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Fungal removal of cyanotoxins in constructed wetlands: The forgotten degraders

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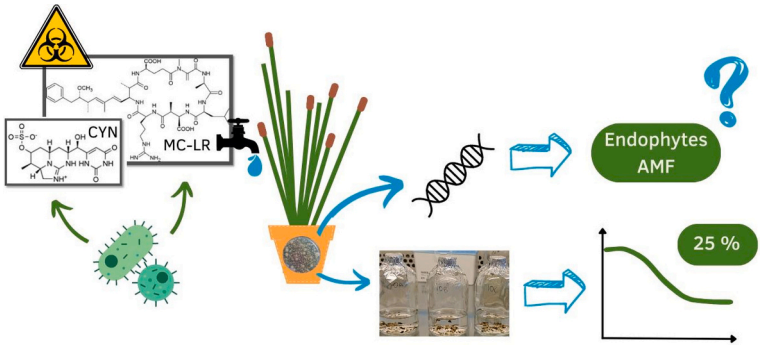
HIGHLIGHTS

- Cyanotoxin removal mechanism in constructed wetlands is likely driven by microbes.
- The degradation ability of seven mesocosms fungal isolates was demonstrated.
- The fungal communities were affected by exposure to cyanotoxins.
- MC-LR glutathione and cysteine conjugates were not found in extracellular phase.

GRAPHICAL ABSTRACT



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ABSTRACT

Harmful cyanobacterial blooms have increased globally, releasing hazardous cyanotoxins that threaten the safety of water resources. Constructed wetlands (CWs) are a nature-based and low-cost solution to purify and remove cyanotoxins from water. However, bio-mechanistic understanding of the biotransformation processes expected to drive cyanotoxin removal in such systems is poor, and primarily focused on bacteria. Thus, the present study aimed at exploring the fungal contribution to microcystin-LR and cylindrospermopsin biodegradation in CWs. Based on CW mesocosms, two experimental approaches were taken: a) amplicon sequencing studies were conducted to investigate the involvement of the fungal community; and b) CW fungal isolates were tested for their microcystin-LR and cylindrospermopsin degradation capabilities. The data uncovered effects of seasonality (spring or summer), cyanotoxin exposure, vegetation (unplanted, *Juncus effusus* or *Phragmites australis*) and substratum (sand or gravel) on the fungal community structure. Additionally, the arbuscular mycorrhizal fungus *Rhizophagus* and the endophyte *Myrmecridium* showed positive correlations with cyanotoxin removal. Fungal isolates revealed microcystin-LR-removal potentials of approximately 25 % in *in vitro* biodegradation experiments, while the extracellular chemical fingerprint of the cultures suggested a potential intracellular

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metabolization. The results from this study may help us understand the fungal contribution to cyanotoxin removal, as well as their ecology in CWs.

1. Introduction

From promoting the development of oxygen-dependent life on Earth to threatening public health, photosynthetic cyanobacteria have adapted to many environments (Aba et al., 2021; Demoulin et al., 2019). Their growth is favoured in eutrophic waters, where they proliferate into cyanobacterial harmful algal blooms (CyanoHABs) (Cruz et al., 2020). Despite public legislation, nutrient run-off from agriculture is still the main cause of water eutrophication in many countries. Increasing temperatures and heavy rainfalls promote development of CyanoHABs even further (Chorus et al., 2021; Huisman et al., 2018).

Besides decreasing oxygen concentration and altering organoleptic properties of contaminated waters, cyanobacteria often produce cyanotoxins of great concern to human and animal health (Aba et al., 2021). The most frequent are the hepatotoxin microcystin-LR (MC-LR), and the cytotoxin cylindrospermopsin (CYN) (Svirčev et al., 2019). The primary routes of human exposure are through intake of contaminated drinking water and by recreational waters (Cruz et al., 2020). However, cyanotoxins may also reach humans through the food chain by bioaccumulation in aquatic organisms, or by accumulating in the edible parts of plants when cyanotoxin-contaminated water is used for irrigation (Chatziefthimiou et al., 2016; Corbel et al., 2014; Melaram et al., 2022). Hence, it is imperative to properly monitor and treat surface and irrigation waters (Lee et al., 2017; Melaram et al., 2022). Official guidelines to regulate cyanotoxin levels in irrigation waters are not yet available, although the World Health Organization (WHO) and the European Union have suggested a drinking water limit value of 1 μg L^{-1} for MC-LR (European Union, 2020; WHO, 2020a). WHO also contemplates a limit value of 0.7 μg L^{-1} for CYN (Chorus et al., 2021; WHO, 2020b).

Conventional water treatment for CyanoHABs, *e.g.*, use of copper-based algicides or mechanical mixing and circulation, are expensive and not always technically feasible, especially in rural areas where untreated surface water with critical levels of cyanotoxins might be used for crop irrigation (Redouane et al., 2023). In such situations, nature-based solutions like constructed wetlands (CWs) are being explored as an inexpensive alternative to remediate cyanotoxin-contaminated water (Bavithra et al., 2019). These engineered systems mimic natural wetlands, which both purify water by assimilating nutrients, and degrade pollutants (Kadlec and Wallace, 2008). To this end, experimental CW microcosms have demonstrated an effective elimination of MC-LR (>90%) (Bavithra et al., 2019; Cheng et al., 2022; Thyssen et al., 2024; Wang et al., 2022b). Moreover, studies in our research group indicate removal of CYN up to 50% in CW mesocosms operated in batch conditions (Thyssen et al., 2024), and up to 95% and 98% for MC-LR and CYN respectively when operated in continuous mode (Martinez i Quer et al., 2023).

The underlying mechanisms for cyanotoxin removal, as well as the general microbial ecology of CWs, are far from understood (Farooq et al., 2023). In terms of cyanotoxin biodegradation, most studies focus on bacteria, with one bacterial MC-LR-biotransformation pathway already described (Bourne et al., 2001, 1996; Wang et al., 2018). Although there is no literature available on fungal MC-LR degradation in CWs, some fungal species such as *Trichaptum abietinum* or *Trichoderma citrinoviride* have been shown to remove MC-LR below limits of detection at laboratory conditions (Jia et al., 2012; Mohamed et al., 2021, 2014). Fungal MC-LR-biotransformation routes are unknown. However, glutathione S-transferase (GST), a detoxifying enzyme involved in phase II metabolism, may be relevant in this respect (Esterhuizen-Londt et al., 2017). Fungi are known for their general ability to degrade recalcitrant compounds by the activity of various exoenzymes with a broad substrate specificity (Esterhuizen-Londt et al., 2017; Spennati et al., 2021). Fungal proteases and peroxidases could prove useful in the biodegradation of cyanotoxins (Akhtar and Mannan, 2020). Bacterial and fungal enzymatic pathways for CYN transformation are unknown.

Information on the presence and ecology of fungi in CWs is very scarce (Usharani, 2019), as are their abilities to degrade pollutants (Hu et al., 2021a, 2022). To the best of our knowledge, there are no reports on fungal contribution to cyanotoxin removal in CWs. Therefore, the present study aimed at investigating; a) the effect of MC-LR and CYN exposure on the structure of CW fungal communities using an experimental design where various CW parameters were taken into consideration, and b) the potential of CW fungi to biotransform MC-LR and CYN. It was hypothesized that the fungal community composition would be influenced by cyanotoxin exposure, namely enhancing the abundance of taxa capable of tolerating/degrading the toxins; and that some CW fungal isolates would be able to transform MC-LR and CYN by enzymatic pathways.

2. Materials and methods

2.1. Cyanotoxin extraction and analysis by LC-MS/MS and LC-QTOF

Two cyanobacterial strains were used for production of cyanotoxins, *Microcystis aeruginosa* LEGE 91094 and *Chrysosporum ovalisporum* LEGE x-001, which produce MC-LR and CYN, respectively. The strains were obtained from the Blue Biotechnology and Ecotoxicology Culture Collection (LEGE-CC) and maintained in liquid Z8 medium optimized for cyanobacterial growth (Kotai, 1972).

