MC-LR-degrading bacteria might also be slow growing, with low initial abundance, requiring time before MC-LR degradation initiated. This is supported by the very low levels of DNA at the end of the in vitro experiment and the increased relative abundance of potential degrading phyla at the end of the CWs mesocosms experiment.

S. microcystinivorans Y2 degraded MC-LR rapidly without a lag phase (Fig. 1A), confirming that the experimental setup was suited to measure MC-LR degradation. *S. microcystinivorans* Y2 degradation in this study followed a first-order kinetics, differently from the zero-order (linear degradation) previously observed by Park et al. (2001) for the same strain in batch incubations. Therefore, no direct comparison of the degradation rates was possible. Park and colleagues used an optimal temperature of 30 °C and an initial concentration of 18 mg L^{-1} which probably explained the lack of lag phase in their study. However, half-life of the MC-LR was found to be shorter in this study (0.18 days) in comparison to the one calculated by Park et al. (2 days), indicating that even not having optimal environmental conditions this experimental set up brought the conditions for the strain to optimally eliminate MC-LR. Moreover, in the present study there was no intention of in vitro growth optimization of the strain but rather exposing the *S. microcystinivorans* Y2 to environmental relevant conditions (15 °C and initial concentrations of 100 μg L^{-1}). As a result, it can be observed that the difference in the MC-LR degradation pattern, the amount of TOC, and TN removed, suggests that single-strains with MC-LR-degrading capabilities and complex communities operate differently during MC-LR degradation at similar experimental conditions.

In contrast to the nutrient limitations in the in vitro incubations, the CW mesocosms had a high continuous influx of TOC and TN, similar to when CWs are operated to treat eutrophic water (Zhong et al., 2018). With the CW mesocosms, it can be observed that MC-LR degradation started immediately after spiking (Fig. 2A), as observed in another CW microcosms study (Wang et al., 2018). In the present study, over 99 % of the spiked MC-LR was removed after the operation period with around 1 μg L^{-1} MC-LR remaining in all mesocosms. The remaining MC-LR concentration is at the threshold value of 1 μg L^{-1} for safe drinking water (WHO, 2020a). Thus, successful treatment of MC-LR was achieved after seven days. Post-treatment concentrations of MC-LR below 1 μg L^{-1} have also been observed in other CW studies (Wang et al., 2018; Bavithra et al., 2020; Cheng et al., 2021) showing that CWs may be an effective bioremediation tool for water contaminated by MC-LR.

