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Saturated constructed wetlands for the remediation of cylindrospermopsin and microcystin-LR: Plants, microbes, and biodegradation pathways

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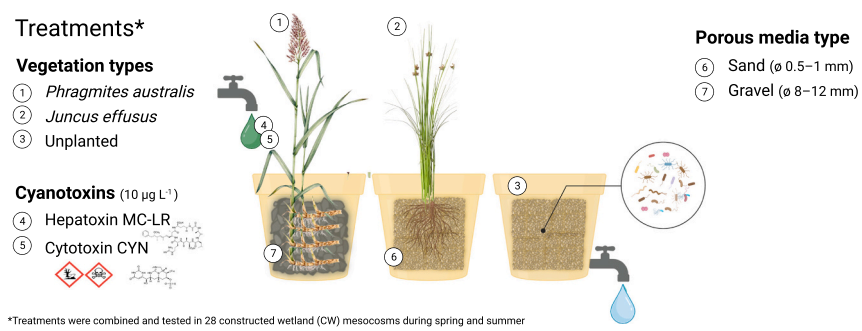
HIGHLIGHTS

- Constructed wetlands display high efficiency in MC-LR and CYN removal.
- Plants boosted removal percentages by 20 % for MC-LR and 45 % for CYN.
- Known cyanotoxins transformation products or genes were not found.
- Differential taxa abundances revealed several bloom-associated bacteria.
- P-k-C* model was used to discuss area requirement for further pilot studies.

GRAPHICAL ABSTRACT



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ABSTRACT

Harmful cyanobacterial blooms will be more intense and frequent in the future, contaminating surface waters with cyanotoxins and posing a threat to communities heavily reliant on surface water usage for crop irrigation. Constructed wetlands (CWs) are proposed to ensure safe crop irrigation, but more research is needed before implementation. The present study operated 28 mesocosms in continuous mode mimicking horizontal subsurface flow CWs. Mesocosms were fed with synthetic lake water and spiked periodically with two cyanotoxins, microcystin-LR (MC-LR) and cylindrospermopsin (CYN), at environmentally relevant cyanotoxins concentrations (10 µg L^{-1}). The influence of various design factors, including plant species, porous media, and seasonality, was explored. The mesocosms achieved maximum MC-LR and CYN mass removal rates of 95 % and 98 %, respectively. CYN removal is reported for the first time in CWs mimicking horizontal sub-surface flow CWs. Planted mesocosms consistently outperformed unplanted mesocosms, with *Phragmites australis* exhibiting superior cyanotoxin mass removal compared to *Juncus effusus*. Considering evapotranspiration, *J. effusus* yielded the least cyanotoxin-concentrated effluent due to the lower water losses in comparison with *P. australis*. Using the P-k—C* model, different scaling-up scenarios for future piloting were calculated and discussed. Additionally,

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bacterial community structure was analyzed through correlation matrices and differential taxa analyses, offering valuable insights into their removal of cyanotoxins. Nevertheless, attempts to validate microcystin-LR biotransformation via the known *mlrA* gene degradation pathway were unfruitful, indicating alternative enzymatic degradation pathways occurring in such complex CW systems. Further investigation into the precise molecular mechanisms of removal and the identification of transformation products is needed for the comprehensive understanding of cyanotoxin mitigation in CW. This study points towards the feasibility of horizontal sub-surface flow CWs to be employed to control cyanotoxins in irrigation or recreational waters.

1. Introduction

Our mechanistic understanding of cyanobacterial bloom dynamics in eutrophicated waters is greater than our capacity to prevent them. Regulations in the U.S. and Europe, implemented in the 1970s to control nutrient discharge into the environment (Ryther and Dunstan, 1971; Brattberg, 1986) have proven insufficient given the actual health status of our water bodies (Zamparas and Kyriakopoulos, 2021). Climate change is counteracting this policy further, by increasing the risk for harmful cyanobacterial blooms events in freshwater and coastal marine ecosystems (Paerl and Huisman, 2008; Smith and Schindler, 2009; Taranu et al., 2015). Such blooms may damage the ecosystem for several reasons, not least by the production of toxic secondary metabolites called cyanotoxins. Cyanotoxins exhibit diverse chemical structures and toxicity and have the potential to induce a variety of serious pathologies in humans and animals upon ingestion or even by direct contact (Merel et al., 2013).

Given that crops account for 70 % of total water use and are anticipated to rise (Nechifor and Winning, 2017), the exposure of crops to cyanotoxin-polluted water is also expected to increase. In rural areas experiencing water shortages, levels of cyanotoxins in crops have been recorded at >50 times the threshold for the recommended maximum daily intake (Redouane et al., 2023). Furthermore, the absence of official national and/or local guidelines for the quality of water used for irrigation generally results in the use of untreated water for irrigation. So far, treatment of cyanotoxin-contaminated water has only been addressed in drinking water treatment, with limited attention given to recreational waters and even less to irrigation waters (Martinez i Quer et al., 2024).

The present study analyzes the use of nature-based solutions, namely Constructed Wetlands (CWs), for treating cyanotoxins-polluted surface water for crop irrigation usage. CWs for water purification is a well-established technology that has proven effective for treating wastewater, including the removal of micropollutants (Vymazal, 2011a, 2011b; Gorito et al., 2017). However, using CWs to remediate surface water for harmful cyanobacterial blooms or cyanotoxins is relatively novel (Fang et al., 2016). A few studies have revealed promising results for this technology, as reviewed by Martinez i Quer et al. (2024). For example, microcystin-LR (MC-LR) removal rates were higher than 80 % in a CW microcosm operated as fill -and -drain batch systems with hydraulic retention time (HRT) of five days for three consecutive cycles (Cheng et al., 2021). So far, mainly MC-LR has been studied, with only one study including cylindrospermopsin (CYN). Thyssen et al. (2024) demonstrated that CYN was effectively removed in CWs operated in batch and unsaturated conditions, eliminating up to 50 % of the total initial concentration with a half-life of approximately one day. However, all the existing CW studies focused on the volumetric removal of cyanotoxins, neglecting evapotranspiration (ET) by plants, even though they play a crucial role in the effective functioning of CWs (Kadlec and Wallace, 2008). Warmer temperatures will coincide with cyanobacterial blooms and enhance CW ET rates (recorded up to 6.8 mm d^{-1} in full-scale systems (Papaevangelou et al., 2012)). ET can lead to underestimation of cyanotoxin removal performance, or higher concentrations in the treated effluent, in comparison to systems where water loss is not considered.

The capability of CWs to purify MC-LR- and CYN-contaminated water has been demonstrated by only a few studies with the CWs operated in batch mode (Wang et al., 2018; Bavithra et al., 2020; Cheng et al., 2021, 2022; Thyssen et al., 2024). Bacterial degradation has been proposed as the main removal mechanism occurring within CW in four of the six studies reviewed by Martinez i Quer et al. (2024). MC-LR bacterial degradation through the *mlrA-D* gene cluster was proven, from 25 bacterial isolates capable of degrading MC-LR, and where 17 carried the *mlr* gene (Li et al., 2017). However, other studies suggested other unknown bacterial mechanisms, as it is known that there are several mycrocystin degraders lacking *mlr* genes (Lezcano et al., 2016, 2017). Conversely, CYN bacterial degradation is a lesser-known phenomenon. To date, only one study by Wang et al. (2018) reported the presence of the *mlrA* gene in the porous media of CW microcosms eliminating MC-LR. Martínez-Ruiz et al. (2020a, 2020b) proposed manganese-oxidizing bacteria to eliminate CYN through unspecific reactive biogenic manganese oxidation. Consequently, more knowledge needs to be gathered to unveil the significance of these pathways within CWs.

In terms of CW design, three issues are particularly important for implementing CWs; the plant species, the porous media type, and the operation mode. To this end, the type of cyanotoxin to be controlled may require different removal mechanisms and consequently specific CW designs. The current understanding of the impact of plants on cyanotoxin removal is inconsistent. Only one study reported a significant plant effect using *Arundo donax* (Cheng et al., 2021); other studies found no influence of plant species on the removal rates (*Iris pseudacorus* and *Juncus effusus*) (Wang et al., 2018; Thyssen et al., 2024). Regarding porous media, gravel is widely used in CWs (Hartshorn et al., 2016; Wang et al., 2018; Cheng et al., 2021). This type of media is favored in CWs due to its high porosity and minimal maintenance requirements. However, alternative materials, such as sand, could potentially enhance surface area and contact time, leading to improved cyanotoxin degradability.