Cyanotoxins were extracted from the cultures using a three-phase sequential extraction method without previous purification, as previously described (Thyssen et al., 2024). The cyanobacterial extracts were lyophilized (Ole Dich Instrument makers, Denmark) to remove methanol, resuspended in 10 mL of water, and filter sterilized (0.2 μm). It should be noted that the extraction procedure was not specific for the selected cyanotoxins. Therefore, the crude extracts contained additional cell metabolites, including other MC and CYN variants.

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 was optimized for the two cyanotoxins as previously detailed (Thyssen et al., 2024). Briefly, 10 μL of water samples were injected into the LC system and separated on a Synergy Polar RP column with water and methanol, both with 0.2% formic acid, as mobile phases. 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.

Suspect screening of known MC-LR and CYN transformation products, as well as chemical fingerprinting were pursued by LC-QTOF. The same vials prepared for the LC-MS/MS were run by 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, United States) was used for the 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 before (Tisler et al., 2021). The samples (10 μL) were injected directly into the LC-QTOF and measured in data-dependent acquisition mode (DDA), with a TOF mass range of 100–1200 Da and 100–1400 Da as previously described (Rodriguez-Dominguez et al., 2021). The samples were measured in positive ionization mode with an electrospray ionization (+5300 V ion spray voltage).

2.2. Effect of CW variables on fungal communities

2.2.1. Experimental setup for CW variables' effect on microbial community

Samples were collected from 28 CW mesocosms from a two-year cyanotoxin-degradation ongoing experiment (Martinez i Quer et al., 2023). Each of the horizontal-flow systems consisted of a 12-L black plastic container (22.5 cm diameter, 29 cm height), with a surface area of 0.03 m^2. A four-cm layer of coarse gravel (ø 8–12 mm, 1900 g) covered the bottom, followed by a geotextile and a 10 cm layer of the corresponding substratum, leaving an effective volume of 1.25 L. The mesocosms were kept in a greenhouse, rain protected but subject to seasonal light and temperature variations.

The experimental variables investigated included: substratum (sand (ø 0.5–1 mm, porosity 37%) or gravel (ø 8–12 mm)); vegetation (*Juncus effusus*, *Phragmites australis,* or no plant); season (sampling in spring (April 2022) and summer (August 2022)); and exposure to cyanotoxins (18 mesocosms were fed with water spiked at 10 μg L^{-1} with both MC-LR and CYN, while 10 mesocosms were used as controls receiving water free of cyanotoxins). Sterile Schott bottles and metal spoons were used to obtain three subsamples (5 cm depth) that were pooled in a single 150-mL sample. Excess water was decanted, and samples were thoroughly homogenized by shaking before freezing at −20 °C until further processed for DNA extraction. Physico-chemical parameters were measured as previously described (Martinez i Quer et al., 2023).

2.2.2. DNA extraction and ITS2 amplicon sequencing

DNA was extracted using the FastDNATM SPIN Kit for Soil (MP Biochemicals), following the manufacturer's instructions with slight modifications for the gravel samples (Martinez i Quer et al., 2023). The extracted DNA was used to build fungal amplicon libraries following a previously described protocol (Sapkota et al., 2022). In brief, the fungal ITS2 region was amplified using the ITS7F and ITS4 primers (Ihrmark et al., 2012). Dual indexing was conducted before pooling of samples, which were paired-end sequenced on an Illumina MiSeq platform using the V2 500 cycle chemistry (Illumina, United States) at Aarhus University Sequencing Center (Roskilde, Denmark). Detailed information for DNA extraction and PCR conditions can be found in supplementary material.

2.2.3. Data treatment and bioinformatics

Analysis of demultiplexed sequence reads was performed using the QIIME 2 pipeline, version 2020.8.0 (Bolyen et al., 2019). The DADA2 plugin was used to denoise, filter chimeras and merge paired-end reads (Callahan et al., 2016). Default settings of Vsearch were used for the taxonomy assignment of amplicon sequence variants (ASVs), relying on the UNITE database for fungi version 9 (Abarenkov et al., 2022; Rognes et al., 2016). All remaining analyses were carried out in the R software environment (R Core Team, 2013), primarily using 'vegan' and 'phyloseq' packages for the diversity-based analyses (McMurdie and Holmes, 2013; Oksanen et al., 2020). ASVs unassigned as fungi at the Kingdom level were discarded together with singletons and doubletons.

For beta diversity, the Bray-Curtis dissimilarity metric was used for Principal Coordinate Analysis (PCoA), and variation partitioning was evaluated by a permutational multivariate analysis of variance (PERMANOVA), using the 'adonis' test from the 'vegan' package (Oksanen et al., 2020). Alpha diversity comparisons (Shannon and Chao1 indexes) were made using the unpaired Wilcoxon test (Kim et al., 2017), while the 'DESeq2' and 'pheatmap' packages were used for differential abundance analysis and the corresponding heat maps (Kolde, 2019; Love et al., 2022). Finally, the 'corrr' package was used to create correlation matrices relying on the Pearson coefficient (Kuhn et al., 2022).

2.3. In vitro biodegradation of MC-LR and CYN

2.3.1. CWs' setup for biodegradation studies

Fungal strains were isolated from four CW mesocosms with a similar design to the ones described in Section 2.2.1 but operated in a growth chamber (Thyssen et al., 2024). Briefly, *Juncus effusus* specimens were planted in two of the CWs, whereas the two others remained unplanted. The mesocosms were filled with sand, operated at unsaturated conditions, and kept indoors at 15 °C with a light/dark cycle of 16/8 h, using a light intensity of 22 μmol photons m^{-2} s^{-1}. The systems were intermittently irrigated with synthetic eutrophic lake water (Table S1) that trickled through the substratum to a collection system at the bottom. These mesocosms had been running for a year, spiked with environmentally relevant concentrations of MC-LR and CYN (up to 200 μg L^{-1}) in a series of experiments focusing on bacterial biodegradation (Thyssen et al., 2024). Hereafter, the CW systems were sampled for the present experimental work, to study CW fungal community and their cyanotoxin-degradation potential.

2.3.2. Isolation and identification of fungi from CWs

Sediment samples were collected from three different points of the mesocosms at a depth of five cm. After homogenizing with sterile water, the supernatants were spread on: a) Potato Dextrose Agar (PDA), and b) synthetic eutrophic lake water agar plates spiked with filter-sterilized MC-LR or CYN to a final concentration of 10 μg L^{-1} (Li and Pan, 2014). Additionally, liquid enrichments were set up by adding 20 mL of sand from the mesocosms into 100 mL of filter-sterilized synthetic water spiked with cyanotoxins to a final concentration of 10 μg L^{-1}. After one week, supernatant aliquots were taken from each bottle and spread on PDA (Li and Pan, 2014).

All media were supplemented with chloramphenicol to a final concentration of 25 μg mL^{-1} and incubated at room temperature in the dark. Fungal colonies from PDA plates were transferred to new PDA plates, without chloramphenicol, to achieve pure isolates. Subsamples of fungal mycelium were scraped off and disrupted with a TissueLyser (OMNI). The DNeasy® Plant Mini QIAGEN kit (Qiagen) was used for DNA extraction, and the ITS region was amplified by PCR using ITS1F and ITS4 primers (Manter and Vivanco, 2007) (see supplementary material for reaction conditions). PCR products were Sanger sequenced (Macrogen) and the resulting sequences were trimmed to remove low-quality sequencing regions using the Geneious Prime software version 2022.2.2. After quality control, high-quality DNA sequences were aligned against the NCBI nucleotide collection (nr/nt) using the BLAST (Altschul et al., 1990), with previously proposed cutoff values to assign fungi at genus and species level (Raja et al., 2017).