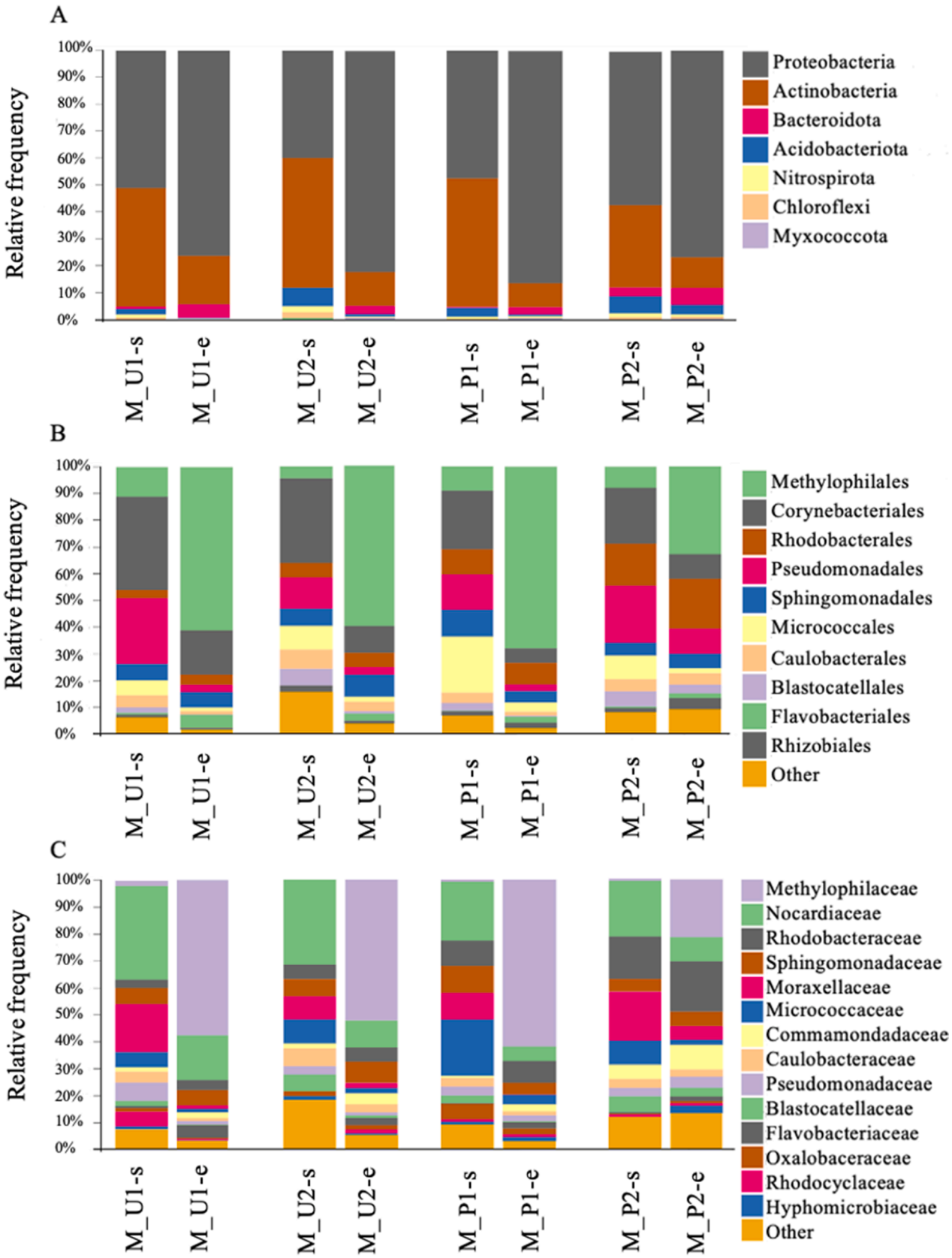

Fig. 4. Relative abundance of microbial community taxa at A) phylum, B) order, and C) family level. Each bar is a mean of $n = 2$. M_U: unplanted mesocosms; M_P: planted mesocosms. Bars placed closely together represent the same mesocosms at the start (s) and at the end (e) of the experiment.

4.2. CYN degradation

As evident by the rapid initial degradation of CYN in the CW mesocosms (Fig. 2B), a high potential for CYN degradation is present in the CWs. However, as the degradation ceases at 52 % degradation after 24 h, the system was not sufficient in alleviating this cyanotoxin. This pattern of rapid initial degradation and reaching a plateau at approximately 50 % before 24 h of incubation has also been observed by colleagues in our lab, using a similar experimental setup with varying concentrations of CYN (8 and 300 μg L^{-1}, unpublished data). Approximately 50 % degradation of CYN was also observed in a single-strain study with *Aeromonas* sp. R6 (Dziga et al., 2016). Two *Bacillus* sp. strains in vitro studies (Mohamed and Alamri, 2012; Mohamed et al., 2022) reported zero-order kinetics degradation constants, while in this study first-order kinetics were best fitted. Although same initial concentration was used (100 μg L^{-1}), those previous experiments were operated in batch and incubation temperatures were almost doubled (28 °C and 30 °C respectively vs present 15 °C). Half-life of CYN was 3.5 and 5.5 days in the past studies, compared with approximetly1 day of half-life for the first 24 h, emphasizing the treatment potential of the CWs mesocosms. We cannot exclude the potential presence of 7-epicylindrospermopsin (an enantiomer of CYN) to be present in the CYN extract, as *C. ovalisporum* has been shown to produce this enantiomer (Banker et al., 2000). An enantiomer could be more recalcitrant towards degradation or require different degrader genes (Frková et al., 2016) and may influence the amount of CYN that we observed to be degraded.

Although some natural consortia have been shown to lack the ability to degrade CYN (Wormer et al., 2008), the microbial assembly present in the vitro CW community cultures was expected to include CYN degraders, as they must have been present in the CW mesocosms. However, no degradation of CYN was found in the in vitro experiment. A major difference between the in vitro CW community and the CW mesocosms is the nutrient supply, and the presence of a substratum to allow biofilm formation. This could suggest that the amount or the presence of specific compounds or e.g. co-factors need to be present before the microbial community can degrade CYN (Horvath, 1972). The CW mesocosms had a high and continuous inflow of nutrients compared to those present in the in vitro incubations (Figs. S7 and S8). This provided a larger fraction of cyanobacterial metabolites in the CWs, and some of these may constitute compounds used during CYN transformation. This would suggest that CYN is degraded through co-metabolic degradation in the CW mesocosms. Co-metabolic degradation of CYN has been observed in a single-strain study with manganese-oxidizing bacteria (*Ideonella* sp. A226 and A228, *Pseudomonas* sp. OF001, *Comamonadaceae* bacterium A210) (Martínez-Ruiz et al., 2020b). Unfortunately, we did not characterize manganese in our experiment. Thus, further studies should look into the mechanisms and relevance of co-degradation of CYN and co-factors involved.