In terms of operation conditions, the removal of cyanotoxins in all mesocosms studies was operated in batch mode, either performing fill and drain (Wang et al., 2018; Bavithra et al., 2020; Cheng et al., 2021, 2022) or recirculating the feeding solution (Thyssen et al., 2024) and there are still many open questions regarding upscaling the technology, in this regard. Among the main CW designs, subsurface horizontal and vertical flow are common (Kadlec and Wallace, 2008), but have not yet been studied. In the present work, we have focused on the potential of CWs with horizontal subsurface flow which are water-saturated and, hence, limited in oxygen diffusion through the porous medium.

In the current study, the elimination of two of the most toxic and commonly occurring cyanotoxins was determined: hepatoxin MC-LR (Pelaez et al., 2010) and cytotoxin CYN (Sinha et al., 2012), with three aims: 1) to determine the effect of vegetation (*J. effusus, Phragmites australis,* and no plant), and porous media (gravel and sand) on the removal of MC-LR and CYN; 2) to assess the influence of ET to comply with different legislative scenarios (in terms of accomplishing thresholds for either removal rates or outlet concentrations); and 3) to validate whether the MC-LR and CYN core removal mechanisms can be observed in CWs mesocosms by employing a multi-parametric analytical approach (metabarcoding of the microbiota, cyanotoxins transformation products (TPs) screening, and degrading genes). The present study was carried out in CW mesocosms, mimicking horizontal

subsurface flow CWs, operated in continuous mode subject to periodic loadings of cyanotoxins at ambient conditions for two consecutive seasons where cyanobacterial blooms are expected (spring and summer). The overall hypothesis was that during the summer season, planted CWs would have enhanced cyanotoxin-removal due to higher temperatures and a more competent microbiota as the cornerstones of the removal process.

2. Materials and methods

Analytical approaches are briefly described in the main manuscript, while full detailed descriptions are provided as supplementary information (SI, sections S1-S8).

2.1. Cyanobacterial and cyanotoxin production

Two cyanobacterial strains were employed to produce the cyanotoxins used for spiking the experimental mesocosms; *Microcystis aeruginosa* LEGE 91094 and *Chrysosporum ovalisporum* LEGE x-001 (formerly *Aphanizomenon ovalisporum),* kindly provided by the Blue Biotechnology and Ecotoxicology Culture Collection (LEGE-CC), Portugal), producing MC-LR and CYN, respectively. The strains were maintained in photobioreactors (Alpha-Aqua, Denmark) with 15-L Z8 media (Kotai, 1972). The cyanotoxin spike solutions used in the present experiment were obtained from the produced cultures using a three-phase sequential extraction method without previous purification, as described by Thyssen et al. (2024). Both toxins were mixed into one single spike solution, given they probably co-occur in nature (Merel et al., 2013; Howard et al., 2021).

2.2. CW mesocosms setup and operating conditions

The present experiment was performed in 28 CW mesocosms operated continuously with a hydraulic loading rate (HLR) of 9.7 cm day^{-1}) via two self-compensating drippers feeding synthetic eutrophic lake water (Table S1) loaded by pulses onto the surface of the mesocosms. Synthetic eutrophic lake water was used to obtain a more controlled environment to investigate the removal processes. Each of the mesocosms consisted of a 12-L black plastic container (22.5 cm diameter, 29 cm height, 0.03 m^2 surface area).

The mesocosms were first set up in August 2020 and have been operated uninterruptedly since. The systems have been used on a long-term operational scheme with different water tables in two stages: stage 1) unsaturated, from August 2020 to December 2021; stage 2) saturated, from December 2021 to December 2022. The present experiment reports stage 2, where water-saturated conditions, mimicking horizontal subsurface-flow CWs, were implemented in December 2021, and sampling campaigns were carried out in the spring and summer of 2022.

The experimental treatments included two types of porous medium (sand, ø 0.5–1 mm, porosity 37 % or gravel, ø 8–12 mm); plants (*J. effusus, P. australis,* and unplanted); season (sampling in April 2022 and August 2022, with an averaged air temperature difference between the two seasons of 11 °C). Porous media and plant variables were tested in triplicate for the eighteen treatment (spiked) mesocosms and in duplicate for the ten control mesocosms, besides the control unplanted systems in which only one replicate was present. During the sampling campaigns, the treatment mesocosms were fed cyanotoxin-spiked water aiming at an environmentally relevant concentration of 10 μg L^{-1} for both MC-LR and CYN, while the remaining control mesocosms were receiving synthetic eutrophic lake water without cyanotoxins. Cyanotoxins were spiked 3 days prior to the sampling campaign (feeding tank ensured 9 days of feed with spiked synthetic lake water) due to i) the cost and accessibility to large quantities of cyanotoxins, and ii) as with ambient conditions where blooms are sporadic events. The experimental system, including the porous media, had no intrinsic sorption capacity to the cyanotoxins (Thyssen et al., 2024). The mesocosms were kept in a greenhouse at Påskehøjgaard, Aarhus (Denmark), avoiding precipitation and controlling seasonal ambient temperatures, humidity, and light conditions. A scheme of the complete setup is illustrated in Fig. 1. More details on the experimental setup in SI S1.

2.3. CWs mesocosm sampling and analysis

After three days of continuous inflow of cyanotoxin-spiked water, influent water, effluent water, and porous media (sand and gravel) were sampled. Water sampling was performed on three consecutive days: three replicate grab samples. Water samples were collected both from the inlet (feeding tank and dripper) and the outlet of each mesocosm. Subsamples were immediately frozen for nutrient measurements and cyanotoxin analysis (including quantification and TPs screening).

Porous media was also collected on day three, as the last sampling step, to minimize the disturbance of the mesocosms. Autoclaved Schott bottles and sterile spoons were used to obtain 150 mL of pooled porous media from three places from each mesocosm, at approximately 5 cm of depth, to ensure a representative sample within the heterotrophic area (Wu et al., 2018). A sub-sample was immediately processed to prepare a suspension to measure enzyme activities and flow cytometry (Section 2.3.3), while the remaining fraction was kept at −20 °C for DNA extraction.

2.3.1. Measurements in the water matrix

2.3.1.1. Target analysis of MC-LR and CYN by LC-MS/MS and suspect screening of transformation products by LC-QTOF. 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) coupled to a 5500 QTRAP-MS (Sciex). The method is detailed in Thyssen et al. (2024). Briefly, 10 μL of water samples were injected into the LC system, a Synergy Polar RP column was used with water and methanol with 0.2 % formic acid as mobile phases for the separation. The MS/MS method was optimized for the two cyanotoxins. The isotope dilution calibration (using MC-LR d_7, CYN $^{15}N_5$) enabled limits of quantification (LOQs) of 0.5 μg L^{-1} and 0.1 μg L^{-1} for MC-LR and CYN, respectively.

For the suspect screening of known MC-LR and CYN TPs, similar samples (as prepared for the LC-MS/MS) were injected directly (10 μL) in the LC coupled to a 6600 quadrupole-time-of-flight (QTOF) instrument (SCIEX). An Acquity UPLC BEH Shield RP18 1.7 μm column (2.1 mm × 30 mm) (Waters Corporation) was used for chromatographic separation. Eluents A and B were water and methanol, both with 0.1 % formic acid. The chromatographic conditions, as well as the detailed parameters of the instrument, were similar to those used by Tisler et al. (2021). Measurements were carried out at data-dependent acquisition mode (DDA), with a TOF mass range of 100–1200 Da in positive ionization mode with electrospray ionization (+5300 V ion spray voltage). The software SciexOS (SCIEX) was used to screen all the known biological TPs as listed by Martinez i Quer et al. (2024).

2.3.1.2. Water quality parameters and nutrient concentration. Physical-chemical parameters (pH, electrical conductivity, temperature, dissolved oxygen, and redox potential) were measured in situ using portable calibrated probes (Multi-Parameter Meter HQ40d, and sensION + EC5, HACH). Total Organic Carbon and Total Nitrogen were measured by a TOC-V analyzer equipped with a TNM-1 unit (Shimadzu). NH_4-N, NO_3-N, and PO_4-P concentrations were determined via QuikChem Methods® (10–107–06-3-D, 10–107–04-1-C, and 10–115–01-1-A, respectively) using an automated flow injection analyzer (QuikChem FIA + 8000 Series, Lachat instruments).