2.3.3. Experimental setup for in vitro biodegradation studies

The 28 fungal isolates obtained from the CW mesocosms (Section 2.3.2) were screened on two types of media (Mineral Salts Media and synthetic eutrophic lake water) spiked with CYN and MC-LR to a final concentration of 1 mg L^{-1} each. Based on these preliminary results (Figs. S10 and S11) six isolates were selected for their potential to degrade the cyanotoxins. Mycelium from pure fungal PDA cultures was added to sterile serum bottles containing 30 mL of synthetic eutrophic lake water spiked with CYN and MC-LR to a final concentration of 1 mg L^{-1} each. After incubation on a shaker (150 rpm) for seven days in the dark at 15 °C, the biomass in the liquid cultures was collected and washed twice with a modified Mineral Salts Media with no carbon source (see Table S2 for details). Three mL of each hyphal suspension was inoculated into 120-mL serum bottles containing 30 mL of Mineral Salts Media spiked with MC-LR and CYN to a final concentration of 1 mg L^{-1} each, simulating environmentally relevant concentrations in a severe HCB (Melaram et al., 2022). The experiment was carried out in triplicates, using aluminium foil-capped flasks to enable gas exchange.

In addition to the fungal isolates, a positive, an abiotic, and a heat-inactivated control were also included. The positive control was a well-known MC-LR bacterial degrader strain, *Sphingosinicella microcystinivorans* Y2, previously grown in R2A medium (Park et al., 2001). The abiotic control, used as distinction from the biotic degradation, consisted of media only. Meanwhile, the heat-inactivated control,

introduced to investigate the influence of adsorption on cyanotoxin removal, included the mycelial suspension of one isolate pasteurized at 85 °C for one hour (*Cladosporium cladosporioides*, which had revealed one of the highest MC-LR removals during the preliminary tests (Fig. S10)). All flasks were kept in the dark at 15 °C, on a shaker (150 rpm). Samples were collected under sterile conditions at 0, 3, 24, 48, 96, 144, 240 and 504 h after inoculation. The samples were centrifuged (5 min, 13,000 rpms), and the supernatant was stored at 4 °C until analyzed by LC-MS/MS and LC-QTOF.

2.3.4. Data treatment and statistical analysis for in vitro biodegradation studies

Results from the *in vitro* biodegradation experiments were visualized using R (R Core Team, 2013). MC-LR biodegradation rate constants were calculated using a 1st order kinetics model as previously described (Thyssen et al., 2024). For the statistical analysis, the SPSS Statistics software (IBM Corp., 2022) was chosen, using a p-value <0.05 to determine statistical significance. Initially, Shapiro-Wilk's and Levene's tests were used to investigate the normality and homogeneity of variance of the data, followed by a one-way analysis of variance (ANOVA) and a Tukey's Honest Significant Difference for post-hoc pairwise comparison.

2.3.5. LC-QTOF data treatment

The high-resolution MS data (100–1400 Da) from the fungal biodegradation time series was screened using the software SciexOS (SCIEX), for the only two known MC-LR transformation fungal products conjugates; cysteine (m/z = 1115.5685) and glutathione (m/z = 1301.6326) (Esterhuizen-Londt et al., 2017). Since no standards are available, published molecular formulas and product ion spectra for both products were used for comparison.

Additionally, the high-resolution MS data (100–1200 Da) was processed with MarkerView™ (SCIEX) for the molecular feature extraction (the feature is defined by exact m/z and retention time) based on the method of (Tisler et al., 2021). Briefly, one feature was only considered if present in 3 out of the 3 injected replicate samples with a mass tolerance of 10 ppm and a retention time tolerance of 0.1 min. All features present in methanol, water, standards, abiotic and inactivated samples were filtered when present 10-fold lower than in any other sample. Using the remaining features, PCA with Pareto scaling was performed. For visualization, a score plot of the Log transformed PCA scores data belonging to the two main principal components (PC1 and PC2) for each time point was generated.

3. Results

3.1. Effect of CW variables on fungal communities

Metabarcoding of the ITS2 region resulted in 1,798,123 reads. Excluding non-fungal reads, which comprised nearly 55% of the total sequences, the remaining reads were clustered into 853 ASVs. Fungal communities in both control and treatment samples were dominated by Ascomycota, followed by Basidiomycota and Rozellomycota (Fig. 1). Glomeromycota fungi appear to be more prevalent in the planted systems during the spring (especially in *Phragmites*), compared to a lower abundance in the planted mesocosms during summer. Certain genera belonging to the Mucoromycota phylum were identified in 20% of the systems exposed to cyanotoxins, while they were only represented in one of the controls mesocosms.

The PCoA of the fungal ASVs (Fig. 2A) showed clustering of samples according to the seasons. This was confirmed by a PERMANOVA test, which revealed that approximately 11% of the variation in fungal community structure was explained by seasonality (p-value < 0.001), while only 8% was due to cyanotoxin exposure (p-value < 0.001). By performing separate PCoAs with the spring and summer data subsets, clustering of samples according to vegetation (in spring) and cyanotoxins (in summer) was evident (Fig. 2B and C, respectively).

A PERMANOVA test for each season data set showed that during summer, cyanotoxin exposure explained 24% of the variation, while it only explained 5% in the spring samples (Table 1). The presence of the two plant species was also a strong driver in shaping fungal community structure, explaining 20% of the variation in summer, while the substratum only accounted for 6%. Moreover, we also observed that interaction between plant and cyanotoxin explained 11% of the variation. On the other hand, the spring data uncovered the presence of the two plant species as the main factor contributing to the fungal community structure (22%). While the substratum explained approximately 14% of the difference, the plant-substratum interaction accounted for 11% of the variation (Table 1).

Maintaining the seasonal division, the relationship between fungal

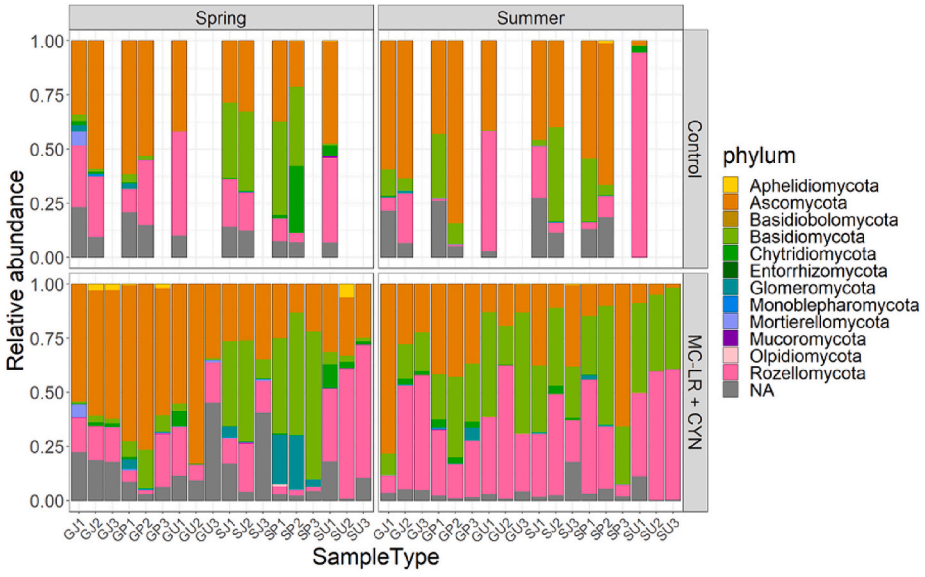

Fig. 1. Relative abundance of fungal taxa in CW samples, according to season and type of cyanotoxin treatment (control in the upper row, and spiked with cyanotoxins bottom row). Suffixes denote: G (gravel), S (sand), J (*Juncus*), P (*Phragmites*), and U (unplanted). The spiked systems (MC-LR + CYN) were run in triplicate, while the control mesocosms were only in duplicates (Control).