Biological active sand filters, or natural ambient consortia removing MC-LR and CYN, may show lag phases before cyanotoxin degradation is initiated (Ho et al., 2006, 2010; Smith et al., 2008; Hoefel et al., 2009). However, lag phases can be avoided or substantially reduced if the systems have been exposed previously to cyanotoxins (Christoffersen et al., 2002; Smith et al., 2008), maintaining cyanotoxin degraders. The mesocosms used in the present study were pre-conditioned with lake water from Lake Arresø with a substantial phytoplanktonic growth (Søndergaard et al., 1992) but no traces of MC-LR and CYN when collected (data not shown). Nevertheless, the native microbial community was able to degrade these cyanotoxins, as the CW mesocosms commenced MC-LR and CYN degradation without a lag phase (Fig. 2). Even though the microbiota was seemingly not exposed to MC-LR or CYN at the time sampling of Lake Arresø was performed, it is well known that its water sometimes contains cyanotoxins (Holst et al., 2003) and they seem to possess the genetic potential to carry out degradation of the cyanotoxins.

4.3. Bacterial response to MC-LR, CYN, and cyanobacterial metabolites

Looking into the bacterial community of the CWs, it is evident that changes in the structure are occurring after loading of the cyanotoxin extracts (Fig. 4). The overall number of ASVs and Shannon diversity index increased for most CW mesocosms, indicating that the cyanotoxin extracts generally selected for a more diverse bacterial community (Fig. S10). The community responded to the cyanotoxin extracts whether that being the cyanotoxins or other cyanobacterial cell constituents. This situation is similar to when treating cyanobacterial blooms in surface water, cyanobacterial cell constituents will also be present in the CWs. Therefore, we consider our experimental setup to be close to real environmental conditions during blooms.

Numerous taxonomic orders have been described to contain MC-degrading bacteria such as: Methylophilales, Corynebacteriales, Pseudomonadales, Sphingomonadales, Micrococcales, Caulobacterales, Flavobacteriales, Rhizobiales, Xanthomonadales, Bacillales, Bifidobacteriales, Enterobacterales, Burkholderiales, Aeromonadales, Lactobacillales, and Rhodospirillales (Massey and Yang, 2020). Knowledge of CYN-degrading bacteria is very limited as only a few species have been confirmed to degrade CYN. Orders with confirmed CYN degraders are: Bacillales (Mohamed et al., 2022; Mohamed and Alamri, 2012), Aeromonadales (Dziga et al., 2016), Burkholderiales, and Pseudomonadales (Martínez-Ruiz et al., 2020b). In the present study, the CW bacterial community consisted of multiple of these orders with known cyanotoxin-degrading abilities: Methylophilales, Corynebacteriales, Pseudomonadales, Sphingomonadales, Micrococcales, Caulobacterales, Flavobacteriales, and Rhizobiales. In particular, the order Methylophilales is of interest, as this order was the most abundant after the introduction of the cyanotoxin extracts (Fig. 4B). The number of 16S rRNA gene copies increased during the experiment (Fig. 3). Therefore, the change in the relative community abundance was due to the increased proliferation of species potentially involved in the degradation of the cyanotoxin extracts. Recent studies have shown that bacterial communities degrading MC often have a large enrichment of Methylophilaceae (Methylophilales) (Mou et al., 2013; Wang et al., 2018; Cheng et al., 2021; Santos et al., 2022) supporting the finding in the present study (Fig. 4C). This implies that Methylophilaceae was probably involved in the degradation of MC-LR and other cyanobacterial metabolites in the CWs. Two described species; Methylobacillus sp. (Hu et al., 2009) and Methylotenera sp. (Mou et al., 2013) within Methylophilaceae are so far described to degrade MC-LR and these may be contributing to the efficient degradation of MC-LR in the CWs. In contrast to Methylophilales, other orders with species described to have MC-LR-degrading abilities; Corynebacteriales, Pseudomonadales, and Micrococcales dropped heavily in relative abundance during incubation, indicating that bacteria within these orders were not involved in MC-LR degradation. Sphingomonadales remained at constant relative abundance throughout the experiment. As the 16S rRNA gene copy count increased during the treatment period (Fig. 3), this order proliferated at a rate that did not change the relative abundance. Meaning they were actively dividing although at a slower rate than those that increased in relative abundance. Numerous species within this order (Sphingomonas, Sphingopyxis, Novosphingobium, Sphingobium, and Sphingosinicella) can degrade MC-LR efficiently (Li et al., 2017) and the degradation is mainly attributed to the enzymatic mlrA pathway (Wang et al., 2018). Sphingomonadales species were present in high abundance (Fig. 4B; approximately 5 %) and may also have contributed to the degradation of MC-LR in the CW mesocosms. However, few to no mlrA genes were detected (Fig. S) indicating that the Sphingomonadales were not involved in the MC-LR degradation or that this order also utilizes alternative pathways.