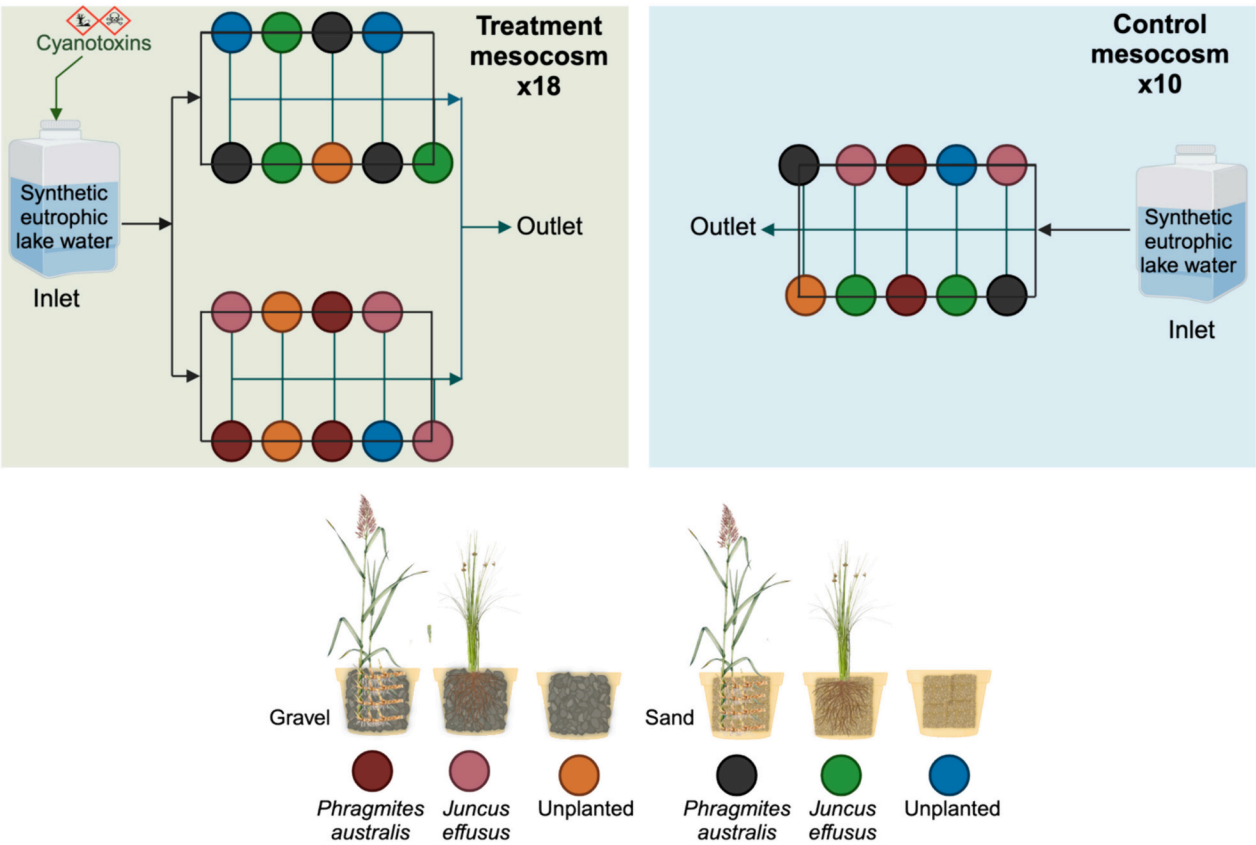

Fig. 1. . Illustration of the mesocosms setup. The colored circles represent the six different types of mesocosms used in triplicates for the cyanotoxin-treated systems and duplicates for the control mesocosms (except the unplanted with only one replicate). The black arrows represent the inflow tubing, while the blue arrows represent the outflow tubing. Figure created with biorender.com.

2.3.2. Microbiological analyses

2.3.2.1. DNA extraction. Porous media samples were homogenized in a sterile petri dish to ensure higher sample representativity and DNA was extracted using FastDNA™ SPIN Kit for Soil (MP biomedicals). For sand, we used ≈0.7 g of sand, whereas 20 g of gravel material was pre-extracted using vortex due to the danger of using gravel stones in the TissueLyser (OMNI) (see Supplementary information, Section S2). DNA concentrations were measured by Qubit (Thermo Fischer Scientific) using the Qubit dsDNA HS assay kit.

2.3.2.2. 16S rRNA amplicon sequencing and bioinformatic analysis. Amplicon libraries were built by a two-step PCR approach as described by Gobbi et al., 2019 with the modification that PCRBIO Ultramix polymerase (PCR Biosystems) was used for both amplification and indexing PCR reactions, carried out using a T100 Thermal Cycler (Bio-Rad).

The library was sequenced using the V2 500 cycle reagent kit on the Illumina MiSeq instrument (Illumina) at the Aarhus University Sequencing Centre (Roskilde, Denmark) as described in Thyssen et al. (2024).

Analysis of the paired-end demultiplexed sequence reads was performed using the QIIME 2 pipeline, version 2020.8.0. A total number of 1149 reads were used for normalization of sequencing data depth for further bioinformatic analysis. Analyses and visualizations were carried out in the R software (R Core Team, 2021). More detailed information about the bioinformatics pipeline can be found in SI, section S3.

2.3.2.3. Real-time qPCR of 16S rRNA and mlrA. Quantification of bacterial 16S rRNA for total prokaryote density, and *mlrA* genes as an indicator of the only described MC-LR degradation pathway (Bourne et al., 1996), 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 and *mlrA* were performed following protocols described elsewhere (Gobbi et al., 2019; Thyssen et al., 2024). All qPCR samples were run in technical triplicate. Information about the method efficiencies and run characteristics can be found in the SI, Section S4.

2.3.3. Extraction of the biofilm suspension

To analyze deeply the microbial community inhabiting the mesocosms' porous media, the biofilm was extracted to perform DNA-independent microbiological analysis. A biofilm suspension for each mesocosm was prepared by mixing 100 mL of sterile phosphate buffer solution (PBS) and 50 mL of its porous medium. The biofilm was detached by sixty seconds of sonication (Bransonic® CPXH Series Baths (United States) followed by two hours of horizontal agitation at room temperature in the dark, using a protocol adapted from Ramírez-Vargas et al. (2020). More detailed information can be found in SI, Section S5. The resulting biofilm suspensions were used for the following analysis: extracellular enzyme activity and flow cytometry.

2.3.3.1. Extracellular enzyme activities. The extracellular enzymatic activities involved in carbon processing (β-glucosidase; EC 3.2.1.21), protein degradation and nitrogen cycling, (leu-aminopeptidase, EC 3.4.11.1), and phosphorus acquisition (phosphatase, EC 3.1.3.1–2) were analyzed in all biofilm suspensions. The activity of the three enzymes was measured by fluorometry using methylumbelliferone (MUF)-linked fluorogenic substrates, following Hill et al. (2006) and Pastor et al. (2019). Briefly, suspensions were incubated in the dark for 1 h at room temperature at 0.8 mM final substrate concentration. The incubation

was terminated with the addition of glycine buffer (pH 10.4, 1:1). MUF standards and blanks of PBS without biofilm suspension were run using the same conditions.

2.3.3.2. Flow cytometry. Samples for measuring the abundances of bacteria, heterotrophic nanoflagellates (HNF), and phytoplankton were collected directly from the biofilm suspensions and fixed with 0.5 % glutaraldehyde and kept at −80 °C. Abundances were determined on an Attune flow cytometer (Applied Biosystems by Life Technologies) with a syringe-based fluidic system and a 20 mW 488 nm (blue) laser. Phytoplankton populations were discriminated based on their pigments. Heterotrophic cells were stained with SYBR Green I DNA stain and identified based on their red and green fluorescence and side scatter. The increase in the ratio between high nucleic acid (HNA) bacteria and low nucleic acid (LNA) bacteria was used as an indicator of bacterial activity. The method was adapted from Meire et al. (2023).

2.3.4. Data treatment and statistics

Cyanotoxin removal rates (volumetric) were calculated from the concentrations at the inlet and the outlet. For determining mass removal rates, mass loads were calculated using water balances and ET rates (Section S6, Eqs. S1-S3, and Table S2). Moreover, areal weathering rates (K_A) and area estimations were calculated using the PKC* model. All equations used are detailed in the SI (Section S7, Eq. S4, and Table S3).