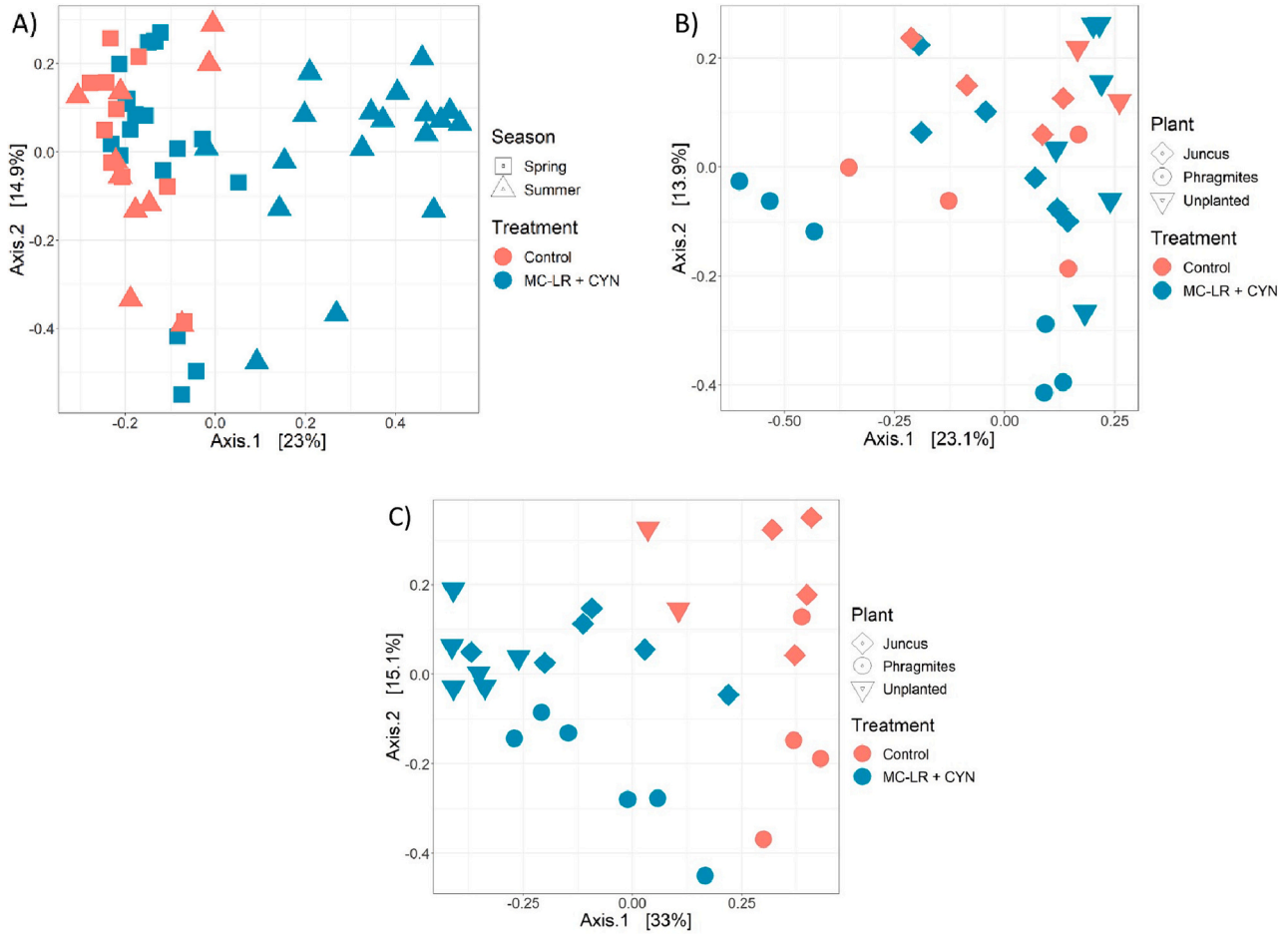

Fig. 2. PCoA plot of the fungal community using Bray-Curtis dissimilarity matrices, showing the distribution of samples based on treatment and season (A). PCoA plot of the spring (B) and summer (C) seasons, showing clustering based on plant species and cyanotoxin treatment as main variables.

Table 1
PERMANOVA test using "adonis" test on Bray-Curtis dissimilarity matrices of the summer and spring data subsets shows the variation in fungal community composition that can be explained by each of the variables and their interactions.

Variables	Effect size in spring (R2)	Effect size in summer (R2)
Cyanotoxin	0.052**	0.235***
Plant	0.223***	0.196***
Substratum	0.14***	0.063**
Cyanotoxin × Plant	ns	0.105**
Cyanotoxin × Substratum	ns	ns
Substratum × Plant	0.111***	ns
Cyanotoxin × Substratum × Plant	ns	ns
Residual	0.344	0.323

ns represents no statistically significant effect.
*** Denotes statistical significance below 0.001.
** Below 0.01.

taxa and physico-chemical parameters or cyanotoxin removal was analyzed based on Pearson coefficients. No significant (p-value > 0.05) positive associations between the identified fungal taxa and cyanotoxin removal were found in the spring campaign (Fig. 3A). However, several significant (p-value < 0.05) correlations were observed in the summer (Fig. 3B). For example, the genus *Rhizophagus* was positively correlated with MC-LR removal, while *Myrmecridium* showed a positive correlation with both MC-LR and CYN removal. Additionally, certain genera such as *Fusarium* and *Rhizophagus* also tended to have positive interactions with

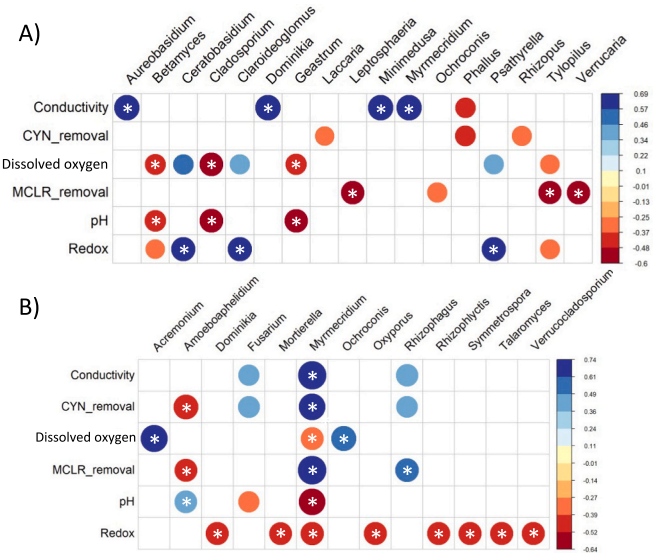

Fig. 3. Correlation matrices based on Pearson coefficients between fungal genera and physico-chemical parameters for the spring (A) and summer (B) sampling campaigns (p-value < 0.1). * denotes significant correlations (p-value < 0.05) between fungal genera and the selected variables. Vermilion tones designate negative correlations, while blue are positive.

CYN removal (p-value < 0.1) (Fig. 3B). Physico-chemical parameters and cyanotoxin removal data are detailed elsewhere (Martinez i Quer et al., 2023).

3.2. In vitro biodegradation of MC-LR and CYN

After characterizing the native fungal communities of the present CWs exposed to cyanotoxins, the aim was to study if some of the fungal isolates could degrade cyanotoxins. For this, a second setup was used and twenty-eight fungal isolates, assigned to 13 genera and 17 different species, were obtained (Table S3). This included several potential endophytes, saprotrophs, yeasts (*e.g.*, *Rhodotorula* and *Debaryomyces*) and other soilborne fungi. Plant pathogens, such as *Fusarium*, as well as two mycoparasites belonging to the *Cosmospora* genus were also identified. Among these isolates, widely distributed within the fungal clade, six strains were picked for the biodegradation experiment (*Cladosporium cladosporioides, Dydimella sp., Furcasterigmium furcatum, Penicillium brevicompactum, Rhodotorula diobovata, Trichoderma sp.*) based on preliminary screening results (Figs. S10 and S11).

Degradation of MC-LR was observed for all six selected isolates (Fig. 4), ranging from 20 to 35% removal within a week. The positive control (*S. microcystinivorans* Y2) removed all MC-LR (>99.9%) within three hours. There were negligible differences between the concentrations of the abiotic and heat-inactivated controls, whereas for all the isolates, the final MC-LR concentration was significantly lower that the starting concentration (ANOVA, p-value < 0.05). In the case of *R. diobovata*, a significant removal of MC-LR was observed after three hours, while *C. cladosporioides* and *Trichoderma* required 24 h (Fig. 4). These three isolates presented the highest degradation rate constants (Table 2).