Few to no *mlrA* gene copies were detected throughout the experiment (Fig. S9), suggesting that additional degradation mechanisms were in play in the CWs. Analysis performed by colleagues, looking into the transformation products formed during the treatment period, did not

find the linearized MC-LR or the Adda moiety at any time point during the incubation period (unpublished data). These two transformation products are often observed in vitro studies with single-strains having the *mlrA-D* gene cluster (Bourne et al., 2001; Yang et al., 2020). Thus, we suspect that the major degradation of MC-LR in the CW mesocosms is attributed to other degradative pathways than the MlrA-D enzymatic pathway. In support, Lezcano et al. (2017) found a higher correlation between MC concentration and *mlr*-lacking bacteria compared to those with *mlr* genes during a cyanobacterial bloom. This also suggests that alternative degradation routes may be important for MC degradation in the ambient environment. Therefore, it is crucial to obtain more information regarding which pathways are being employed and which transformation products are being formed by the *mlr*-lacking bacteria to assess the toxicity of these transformation products. On this note, few studies have described CYN degradation by bacteria (Smith et al., 2008; Klitzke et al., 2010; Mohamed and Alamri, 2012; Dziga et al., 2016; Martínez-Ruiz et al., 2020b; Mohamed et al., 2022), and only one study has investigated the biotransformation products of CYN (Martínez-Ruiz et al., 2020a). A large knowledge gap exists regarding CYN transformation products which needs to be explored as well, to ensure that CYN degradation equals detoxification.

4.4. From biodegradation to CW technology

In the present study, it is indicated that CWs constitute a potential treatment application for cyanotoxin-containing water. Only one type of experimental condition for the CW mesocosms was used in the present study. Usage of different types of porous media has been shown to influence the degradation efficiencies of MC-LR in CW microcosms, providing a parameter that can be optimized (Cheng et al., 2021; Cheng et al., 2022a). The present study obtained satisfying degradation of MC-LR at 15 °C which is relevant for northern temperate climate zones. Optimization of parameters such as the hydraulic loading rate and retention time, type of porous media, and various plant species, customized to which climate the system is operated under, can potentially provide more efficient degradation of cyanotoxins (Wang et al., 2018; Cheng et al., 2021, 2022a) . Also, CWs in the environment are typically fed continuously, therefore, the degradation efficiencies of MC-LR and CYN need to be explored with this type of loading.

5. Conclusion

The present study shows that the microbial community present in CW mesocosms can efficiently remove both MC-LR and CYN from synthetic lake water, with similar degradation constants. CWs are more optimal for degradation of MC-LR and CYN compared to similar microbial communities, operated at batch culture conditions. CW mesocosms removed 99 % of the MC-LR and the final concentration was at 1 μg L^{-1}, meeting the guideline value for safe drinking water. A maximum of 52 % of CYN was removed, showing incomplete degradation of this cyanotoxin. Proteobacteria was the phylum dominating at the start but also increased in abundance during the experiment. *Methylophilaceae* was rare before spiking the CWs, but predominant at the end of the treatment period, indicating a strong response to the presence of cyanotoxins and may be main responsible for cyanotoxin removal, also taking their documented cyanotoxin-degradation potential into account. Future studies should focus on the biotransformation pathways of CYN, including transformation products identification, in CWs to optimize the operating conditions to achieve more complete elimination of this cyanotoxin.

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Declaration of Competing Interest

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Data availability

The sequence data have been deposited and are publicly available from NCBI database, BioProject ID: PRJNA986856 (https://www.ncbi.nlm.nih.gov/sra/PRJNA986856).

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.hal.2023.102549.

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