All statistics were performed using the R language (R Core Team, 2021) in RStudio (Posit Team, 2022). The significance test of the variance was evaluated using two- and three-way ANOVA tests (Tables S4-S9). For prokaryotic Community structure variance, Permutational Multivariate Analysis for Variance (PERMANOVA) was used (Tables S10-S12) using the Vegan package (Oksanen et al., 2022). Non-metric multidimensional scaling (NMDS) representation of the amplicon sequence variant (ASVs) data was calculated using Bray-Curtis distances matrices from the default options of the phyloseq package (McMurdie and Holmes, 2013). Differential taxa abundances expressed as Log2 fold change were calculated using the geometric means prior to estimating the size factors, default Benjamin-Hochberg procedure for multi-inference correction, and Wald test for p-adjustment of the final values using the DESeq2 settings package (Love et al., 2014). Principal Component Analysis (PCA) was performed by using the environmental variables as vectors, scaling to unit variance before the analysis in the package's embedded function, and plotting final biplot graphs using the default options of the FactoMineR package (Lê et al., 2008).

3. Results and discussion

3.1. Cyanotoxin removal efficiencies by the CWs mesocosms

Considering the two sampling seasons, mass removal rates were in the range of 17 % to 95 % for MC-LR (Fig. 2 A) and 0 % to 98 % for CYN (Fig. 2B). Notably, the removal rates were 20 points lower for MC-LR (Planted 80 ± 13 % and Unplanted 60 ± 18 %), and 45 points lower for CYN (Planted 67 ± 16 % and Unplanted 21 ± 19 %) in unplanted mesocosms, compared to the treatments with plants (ANOVAs p-value$_{MC\text{-}LR}$ = 1.53 × 10^{-14}, p-value$_{CYN}$ = 1.41 × 10^{-15}). Moreover, the planted mesocosms delivered higher CYN removal rates for both seasons (Planted$_{Spring}$ 57 ± 12 % and Unplanted$_{Spring}$ 17 ± 19 %, Planted$_{Summer}$ 77 ± 14 and Unplanted$_{Summer}$ 26 ± 17 %), while for MC-LR it was only higher in the spring (Planted 83 ± 6 % and Unplanted 63 ± 11 %) (ANOVAs p-value$_{MC\text{-}LR\text{-}Spring}$ = 3.33 × 10^{-11}, p-value$_{CYN\text{-}Spring}$ = 8.27 × 10^{-13}, p-value$_{CYN\text{-}Summer}$ = 8.31 × 10^{-8}). *P. australis* planted mesocosms had less variance in their removal rates in comparison to the other treatments, delivering more consistent removal rates in the three consecutive sampling days (Fig. 2). Gravel mesocosms had generally higher removal rates, significant for CYN (Gravel 55 ± 25 % and Sand 48 ± 29 %) (ANOVA p-value$_{CYN}$ = 2.44 × 10^{-4}), but not for MC-LR.

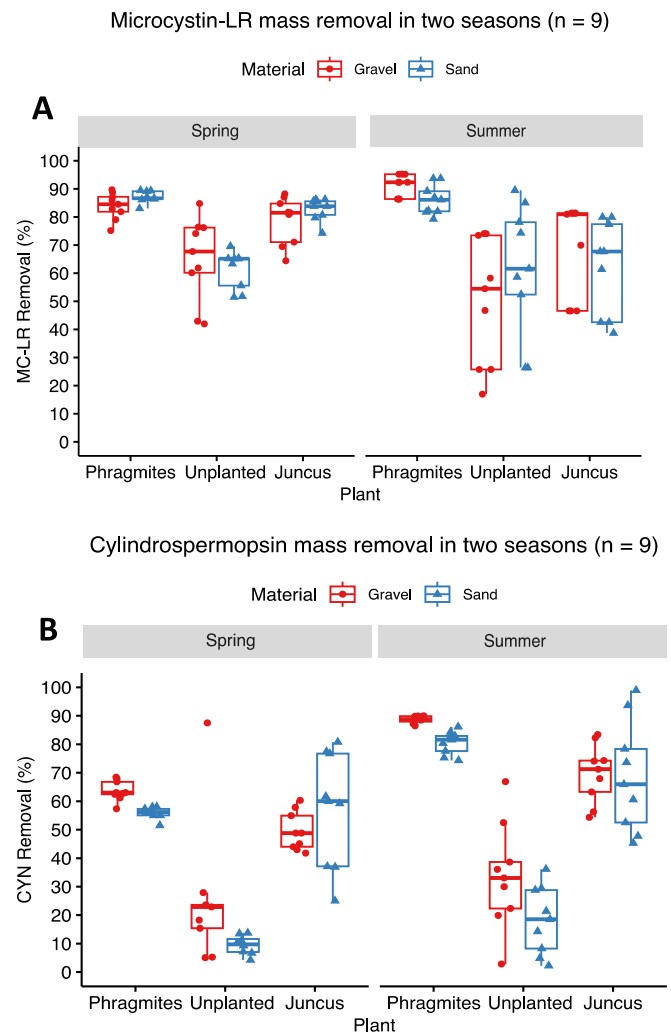

Fig. 2. . Box plot graph showing MC-LR (A) and CYN mass (B) removal grouped by plant treatment. In the left panel, the data shows the spring campaign (April 2022) while the right panel represents the summer (August 2022). The boxes show the distribution of three days measurements in the experimental triplicates ($n = 9$), in the middle of the boxes the median is marked with a heavy line, 25th and 75th percentiles are shown as the boxes together with the maximum and minimum values in both extremes of each box's line.

Regarding the effect of the seasons, summer had higher removal rates for CYN and lower for MC-LR (MC-LR$_{Spring}$ 77 ± 12 % and MC-LR$_{Summer}$ 70 ± 22 %, CYN$_{Spring}$ 43 ± 24 % and CYN$_{Summer}$ 60 ± 28 %,) (ANOVAs p-value$_{MC\text{-}LR}$ = 4.54 × 10^{-2}, p-value$_{CYN}$ = 2.12 × 10^{-5}), but also higher variation.

The MC-LR removal efficiencies observed in the present study were similar to what has been reported in comparable mesocosm studies operated in continuous batch mode (>99 % removal in one week (Thyssen et al., 2024)), or in sequential batches using fill and drain loading system (>90 % in three days, (Wang et al., 2018), >99.93 % removal in one week (Bavithra et al., 2020)), >80 % removal in five days, >75 % in three days (Cheng et al., 2022). However, from the studies where the HLR has been reported, the present work operated with a higher HLR (9.7 cm day^{-1}) (Wang et al., 2018; Bavithra et al., 2020; Cheng et al., 2021, 2022). Where other studies failed to prove CYN biodegradation, using natural bacterial consortia (Wormer et al., 2008), our study is the first to demonstrate the elimination of CYN in CW mesocosms operating at water saturated conditions in continuous mode - mimicking horizontal sub-surface flow CWs. Elimination in the present study is mostly due to bacterial degradation, given that i) sorption of the

cyanotoxins to both porous media used in this experiment was characterized and verified to be negligible and ii) photodegradation does not occur in subsurface flow systems. Plant uptake cannot be disregarded (Lyu et al., 2018). However, if occurring, cannot solely explain the mass removal rates observed.

In the present study, the strongest variable driving the removal efficiency was the presence of plants. Broadly speaking, the toxicity of MC-LR and CYN to plants at environmentally relevant concentrations has been rejected (Machado et al., 2017). To this end, some plant species have detoxifying mechanisms through removal of cyanotoxins. Pflugmacher et al. (2001) reported MC-LR removal via glutathione S-transferase in *P. australis,* using an initial concentration of 0.5 μg L^{-1}, although the plant contribution to the overall removal rate of the CW system was not measured. In comparison, Wang et al. (2018), and Thyssen et al. (2024) did not observe any significant difference in MC-LR removal between *Iris pseudacorus* or *J. effusus* planted, and unplanted mesocosms. In support of our study, Cheng et al. (2021) found that CW mesocosms planted with *A. donax* delivered higher MC-LR-removal rates than unplanted CWs. However, Cheng et al. (2021) suggested that the plant effect was indirect, providing a suitable environment for MC-LR degrading bacteria to thrive. Also, Thyssen et al. (2024) studied four experimental mesocosms; two unplanted and two planted with *J. effusus*. They found that one of the *J. effusus*-planted systems had a significantly dissimilar prokaryotic community compared to the rest. That system also removed MC-LR more efficiently than the other systems. The indirect effect, that the plant provides in the removal rates, can explain the results' disparity in the existing literature and yet emphasize the knowledge gap on this issue.