Compared to the negative controls, *F. furcatum* and *P. brevicompactum* were able to significantly reduce the concentration of MC-LR within two days. No significant difference could be observed for the *Dydimella* strain until day four. Moreover, none of the two known MC-LR transformation fungal product conjugates, cysteine (m/z = 1115.5685) and glutathione (m/z = 1301.6326) (Esterhuizen-Londt et al., 2017), were detected. None of the fungal isolates nor *S. microcystinivorans* Y2 were able to degrade CYN (Fig. 5).

Table 2
MC-LR degradation rate constants (day^{-1}) for all isolates during *in vitro* biodegradation experiments.

Isolate	Rate constant (day^{-1})
Rhodotorula diobovata	0.06
Cladosporium cladosporioides	0.07
Dydimella sp.	0.04
Trichoderma sp.	0.06
Furcasterigmium furcatum	0.05
Penicillium brevicompactum	0.05

3.2.1. Temporal environmental chemical fingerprint of the cultures using LC-QTOF data

The complexity of the LC-QTOF dataset was summarized by performing a PCA analysis, shown in Fig. 6 and Fig. 7 and Figs. S5-S9. In the first 48 h of the experiment, the distribution of the cultures was mainly clustering on the left side without displaying any trend (Figs. S5-S9), indicating a randomized similar metabolic environment among the fungal isolates. At 96 h, *R. diobovata* cultures, together with the positive control, started having a distinct metabolic environment in comparison to the rest of the samples (Fig. 6). Two days after (144 h), the cultures were divided along the PC1 axes, differentiating two groups: *Trichoderma sp., Didymella sp.* and the positive control *versus R. diobovata*, *P. brevicompactum*, *C. cladosporioides* and *F. furcatum* (Fig. S8). After 240 h, *P. brevicompactum*, *F. furcatum* and *Trichoderma sp.* start diverging from the positive control, *R. diobovata, C. cladosporioides* and *Didymella sp.* (Fig. 7). At the end of the experiment, the cultures grouped together again (Fig. S9). Overall, this group differentiation over time matched the difference in the observed MC-LR removal rates.

4. Discussion

4.1. Effect of CW variables on fungal communities

The present study reinforced our expectation that CW systems support rich fungal communities, where Ascomycota species appeared to dominate (Fig. 1), like previously observed (Kharitonov et al., 2022; Ping et al., 2019). This group, as well as the Basidiomycota phylum, are known to include many endophytic and saprotrophic fungi, which are

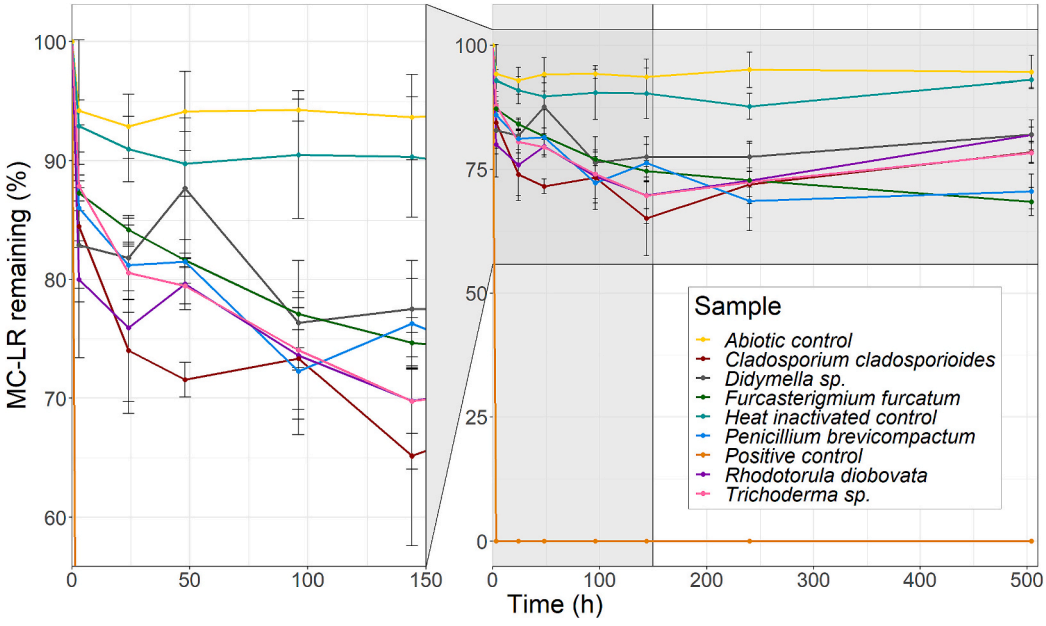

Fig. 4. *In vitro* biodegradation of MC-LR by six fungal isolates and a positive control (*S. microcystinivorans* Y2, known MC-LR degrader strain). An abiotic and a heat-inactivated control were included. The bars indicate the standard deviation at each time point (n = 3). A close-up window of the first week's results is shown to the left.

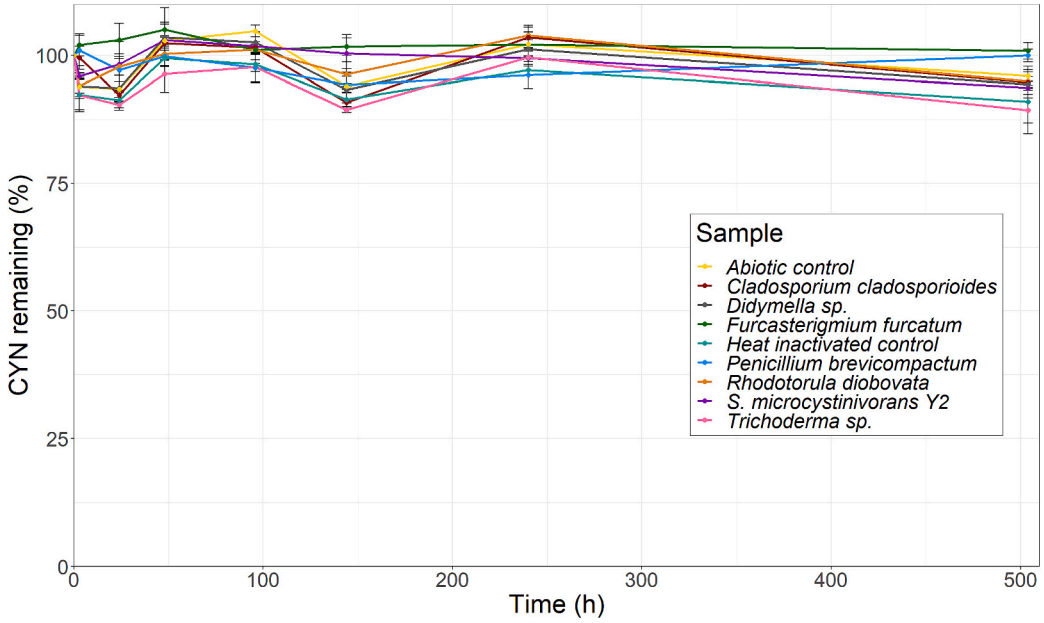

Fig. 5. *In vitro* biodegradation of CYN by six fungal isolates and *S. microcystinivorans* Y2. An abiotic and a heat-inactivated control were included. The bars indicate the standard deviation at each time point (n = 3).