Although weaker than the plant treatments, the porous media also showed an impact on the removal of the cyanotoxins. Gravel-built mesocosms removed CYN more efficiently, while for MC-LR no significant difference between the two media types was observed. Using either gravel or sand offers different hydraulic advantages. CWs based on gravel exhibit lower risks of clogging and higher hydraulic conductivity, enabling higher HLR. In the present study, the volume of treated water was ten times higher in the gravel mesocosms in comparison with the sand-filled mesocosms. While in a horizontal subsurface flow context, gravel would result in longer HRT and consequently higher removal rates, it is well known that gravel CWs may show lower pollutant-removal efficiency due to lower bacterial density and surface contact area. In contrast, sand-filled CWs, generally have higher surface contact area and higher biofilm density due to their smaller pore size, delivering higher removal rates (Akratos and Tsihrintzis, 2007). In the present study, the longer retention time offered by the gravel material delivered higher removal rates for CYN, than the surface contact area or bacterial densities offered by the sand material.

In the current investigation, an effect of season, combined with an effect of medium porosity, was observed. In Figs. 2 A and 2B, during the spring campaign, there was no statistical difference in cyanotoxin removal between media for any of the cyanotoxins. In contrast, in the summer campaign, gravel was significantly higher for both cyanotoxins (MC-LR 70 ± 23 % and CYN 64 ± 26 %) (p-value$_{MC\text{-}LR}$ = 0.0046 and p-value$_{CYN}$ = 0.011). Hence, gravel seems to promote cyanotoxin removal, besides having hydraulic and non-clogging benefits.

Lastly, the effect of season was only statistically significant for CYN removal (Spring 43 ± 24 % and Summer 60 ± 28 %) (p-value$_{CYN}$ = 0.00002). The approximate temperature of the outlet in spring was around 21 °C and 25 °C during summer (data not shown). In line with our findings, Smith et al. (2008) found optimal CYN removal at 25 °C, by in vitro bacterial consortia. Contrary to the primary hypothesis, the season effect was only evident for CYN removal.

MC-LR outlet concentrations reached below 1 μg L^{-1} for all mesocosms in the summer (0.5 ± 0.4 μg L^{-1}) and were around 1 μg L^{-1} except for the unplanted mesocosms in spring (Fig. 3A). Although aiming for an initial concentration of 10 μg L^{-1}, working with live cyanobacterial cultures, there is inherently a variation within their cyanotoxin production and the extraction efficiency of the procedure, leading to fluctuations in spiking concentration, as observed here. One should keep in mind that cyanobacteria density during a bloom will vary in time and the occurrence of the blooms is sporadic, lasting from a few days to several weeks (Wu et al., 2013). Notably, in the summer campaign of the current study, an inlet MC-LR concentration of 1.3 μg L^{-1} was already close to the drinking-water threshold of 1 μg MC-LR L^{-1} (WHO, 2020a), while the initial CYN concentrations were closer to the intended spiking level. In spite of the high mass removal rates observed, none of the outlet concentrations complied with the drinking water guidelines of 0.7 μg CYN L^{-1} (WHO, 2020b) (Fig. 3B) probably due to the high evapotranspiration rates recorded (Table S2). Drinking water guidelines are used, in the present report, as a reference value to contextualize the meaning of the concentration range shown - the aim of this study was not complying with them. Outlet CYN concentrations in *J. effusus* planted mesocosms complied with the recreational guidelines (5.67 μg CYN L^{-1}) in both seasons.

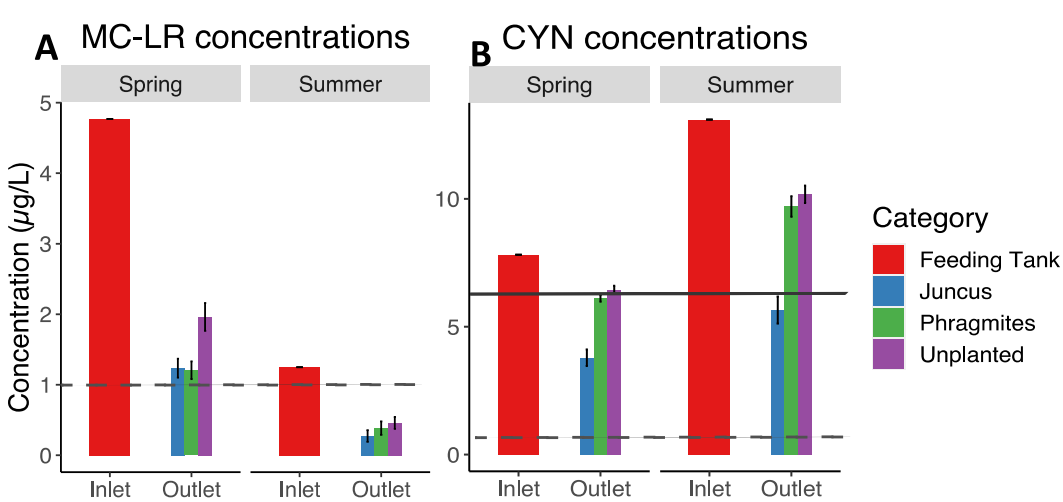

Fig. 3. . Concentration at the Inlet feeding tank ($n = 3$) and outlet ($n = 9$) of the different mesocosms organized by plant type (*Juncus effusus*, *Phragmites australis,* and unplanted) and campaign season Spring (April 2022) and Summer (August 2022), for MC-LR (A) and CYN (B). The dashed lines represent the WHO drinking threshold values for reference purposes (1 μg L^{-1} for MC-LR and 0.7 μg L^{-1} for CYN). The solid lines represent the WHO recreational value threshold for reference purposes for CYN (5.7 μg L^{-1}). The error bars represent standard error.

3.2. Prokaryotic community composition of the CWs

A total number of 458,767 reads was obtained in 55 samples resulting in highly diverse prokaryotic communities with 3124 different taxa detected across all samples. Although the mesocosm communities were highly diverse among their replicates and seasons, some general trends were observed at a higher phylogenetic level. The most relatively abundant phyla (Fig. S1) in the mesocosms, in order of overall relative abundance, were: Proteobacteria, Cyanobacteria, Actinobacteria (Spring), Chloroflexi, Acidobacteria and Gammatimonadota (Summer). This phylum composition was in accordance with a full-scale CWs system studied by Wu et al. (2016).

When the combined data from both seasons were analyzed at the ASV level (Fig. 4A), all four treatment variables (plant, cyanotoxin exposure, porous media, and sampling season) influenced the prokaryotic community structure (PERMANOVA *p*-values = 0.001 for each of the four variables, Table S10). Generally, in both seasons and for both cyanotoxins, removal performance was largely influenced by the presence of plants (Fig. 2). Nonetheless, the prokaryotic community was equally shaped by the different variables ($R^2_{Season} = 0.07$, $R^2_{Cyanotoxin} = 0.05$, $R^2_{Plant} = 0.07$, $R^2_{Media} = 0.08$) (Fig. 4A and Fig. S10). Meanwhile, alpha-diversity represented as a Chao1 index was not significantly different when comparing the two types of porous media, while it was significantly different for the plant type (summer only) (Fig. S2B-C), meaning that the planted systems promoted significantly higher alpha-biodiversity compared to the unplanted systems.

The effect of the season itself also affected the alpha-diversity Chao1 index, being significantly lower in spring compared to summer in all types of mesocosms (Fig. S2A). Moreover, total bacterial counts (Fig. S4A), 16S rRNA gene counts (Fig. S5), and extracellular enzymatic activities (Fig. S6) were notably decreased in the spring campaign. Indicating that the overall prokaryotic population increased during summertime. To the best of the author's knowledge, a report with such a range of variables, describing porous media microbial ecology, is not presented previously, which hardens the comparison with existing literature.

In Fig. 4B and C, looking at the seasons individually, porous media and cyanotoxin presence similarly influenced the prokaryotic community ($R^2_{Spring\text{-}Media} = 0.14$; p-value = 0.001, $R^2_{Spring\text{-}Cyanotoxin} = 0.07$; p-value = 0.001, $R^2_{Summer\text{-}Media} = 0.11$; p-value = 0.001, and $R^2_{Summer\text{-}Cyanotoxin} = 0.09$; p-value = 0.001). The main difference between the two campaigns was the variance explained by the plants in shaping the community ($R^2_{Plant\text{-}Spring} = 0.09$; p-value = 0.002 and $R^2_{Plant\text{-}Summer} = 0.15$; p-value = 0.001). Plants also promoted higher alpha-diversity levels, only significant for summertime (Fig. S2B), supporting the interaction effect of plants and season towards the distribution of the prokaryotic communities.