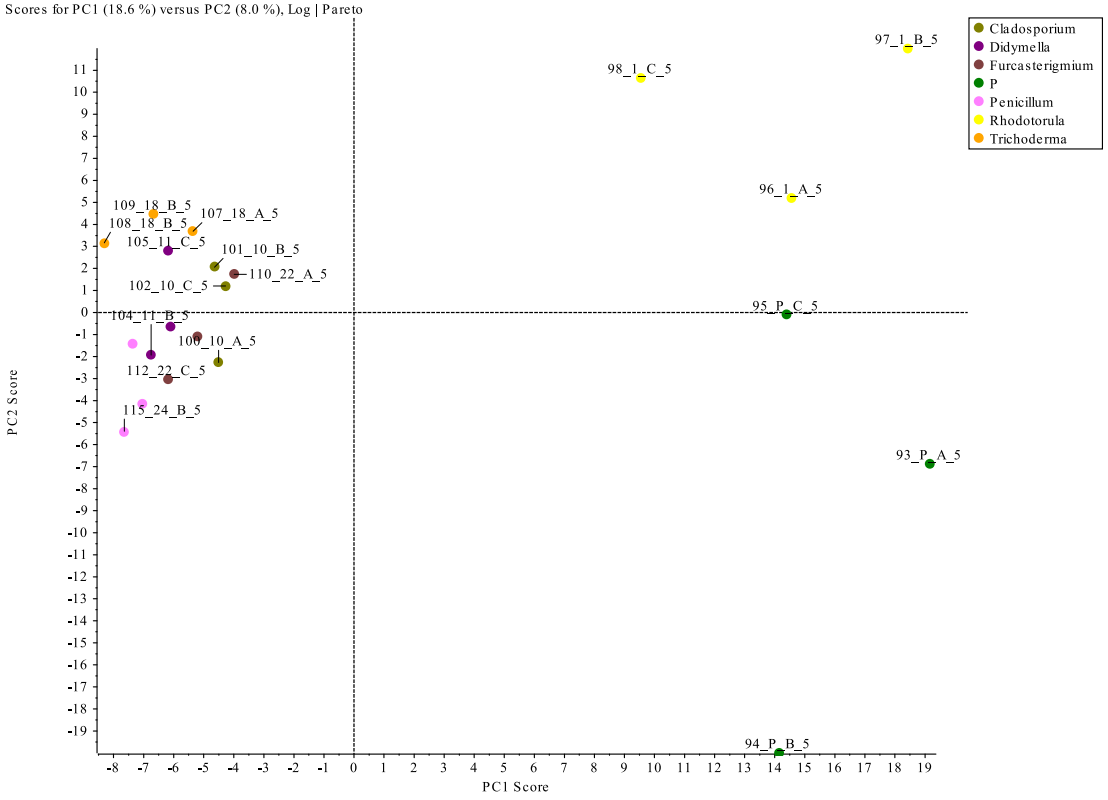

Fig. 6. PCA showing the two principal components PC1 and PC2. In color the different fungal strains triplicates after 96 h of incubation. Number of features = 264. In the top right corner, a legend with the different fungal strains: Cladosporium (*Cladosporium cladosporioides*), Didymella (*Didymella* sp.), Furcasterigmium (*Furcasterigmium frucatum*), P (positive control), Penicillum (*Penicillum brevicompactum*), Rhodotorula (*Rhodotorula diobovata*), Trichoderma (*Trichoderma* sp.).

prominent in CWs (Kharitonov et al., 2022; Vasundhara et al., 2019). Another interesting phylum identified in the amplicon sequencing was Mucoromycota, known to establish associations with plants. This includes *M. hiemalis,* already described to degrade MC-LR (Balsano et al., 2017; Bonfante and Venice, 2020; Esterhuizen-Londt et al., 2017).

Despite some studies suggesting the potential of certain plant species like *P. australis, J. effusus* or *Arundo donax* to remove MC-LR in CWs (Bavithra et al., 2019; Cheng et al., 2022; Martinez i Quer et al., 2023), other studies with *J. effusus* did not reveal this effect (Thyssen et al., 2024). Therefore, no conclusive evidence has been found on the ability of phytoremediation to remove MC-LR, and further research is needed to draw stronger conclusions in this respect. However, even if vegetation

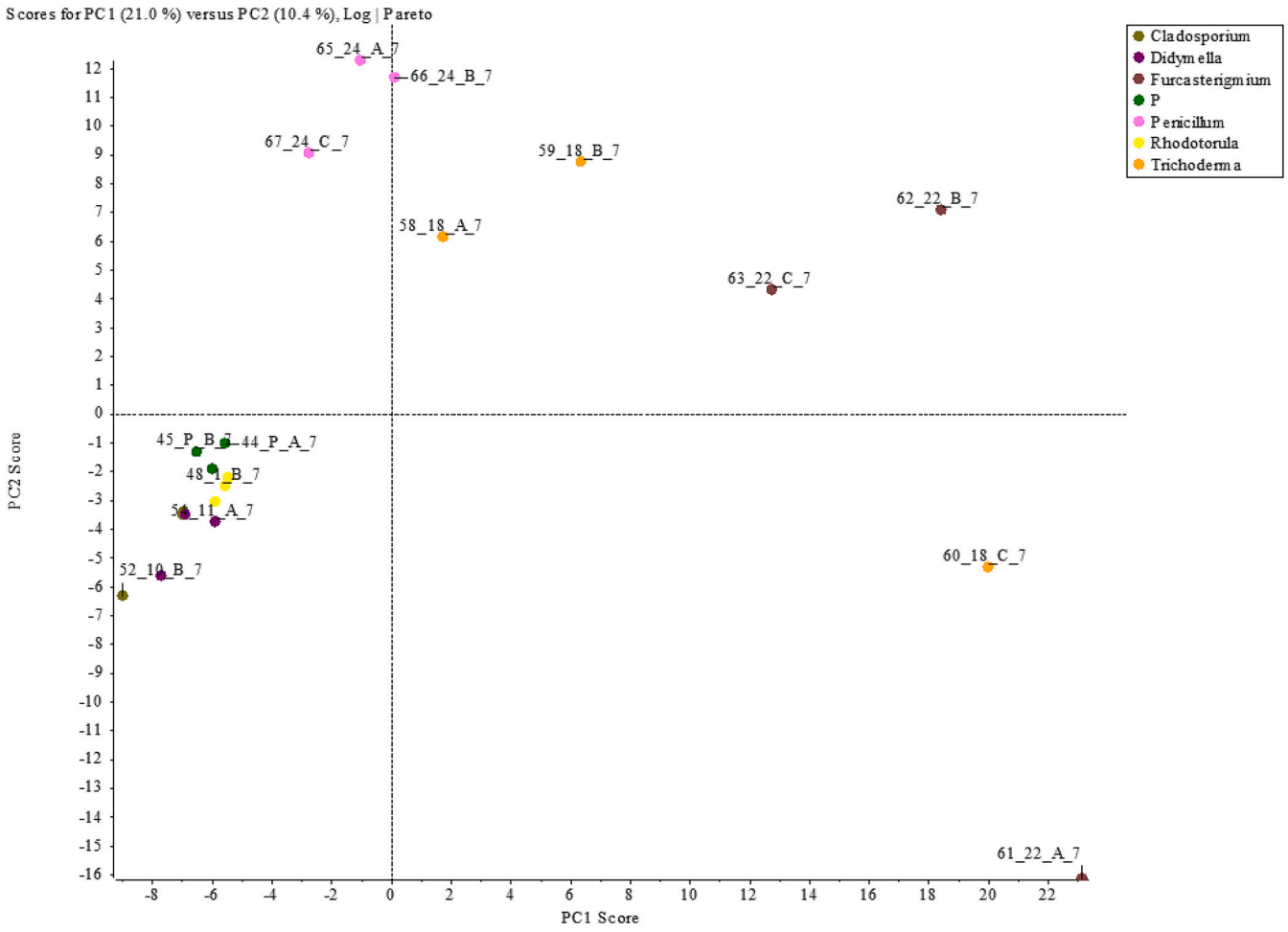

Fig. 7. PCA showing the two principal components PC1 and PC2. In color the different fungal strains triplicates after 240 h of incubation. Number of features = 426. In the top right corner, a legend with the different fungal strains: Cladosporium (*Cladosporium cladosporioides*), Didymella (*Didymella* sp.), Furcasterigmium (*Furcasterigmium frucatum*), P (positive control), Penicillum (*Penicillum brevicompactum*), Rhodotorula (*Rhodotorula diobovata*), Trichoderma (*Trichoderma* sp.).

does not directly contribute to the removal of these toxins, endophytic fungi can still prove beneficial. By protecting the plants against stress, these fungi help maintain a healthy environment, which can increase pollutant biodegradation by promoting the growth of a wider variety of fungi and bacteria in the rhizosphere (Dang et al., 2021; Sapkota et al., 2022; Zhang et al., 2016).