In springtime, with an average water temperature of 21 °C, *P. australis* normally initiates growth of new shoots, after two months of senescence. Even though *J. effusus* is a perennial plant decreases its metabolic activity during cold periods, decreasing also the associated microbial community (Mann and Wetzel, 2000). Hence, this could explain why the plant variable exhibits a higher effect on the community in summertime. In line with our findings, Faulwetter et al. (2013) also found greater abundance and diversity of the prokaryotic community in planted CWs during warmer seasons. By contrast, Wang et al. (2016),

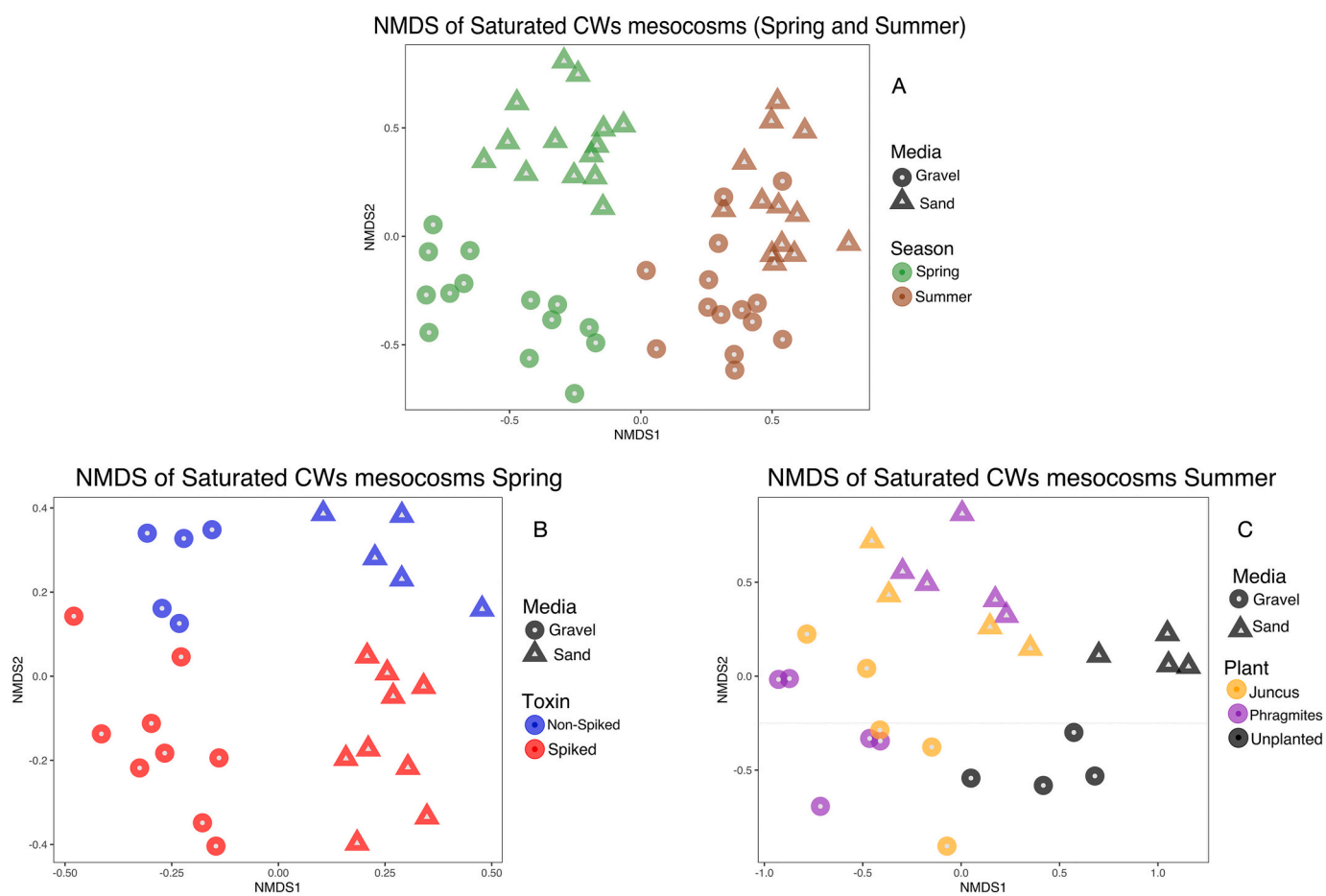

Fig. 4. . NMDS coordinate analyses using Bray-Curtis distances of both seasons (A), only using spring campaign data (B) and only using summer campaign data (C). Each of the geometrical symbols indicates one CW mesocosm system. In each of the graphs, the variables with the highest impact on the ordination of the samples are included.

found that the plant effect is stronger during the winter season, arguing that the plants provide a protected environment that counteracts seasonal temperature fluctuations. However, in the present study, a temperature drop of only 4 °C was experienced, therefore, the buffering capacity of the plants was probably not important.

Consequently, in summertime, when plants are more active, planted CWs may promote a more differentiated prokaryotic community able to degrade cyanotoxins more efficiently. As a result, spiked *P. australis* mesocosms, exhibited the highest removal rates (Fig. 2), higher total bacterial counts and activity (Fig. S4), higher 16S gene counts (Fig. S5), and higher extracellular enzymatic activities (Fig. S6).

Microbial communities are proposed to be the main driver for cyanotoxin (MC-LR) degradation in other CW studies (Wang et al., 2018; Cheng et al., 2021; Thyssen et al., 2024). In the present study, factors such as porous media type, presence of cyanotoxin, sampling season, and plant type shaped the overall prokaryotic community.

3.3. Cyanotoxin removal correlation to specific bacterial taxa

Differential taxonomic analysis (Fig. 5, Table S13) showed a total of 16 bacterial taxa having significantly higher/lower relative abundance in spiked mesocosms (11 taxa higher and 5 taxa lower relative abundance) and belonging to nine genera and five phyla. The most represented phylum was Proteobacteria. The taxa with the highest difference in relative abundance was the only cyanobacterial genus with positive relative abundance in the analysis. This uncultured cyanobacterium has an approximately five times higher relative abundance in the spiked as compared to the non-spiked CW samples (ASV3269, Table S16). In terms of increase in relative abundance (Log2 fold change), this cyanobacterium was closely followed by taxa from the genera *JGI_0001001-H03*, *Hypomicrobium*, *Fimbriimonadacea*, *SWB02,* and one uncultured taxon from the family *Sphingomonadacaea*. Moreover, other taxa with lower log2 fold change than the previously mentioned but with positive increase, belong to the genera *Rhodobacter*, and *Hypomicrobium*, and one uncultured taxon belonging to the family *Caldilineaceae* (Fig. 5, Table S13). Additionally, all the most differentially abundant genera in non-spiked control mesocosms belonged to the Cyanobacteria phylum (except for one; in Fig. 5).

We also observed a relative increase in the occurrence of different bacterial genera associated with cyanobacterial blooms in natural ecosystems, compared to the non-spiked controls, such as four taxa

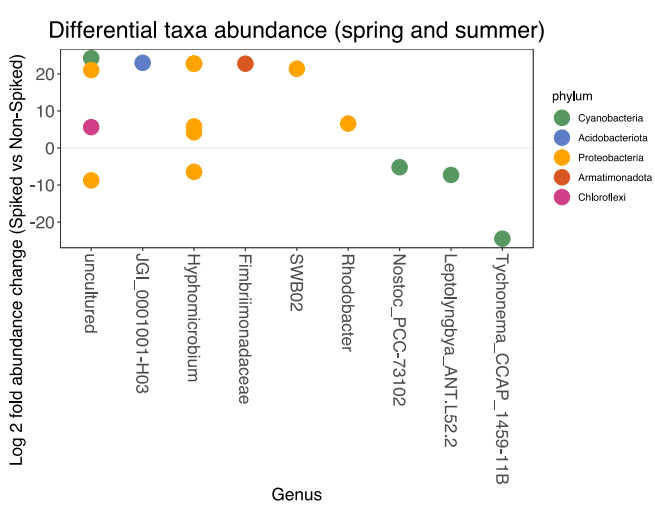

Fig. 5. . Differential taxa abundance of significant genera relatively more abundant in spiked mesocosms in comparison with the control mesocosms, expressed as Log2-fold change. Log2 fold >0 indicates that the specific genus is more present in the spiked mesocosms. Each circle indicates a taxon within the same genus. Only p-adjusted values of the Wald test corrected by multiple testing using the Benjamin and Hochberg method, are shown.

belonging to *Hyphomicrobium* (Briand et al., 2016) and one taxon belonging to *Rhodobacter* which may biologically predate the blooming cyanobacteria (Pineda-Mendoza et al., 2020) (Fig. 5 and Table S13). Furthermore, manganese -oxidizing metabolism is so far the only process proposed for CYN remediation (Martínez-Ruiz et al., 2020a) and is related to the four taxa with increased abundance belonging to the genus *Hyphomicrobium,* which are the main manganese-oxidizing bacteria in freshwater ecosystems (Tyler, 1970).