The effect of plants on the diversity of fungal communities (Figs. S1 and S2), showed that mesocosms with *J. effusus* exhibited a significantly (p-value <0.05) higher alpha diversity than the unplanted mesocosms. With *P. australis*, this difference was only statistically significant during the summer, possibly because it is perennial with a dormant stage during winter, contrary to *J. effusus* (Great Lakes Commission, 2022; Wang et al., 2021). The dormancy may affect the fungal community, as the metabolic activity of the rhizosphere decreases. This effect has not been sufficiently studied in CWs, particularly in relation to fungi, but there are some indications of change in microbial activity during plant dormancy (Zhang et al., 2022).

Plant influence on the fungal community may be due to the additional ecological niche that the rhizosphere provides, with various plant-fungal interactions (Voronina and Sidorova, 2017; Zhang et al., 2016). The results from the PERMANOVA analysis of both seasons confirm this theory, as the presence of the different plant species was identified as one of the main factors shaping fungal communities (Fig. 2, Table 1). Contrary to what was previously observed for the bacterial community (Martinez i Quer et al., 2023), in the present study the plant effect on shaping the fungal community was stronger in spring. This indicates that potential competition for specialized niches is dynamically occurring between both kingdoms.

Another group of valuable plant-associated fungi that were identified through metagenomics in the present study was the Glomeromycota (Fig. 1), which form arbuscular mycorrhizal symbioses with plants (Taylor et al., 2015). Due to their nature as obligate symbionts, the isolation of these fungi by conventional plate cultivation is not feasible (Calheiros et al., 2019; Ferrol and Lanfranco, 2020). Members of Glomeromycotan fungi, also known as Arbuscular Mycorrhizal Fungi (AMF), often improve plant resistance to biotic and abiotic stresses, and are proposed to be involved on the removal of pharmaceuticals and other organic contaminants in CWs (Hu et al., 2021b; Xu et al., 2018, 2016). To the best of the authors' knowledge, no research has been done on AMF from CWs exposed to cyanotoxins. However, it has been suggested that AMF not only enhance the uptake of organic pollutants by plants, but they also improve the conditions in the rhizosphere, which can promote microbial activity in this region and boost biodegradation of pollutants (Hu et al., 2022, 2021b).

Besides vegetation; seasonality, substratum type and cyanotoxin exposure were also identified as drivers of fungal community structure (Fig. 2, Table 1), confirming the first hypothesis. The toxin impact was more pronounced during summer, which could be influenced by the temperature difference. High temperatures (20–30 °C) allow for faster metabolic reactions (Li et al., 2009), which could force fungi to compete for the available carbon sources, making community changes more evident, as species that can gain energy by transforming cyanotoxins

will have an advantage. Previous *in vitro* studies have reported positive effects on the fungal degradation of xenobiotics with increasing temperature (up to 28 °C), attributed to higher solubilities of the compounds and enhanced cellular activity of the fungi (Dhiman et al., 2022).

As for substratum type, the effect on fungal community structure was more pronounced in the spring samples, similar to what was observed for the bacterial community of the same systems (Martinez i Quer et al., 2023). This could indicate that non-optimal environmental conditions (lower temperature) could make the impact of the substratum more evident. Due to distinct particle sizes, gravel and sand systems exhibit differences in oxygen concentration, retention times and surface area available for microbial growth (Ji et al., 2022; Sharma and Brighu, 2013). These properties may have an impact on the microbial community depending on the aeration and growth requirements of each organism. Furthermore, these factors could also affect plant development (Ji et al., 2022), which may explain the significant interaction between plant and substratum seen in spring (Table 1).

4.2. In vitro biodegradation of MC-LR and CYN

Previous studies on constructed and natural wetlands revealed the presence of many saprotrophic and endophytic fungal species (Kharitonov et al., 2022; Park et al., 2021; Xie et al., 2020), which is in agreement with the majority of the isolates obtained from the CW mesocosms in the present study. Species from the *Trichoderma, Fusarium, Cladosporium, Penicillium, Rhodotorula* and *Mortierella* genera have already been described in CWs (Kharitonov et al., 2022; Usharani, 2019), and were also identified through amplicon sequencing in this study. *Sarocladium strictum,* isolated from the planted systems, has been found in natural wetlands in association with the roots of *P. australis* (Wang et al., 2022a), although ITS sequencing didn't reveal its presence in the current project. To the best of our knowledge, there are no previous reports of *Umbelopsis*, *Didymella*, *Cosmospora*, *Furcasterigmium*, *Debaryomyces* and *Akanthomyces* in CWs. Except for *Umbelopsis*, these genera were not reflected in the results from the community analyses of the CWs in the present study. *Umbelopsis isabellina* has been proposed as a candidate for bioremediation due to its heavy metal and organic pollutant removal abilities in liquid culture (Janicki et al., 2018) and studying its role in CWs could unlock new applications for this species.

The six isolates used in the biodegradation experiments accomplished a 20 to 30% MC-LR removal, which is similar to the reported 37% removal by the aquatic fungus *Mucor hiemalis* (Esterhuizen-Londt et al., 2017). Yet, other species, such as *Trichaptum abietinum, Schizophyllum commune* and *Trichoderma citrinoviride,* have shown complete MC-LR removal (total microcystins for *T. citrinoviride*) (Jia et al., 2012; Mohamed et al., 2021, 2014). In this last case, toxin removal by adsorption to the cell wall was ruled out based on the results from *T. citrinoviride*'s heat-inactivated mycelium (Mohamed et al., 2014). The negligible differences between the abiotic and heat-inactivated controls of the present study (Fig. 4) further support the non-significant contribution of adsorption to MC-LR removal. However, it must be considered that the inactivation of mycelia by heat could lead to alterations in the surface of the cells, interfering with the results (Qiu et al., 2018). To overcome this issue, experiments using sodium azide have been proposed (Santos et al., 2020), but not implemented in the current study. Thus, adsorption seems negligeable but should be further confirmed in future studies.

Although fungal pathways for biodegradation of MC-LR are unknown, several studies suggest the importance of GST and antioxidant enzymes for the biotransformation of this cyanotoxin (Jia et al., 2012; Mohamed et al., 2021, 2014). Since microcystins are large water-soluble molecules, their passive diffusion into living tissues is unlikely. Therefore, it has been proposed that some fungi possess an active transport mechanism to translocate MC-LR into the cells, reinforced by measured bioaccumulation of this toxin in *M. hiemalis* (Esterhuizen-Londt et al., 2017). Furthermore, as shown in Fig. 4, the MC-LR concentration in the last two time points (10 days and 3 weeks respectively) increases slightly for four of the isolates. One possible explanation for this is the prolonged culture time without a readily available carbon source, except for the cyanotoxin extract. While fungi were active, MC-LR accumulated and was transformed, but when there was no more substrate growth, the mycelia started to die, releasing part of the accumulated toxin. A similar trend was found in biodegradation experiments with *M. hiemalis* when using the crude cyanotoxin extract (Esterhuizen-Londt et al., 2017). To discard bioaccumulation as the only (or primarily) mechanism for MC-LR removal, future tests should include the determination of the intracellular concentration of the toxin as previously suggested (Esterhuizen-Londt et al., 2017).