From the "uncultured" genera (Table S13), there are two taxa from the *Sphingomonadaceae* family: ASV2461 and ASV811. *Sphingomonadaceae* is one of the most important MC-LR degrading families (Zhang et al., 2020) and with several MC-LR degraders containing the *mlr* gene cluster (Jiang et al., 2011). However, presently, *mlrA* gene copies were not detected, suggesting that *mlr*-dependent degradation did not occur at a significant level. Also, the cyanobactericidal bacterial genus (*SWB02* sp.) (Van Le et al., 2023), appeared approximately five times more often in spiked mesocosms than in non-spiked systems. Two cyanobacterial genera (*Nostoc PCC 72120* sp. and *Leptolyngbyga ANT.L52.2* sp.) were three times decreased in spiked mesocosms (Fig. 5).

Using community analysis such as differential taxa abundance (Fig. 5) indicates the possibility that CWs with a harmful cyanobacterial bloom history have a bacterial community composition that can more readily metabolize cyanotoxins, compared to ambient communities with no harmful cyanobacterial blooms' prehistory. The theoretical residence time of the cyanotoxins in CWs is at least several hours in comparison to the half-life of the cyanotoxin in a natural lake environment, which can be several weeks (Zastepa et al., 2014; EPA, 2015). It indicates the great potential for cyanotoxin remediation in CWs, due to its biodiverse gene pool, having high probabilities for the presence of biodegradation genes in the bacterial community.

3.3.1. Environmental factors affecting the removal performance and bacterial communities

In Fig. 6A, a PCA biplot of the environmental variables in the spiked mesocosms is plotted, including data from both sampling campaigns. The grouping of the mesocosms is mainly determined by plant type (confident ellipses), followed by porous media type. Besides, the mass removal variable for both cyanotoxins contributed heavily to mesocosms grouping in all plots (Figs. 6A-C, Fig. S7), especially driving the ordination of *P. australis* planted mesocosms, iterating the importance of the design. On top of that, variables such as enzymatic activities, conductivity, nutrient removal, total bacteria and phytoplankton density, and volumetric removals were the main drivers separating the planted groups. Unplanted mesocosms were defined by higher pH, oxygen concentration, 16S gene density, and bacterial activity. The position of vectors was consistent throughout spring and summer campaigns (Fig. 6B and C). A bar plot with the different parameters' contributions for both campaigns is presented in Fig. S7. Overall, it can be observed that the same grouping for Fig. 6 was consistent with the one in Fig. 4.

Dissolved oxygen is an important variable differentiating the mesocosms (Fig. 6A, Fig. S7) and it is persistently higher in unplanted mesocosms throughout the sampling campaigns (Figs. 6B and S8). Other mesocosms studies also found higher levels of oxygen in unplanted mesocosms vs. planted ones (Gaget et al., 2017), indicating that heterotrophic metabolism is consuming the oxygen while degrading the available plant biomass and exudates, supported by a higher extracellular enzymatic activity in the planted mesocosms (Fig. S6). The oxygenation effect of plants may be local in the rhizosphere. Oxygen levels in the outlet of the mesocosms range from 0 to 3 mg L^{-1} dissolved oxygen (Fig. S8B), thus, the fluctuations are not so relevant as the mesocosms are designed to be at hypoxic (water -saturated) conditions (Klitzke and Fastner, 2012). Other factors brought by in planted mesocosms, such as the plant itself, the rhizosphere, or the suitable environment that they create for microbial colonization, seem to be more important for the degradation of cyanotoxins.

Extracellular soil enzymes, namely phosphatases, glucosidases, and

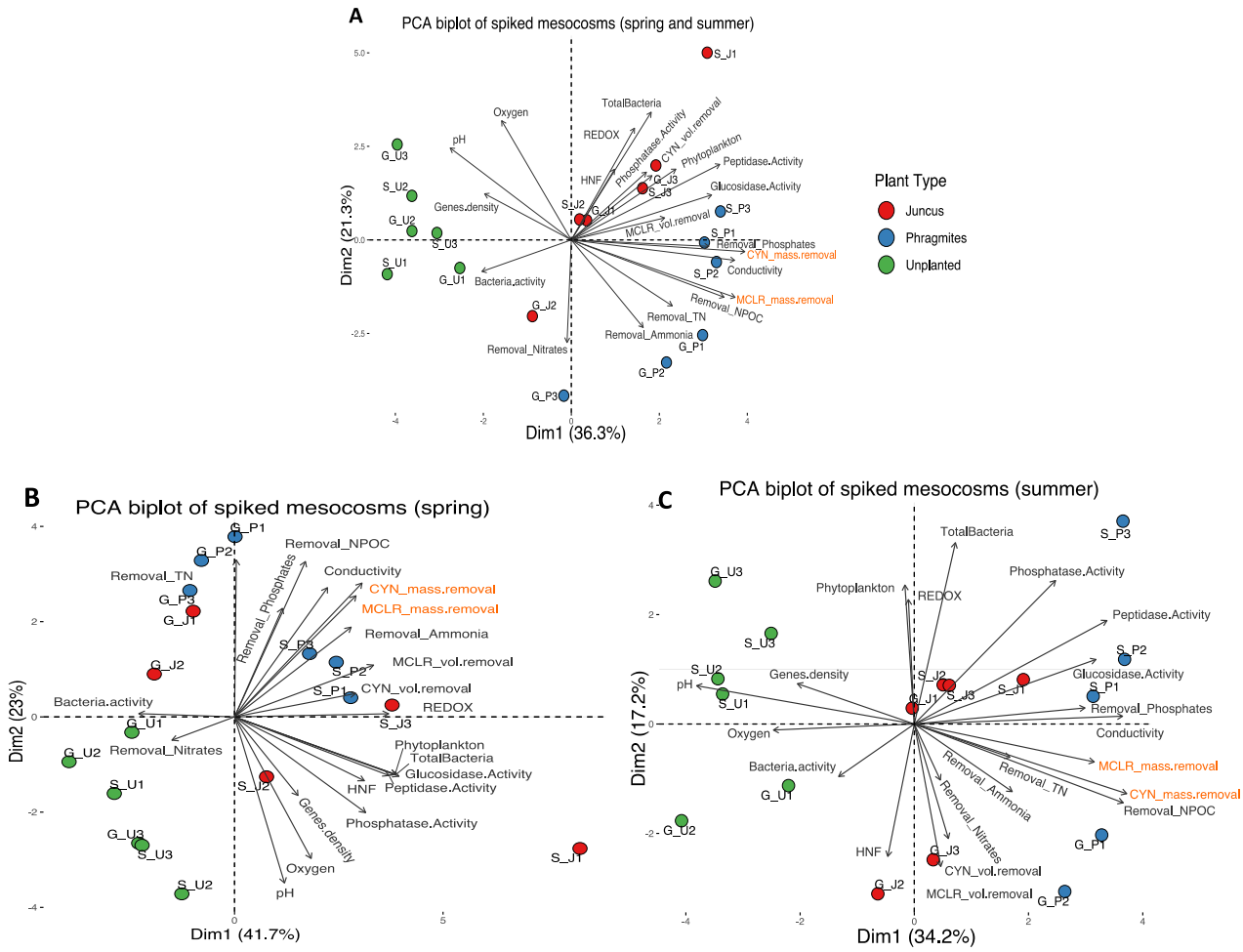

Fig. 6. . PCA biplot with spiked mesocosms, the environmental and microbial variables together in the two principal components (Dim1 and Dim2) for both seasons (A), spring (B), and summer (C). Contribution scores were calculated for each environmental variable and represented as vectors (arrows); the length of each vector represents the contribution to the variance of each dimension. Colored ellipses show the 95 % confidence interval where the different plant categories can be distributed.

peptidases, are used as bioindicators for the removal of different pollutants in CWs (Truu et al., 2009). Peptidases and glucosidases are involved in carbon cycling while phosphatases mobilize phosphorous from organic phosphoric esters (Chróst, 1991). Thus, planted mesocosms had lower concentrations of phosphates and higher carbon availability due to plant biomass decay and phosphorus assimilation by plants (Fig. S9A). Therefore, it seems plausible that the activity of those enzymes was higher in the planted mesocosms (Fig. S6). Other organic micropollutants have been found to increase extracellular enzymes such as proteases and phosphatases, and additionally inhibit enzymes like β-glucosidase (Zhou et al., 2005).