Inside the cells, the GST enzyme has been suggested to catalyze the conjugation of glutathione or cysteine to MC-LR, a phase II metabolic reaction for detoxification (Balsano et al., 2017; Esterhuizen-Londt et al., 2017; Morel et al., 2009; Osman et al., 2018). MC-LR conjugates with glutathione or cysteine have been verified in biodegradation studies using *M. hiemalis* when exposed to pure MC-LR, while only the glutathione conjugate was found in the fungal pellets when using crude cyanotoxin extract (Esterhuizen-Londt et al., 2017). Presently, fungal mycelium was not analyzed and the screening of both conjugates in the aqueous phase was unfruitful. Thus, it is not clear if the conjugates might only be found intracellularly due to detoxification of MC-LR.

Considering the chemical fingerprinting data (Figs. 6–7 and Figs. S5-S9), at the start of the experiment samples revealed no distinct grouping, indicating that no differentiative influence on the extracellular metabolome was observed while extracellular degradation was initiated. After 96 h, the metabolome fingerprint started diverging (Fig. 6) and kept diverging through 144 h (Fig. S8) and 240 h (Fig. 7). This coincides with the time frame where the extracellular MC-LR concentration reached a stable level in all cultures. The most surprising result is that the environmental metabolome of the positive control, even though it is assumed to have a different degradation pathway (*mlrA-D* gene), was subject to a similar development as the fungal cultures. These findings could support the absence of conjugates in the aqueous phase, indicating that the MC-LR metabolism occurs intracellularly, and potentially only secondary metabolites might be released to the medium.

Overall, *in vitro* biodegradation results seemed to indicate that CW mesocosms provided appropriate conditions for microbial interactions that were not met in the *in vitro* tests. Thus, mesocosms (showing maximum removals of 95% and 98% for MC-LR and CYN, respectively (Martinez i Quer et al., 2023)) might allow the necessary cofactors to be produced and used efficiently by various microorganisms for cyanotoxin degradation. The lack of such a microbial synergy and cofactors could explain the incomplete degradation of MC-LR *in vitro*. Joint activity of a microbial consortium has been shown for the degradation of other organic pollutants (Zhang and Zhang, 2022), and in other studies comparing *in vitro* with CW mesocosm studies (Thyssen et al., 2024). The action of such complex consortium interactions must be confirmed with additional experiments, possibly by investigating not only fungal, but also bacterial-fungal and plant-fungal interactions. Moreover, valuable insights can be gained by exploring the cyanotoxin degradation capabilities within CW systems of the six isolates from the present study, as their environmental niche (*e.g.* endophytic lifestyle) might influence their cyanotoxin removal potential.

4.3. Fungal candidates with potential for cyanotoxin removal

Among the strains used for the *in vitro* removal experiments, only *Trichoderma* could be associated with known MC-LR degraders (Mohamed et al., 2014). On the other hand, *C. cladosporioides* has been suggested to inhibit the growth of *M. aeruginosa* during CyanoHABs (Wymer et al., 2022), and has also been identified to produce proteases useful for degradation of organic compounds (Jakovljević and Vrvić, 2018). Thus, it could prove valuable to investigate these two microorganisms further. *R. diobovata* is a nitrophilous yeast which has been

proposed as a bioremediation agent for its ability to efficiently assimilate ammonium, nitrate and nitrite (Civiero et al., 2018). Moreover, other species from this genus are known to degrade polycyclic aromatic hydrocarbons (PAHs) through a process thought to include glutathione S-transferase (GST) (Ide-Pérez et al., 2020; Martínez-Ávila et al., 2021). Therefore, additional studies could allow the implementation of bioaugmentation strategies based on this yeast to control CyanoHABs (Bavithra et al., 2019; Civiero et al., 2018).

In accordance with the correlation analyses of the CW mesocosms variables (Fig. 3), *Myrmecridium* and *Rhizophagus* genera might also be important for cyanotoxin removal in CWs. Members of the first genus (*e.g. Myrmecridium schulzeri*) have been isolated and identified as endophytic fungi associated with the roots of *P. australis* (Salimi et al., 2023). Moreover, unrelated studies have revealed *M. schulzeri* as a producer of extracellular hydrolytic and proteolytic enzymes (Bezerra et al., 2015), which could be involved in the degradation of CYN and MC-LR.

The AMF *Rhizophagus* showed positive correlations with the disappearance of both cyanotoxins, although only the interaction with MC-LR was statistically significant (p-value < 0.05). The large intraspecific genome diversity within strains of this genus enhances their resilience to environmental conditions and facilitates their adaptation to various plant hosts (Chen et al., 2018). Although a direct impact of AMF on the removal of organic pollutants such as PAHs by increased peroxidase activity has been proposed (Joner et al., 2001), most studies have suggested an indirect effect. The suggested mechanisms are stimulation of the microbial community of the rhizosphere, enhancement of plant growth, improved bioaccumulation within the roots, and increased biodegradation activity of roots and their associated microbiome (Gao et al., 2011; Joner et al., 2001; Rajtor and Piotrowska-Seget, 2016). In particular, *R. irregularis* has been suggested to benefit its hosts by improving plant tolerance to PAHs (Lenoir et al., 2017). Since traditional isolation methods are not able to culture AMF, the combination of isolation and molecular methods used in the present study proved useful for exploring different candidates for cyanotoxin removal.

Even though the correlation was not statistically significant, *Fusarium* correlated positively with CYN removal (p-value < 0.1). Several studies have focused on the ability of this genus to degrade various xenobiotics, including aromatic compounds (Zhu et al., 2020). Gene-function predictions and enzyme-activity assays have uncovered a variety of extracellular enzymes in *Fusarium* (Huy et al., 2017; Zhu et al., 2020), which might facilitate degradation of complex molecules such as CYN. Manganese peroxidases are particularly interesting enzymes widely produced by members of this genus (Huy et al., 2017; Nidhi et al., 2020; Zhao et al., 2023). In a preceding study in our lab, cyanotoxins also enhanced manganese-oxidizing bacteria from the *Hypomicrobium* genus (Martinez i Quer et al., 2023). Given that previous research has proposed the unspecific reaction of CYN with reactive manganese species as a possible bacterial degradation pathway (Martínez-Ruiz et al., 2020), this group of enzymes deserves further exploration.

5. Conclusion

Fungal DNA amplicon sequencing revealed that seasonality (spring or summer), cyanotoxin exposure (MC-LR + CYN or control), vegetation (unplanted, *J. effusus* or *P. australis*) and substratum (sand or gravel) were drivers of fungal community structure in CW mesocosms. Since the arbuscular mycorrhizal fungus *Rhizophagus* and the endophyte *Myrmecridium* correlated positively with MC-LR and CYN removal, their involvement in cyanotoxin degradation deserves further examination. *In vitro* biodegradation experiments revealed that six fungal strains (*Rhodotorula diobovata, Cladosporium cladosporioides, Dydimella sp., Trichoderma sp., Furcasterigmium furcatum* and *Penicillium brevicompactum*), isolated from CW mesocosms, were able to remove approximately 25% of the MC-LR. The involvement of GST in fungal biotransformation of MC-LR was proposed to happen intracellularly. On the other hand, CYN was not removed by axenic cultures of any of the fungi. Ultimately, the present project, focusing on fungal communities in CWs, is a starting point to understand the ecology of fungi in CWs and their contribution to cyanotoxin removal. To deliver more precise mechanistic information on micropollutant biotransformation, the combined chemical fingerprinting and ITS amplicon sequencing approach may be further developed.

CRediT authorship contribution statement

Ángela González Álvarez: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Alba Martinez i Quer:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Lea Ellegaard-Jensen:** Writing – review & editing, Validation, Software, Methodology, Data curation. **Rumakanta Sapkota:** Writing – review & editing, Validation, Software, Methodology, Data curation. **Pedro N. Carvalho:** Writing – review & editing, Writing – original draft, Validation, Resources, Project administration, Methodology, Funding acquisition, Data curation, Conceptualization. **Anders Johansen:** Writing – review & editing, Writing – original draft, Validation, Supervision, Resources, Project administration, Investigation, 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. Pedro N Carvalho, 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 NCBI database, BioProject ID PRJNA1019934 (https://www.ncbi.nlm.nih.gov/sra/PRJNA1019934).

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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.2024.172590.

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