In terms of bacterial counts by flow cytometry (Fig. S4), planted mesocosms had the highest total bacteria counts. Opposite and contributing less, 16S gene counts (normalized to the dry weight of porous media), the variation in the dataset does not display a clear tendency besides the marked seasonality (Fig. S5). The enhanced presence of 16S genes does not necessarily mean a higher number of active bacteria, but just more bacterial biomass including dormant or dead bacteria. However, in terms of activity, unplanted mesocosms only had more active bacteria in comparison to the rest during springtime, in agreement with the above-mentioned effect of vegetation dormancy (Fig. S4).

To conclude, planted mesocosms seem to provide a more suitable environment increasing the number of total bacteria counts, and having more activity of nitrogen- and carbon- cycling enzymes (Fig. 6). These enzymes would ultimately provide more available nutrients in the systems, and as a result, a richer porous media independently of its material type. Moreover, the observed higher conductivity levels due to ET were not affecting the basic microbial processes. Redox potential and nitrate concentration were also higher in planted mesocosms. Altogether planted CWs seemed the best suited environment for efficiently removing both MC-LR and CYN, where the plants themselves may have removal mechanisms acting in synergy with their microbial community.

3.4. Biodegradation pathways

Suspect screening analysis of 22 and 7 known TPs for MC-LR and CYN, respectively (Martinez i Quer et al., 2024), was successfully performed. However, only traces of MC-LR-TP11 (Phenylacetic acid, PubChem CID 999 (Yang et al., 2020; Wei et al., 2023)) and CYN-TP1 (TP290 (Martínez-Ruiz et al., 2020a)) were tentatively detected (Martinez i Quer et al., 2024), as the lack of specific product ion spectra of sufficient quality did not allow unequivocal identification. In line with the chemical data, *mlrA* gene quantification by qPCR did not produce measurable values in any of the samples. Hence, alternative degrading mechanisms were in play, removing MC-LR in the mesocosms. Other studies also failed to detect the *mlrA* gene while seeing MC-LR removal (Mou et al., 2013; Lezcano et al., 2016; Thyssen et al., 2024). The genus *Rhodococcus* was relatively more abundant in spiked mesocosms (Fig. 5) and has been described as a potential *mlr*-independant

MC-LR degrader (Zhang et al., 2020), in line with our lack of *mlr* gene detection and associated TPs. Although TPs for CYN have been proposed (Martínez-Ruiz et al., 2020a), degradation pathways for CYN are unknown. Obviously, more work is needed to disclose environmentally relevant degradation pathways, beyond the in-vitro experiments.

3.5. Scaling up

In order to advance the future implementation of CWs to address the problems with cyanotoxins, this section elaborates on potential scenarios and options for piloting. Selection of plant species should take seasonality into account; for instance, perennial reed (*J. effusus*) would be preferred to keep a more protected environment in cold climates (Vymazal, 2011b). Depending on the climate, minimizing ET might be preferred if recycling of water for irrigation, and not the bioremediation performance, is the primary objective. Even though mesocosms systems are more prone to ET effects than full-scale systems, *P. australis* provided the highest mass removal of cyanotoxins in the present study, but lowest effluent quality due to the high water loss. In the lack of a regulatory framework to set design priorities (e.g. removal rate targets or effluent quality), the results of the present work put forward considerations on ET and treated water availability for leading further piloting options.

Another important factor is the hydraulics of the systems and as influenced by the type of porous media. Considering the results from section 3.1, gravel systems resulted in higher removal performances, mainly of CYN. Also, gravel mesocosms showed 10 times higher volume-treatment capacity, while potentially being less prone to clogging. Thus, gravel would often be preferred when scaling up CWs.

The microbial community, as the main driver of MC-LR and CYN removal, will develop in accordance with the growth conditions in any CW. To ensure a quick establishment of competent cyanotoxin-degrading systems with proper degrading genes, a pre-exposure with either a carbon booster or with eutrophicated water that contains the cyanotoxins is recommended. In the current study, the mesocosms used had been previously exposed to MC-LR and CYN prior to the present experiment (Summer and Autumn 2020 and Spring and Summer 2021).

Taking application purposes into account, nutrient monitoring is important as it is usually an issue where most countries have regulation guidelines to prevent eutrophication when irrigating crops. Thus, a balance is needed between exploring the possibility of nutrient recycling from eutrophic waters, cyanotoxin threshold values, and water availability. One of the main limitations of the CW application is its demand for land area. To provide future piloting data, the P-k—C* model (Kadlec and Wallace, 2008) was fit with the experimental data to generate weathering rates (K_A; Section S11) and enable an estimation of area demand according to different legislation scenarios (Table S4). The experimental data in use does not account for ET rates, aiming to be more conservative given the uncertainty of scaling up processes and systems. The first legislation scenario considered was encompassing effluent water using the WHO long-term exposure guideline for drinking water for MC-LR (1 μg L^{-1}) and CYN (0.7 μg L^{-1}) (WHO, 2020a, 2020b), being the most conservative scenario. If designing a system for a daily irrigation need of 1 m^3 water, the area needed for a horizontal subsurface flow CW may vary from approximately 7 to 30 m^2 for MC-LR, and 36 to 415 m^2 for CYN. However, this scenario is very conservative, as CW technology is not used for the purification of drinking water. Nevertheless, it helps to set up a strict benchmark given the lack of proper irrigation standards.

For less strict water usage standards, a smaller CW surface will be required. When treating the water to achieve either PNEC values for MC-LR (5.67 μg L^{-1}) or recreational values for CYN (6 μg L^{-1}), areas vary between approximately 2 to 7 m^2 for MC-LR and 6 to 72 m^2 for CYN. The present study shows how important it is to choose the right CW design, and therefore, the importance of mesocosms studies prior to pilot construction. Blooms can simultaneously consist of a mix of various cyanotoxin species (Merel et al., 2013; Howard et al., 2021) and with MC-LR and CYN, estimates for the highest land area required (CYN) should be used. It should be noted that water for irrigation is not currently regulated. If such guidelines existed, limits might fall in between the concentrations of drinking and recreational waters, as indirect consumption of cyanotoxins occurs when consuming cyanotoxin-irrigated crops. When estimating the most suited design for piloting for a combined treatment of MC-LR and CYN in summertime-like conditions, it will probably require an area between 6 and 36 m^2. This range reflects the difference between the two regulation scenarios and the optimum mesocosm for removal of CYN, the cyanotoxin most hard to eliminate.

4. Conclusions

Mesocosm CWs proved an effective tool for remediating MC-LR and CYN. Planted systems, especially with *P. australis*, were robust through the seasons and the different types of porous media and seasons, delivering high overall removal mass rates. Contrarily, *J. effusus* planted systems provided an effluent of higher quality (lower MC-LR and CYN concentration) due to its lower ET rates. Prokaryotic communities in the mesocosms were shaped by season, porous media type, cyanotoxin presence, and plant species. Several bloom-associated bacteria were found in significantly higher relative abundance in the spiked mesocosms compared to the non-spiked, including four *Hyphomicrobium* genera, manganese-oxidizers in freshwater, and one taxon from *Rhodobacter*. This genus had been related to *mlr* -independent MC-LR degradation. However, we did not detect *mlr* genes in the present work supporting the hypothesis that MC-LR removal was carried out by other microbes in the mesocosms. Neither did we detect currently known TPs for MC-LR and CYN. Therefore, it opens up the possibility for alternative pathways with other TPs occurring in more complex setups, also degrading cyanotoxins. Overall, horizontal subsurface-flow CWs are suggested for future treatment of recreational or irrigation waters contaminated with cyanotoxins.

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CRediT authorship contribution statement

Alba Martinez i Quer: Writing – original draft, Visualization, Validation, Investigation, Formal analysis, Data curation, Conceptualization. **Carlos Alberto Arias:** Writing – review & editing, Supervision, Resources, Project administration, Conceptualization. **Lea Ellegaard-Jensen:** Writing – review & editing, Supervision, Resources, Project administration, Conceptualization. **Anders Johansen:** Writing – review & editing, Supervision, Project administration, Methodology, Conceptualization. **Maria Lund Paulsen:** Writing – review & editing, Visualization, Validation, Methodology, Investigation. **Ada Pastor:** Writing – review & editing, Visualization, Validation, Methodology, Investigation. **Pedro N. Carvalho:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Pedro N. Carvalho reports financial support was provided by Independent Research Fund Denmark. Carlos A. Arias reports financial support was provided by European Commission Marie Sklodowska-Curie Actions. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The sequence data have been deposited and are publicly available from the NCBI database, BioProject ID PRJNA1034523 (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA1034523).

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2024.174745.

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