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Eco-technologies for treatment of water contaminated with cyanobacteria and cyanotoxins Guna Bavithra

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Guna Bavithra
Doctoral degree in Environmental Science and Technology
Chemistry and Biochemistry Department
Faculty of Sciences of the University of Porto
2025

PhD

Eco-technologies for treatment of water contaminated with cyanobacteria and cyanotoxins

Guna Bavithra

Thesis carried out as part of the doctoral degree in Environmental Science and Technology,

Chemistry and Biochemistry Department, Faculty of Sciences, University of Porto

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All the corrections determined by the jury, and only those, were made.

The Supervisor,

Porto, __09__/__01__/_2026____

Dedicated to my mother and my grandmother

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Finally, to myself.

Resumo

O trabalho apresentado nesta tese teve como objetivo potenciar o conhecimento e investigar soluções para melhorar as eco-tecnologias de tratamento da água, através da valorização de recursos locais, da utilização de diferentes espécies de plantas, e da avaliação da eficácia e dos mecanismos envolvidos na remoção de cianotoxinas. A proliferação de cianobactérias tóxicas está a aumentar a uma escala sem precedentes, a nível global, impondo grande pressão sobre os recursos de água doce. As toxinas associadas a estes microorganimos - cianotoxinas -representam riscos ecológicos e de saúde pública significativos, particularmente em contextos descentralizados, onde não é possível a utilização de tecnologias convencionais de tratamento da água, por serem altamente dispendiosas e pouco sustentáveis. As Soluções baseadas na natureza (SBN), como as zonas húmidas construídas (ZHC), surgem como tecnologias alternativas para o tratamento da água podendo, inclusivamente, ser utilizadas para remover contaminantes químicos tais como as cianotoxinas.

Nesta tese investigaram-se ZHC de fluxo vertical sub-superficial, à escala laboratorial, para a remoção de duas cianotoxinas prevalentes no meio aquático - microcistina-LR (MC-LR) e cilindrospermopsina (CIN). As funções dos vários componentes dos sistemas ZHC, incluindo o substrato e as espécies vegetais, bem como o microbioma associado foram avaliados. A investigação iniciou-se com um estudo de comparação das capacidades de adsorção química, entre vários tipos de resíduos agrícolas e substratos de ZHC de referência, como carvão vegetal e argila expandida (LECA). A partir deste estudo, selecionaram-se os materiais mais adequados para o estudo subsequente do funcionamento das ZHC.

Para avaliar a adsorção de cianotoxinas aos materiais de resíduos agrícolas relevantes e substratos de ZHC de referência, os materiais adsorventes foram testados em estudos de imersão, começando com uma triagem inicial da adsorção de MC-LR e CIN em dois períodos de contacto. As percentagens de adsorção observadas foram mais elevadas para o carvão vegetal (>86% MC-LR e >98% CIN) e LECA (78% MC-LR e 80% CIN), e mais baixas para a casca de arroz (<10% em MC-LR e CIN), cortiça (<10% em MC-LR e CIN) e areia (<26% em MC-LR e CIN). Consequentemente, os dois adsorventes com melhor desempenho (carvão vegetal e LECA) foram caracterizados quanto à cinética de adsorção com sete períodos de contacto (1, 2, 4, 8, 16, 24 e 48 h), recorrendo a modelos cinéticos de pseudo-primeira e segunda ordem. As isotermas de adsorção também foram determinadas com três concentrações diferentes de cada cianotoxina (0,1, 1 e 10

mg/L) e três massas diferentes de adsorvente (0,2, 0,5 e 1 g) num período de equilíbrio de 24 h. Os dados experimentais foram ajustados aos modelos de isoterma de adsorção relevantes — Langmuir, Freundlich, Dubinin–Radushkevich (D-R) e Temkin — para compreender a capacidade da adsorção de MC-LR e CIN nos materiais seleccionados. O modelo cinético de pseudo-segunda ordem ajustou-se aos resultados de adsorção de ambas as cianotoxinas (R^2: 0,94 – 0,99), sugerindo que a taxa de adsorção foi limitada pela adsorção química. Os modelos de Freundlich e D-R ajustaram-se à adsorção das cianotoxinas no biocarvão e LECA, devido à capacidade superior de adsorção associado à heterogeneidade significativa da superfície, porosidade (que também foi confirmada pela análise microscópica da superfície usando SEM/EDS) e comportamento de adsorção multicamadas destes materiais. Observou-se que a capacidade de adsorção é maior no carvão vegetal (Kf: MC-LR = 0,05, CIN = 0,16) que em LECA (Kf: MC-LR = 0,02, CIN = 0,01).

As experiências em ZHC foram realizadas utilizando nove microcosmos montados, à escala laboratorial, com camadas sequenciais de cascalho, LECA, carvão vegetal e areia. Na camada superficial de areia, foram transplantadas *Canna indica* ou *Sparganium erectum* ou uma policultura de ambas as espécies (plantas adultas). O ensaio experimental consistiu no tratamento de água de nascente contaminada artificialmente com concentrações ambientalmente relevantes de MC-LR e CIN (2-15 µg/L) durante uma semana, ao longo de ciclos de 10 semanas. Os parâmetros físico-químicos da água (pH, carência química de oxigénio (DQO), potencial oxidação-redução (ORP) e concentração de nutrientes) e o estado das plantas foram monitorizados ao longo do tempo, mostrando que os sistemas funcionaram corretamente, simulando uma ZHC, mesmo na presença de cianotoxinas. Foram determinadas as taxas de remoção de cianotoxinas da água, e a retenção no substrato e a acumulação das toxinas nas plantas, nas diferentes condições de tratamento. O microbioma dos substratos das ZHC também foi investigado. Em todos os sistemas, a remoção de MC-LR da água excedeu 96 %, enquanto a remoção de CIN variou entre 70-98 % ao longo do período experimental, sendo maior na presença de *C. indica*. Não foi observada acumulação de cianotoxinas nos tecidos vegetais, embora não se possa excluir a degradação das toxinas dentro das plantas. Além disso, a *C. indica* em ZHCs da policultura exibiu adaptação ao stress. A retenção no substrato foi baixa (< 8 %), indicando uma potencial biodegradação das toxinas. Durante o ensaio, a exposição às cianotoxinas induziu mudanças marcantes nas comunidades microbianas presentes no substrato, especialmente na rizosfera (substrato em contacto com as raízes das plantas). Embora os genes da via *mlr* associados à degradação da microcistina não tenham sido

detectados no substrato em contacto com as raízes das plantas, no substrato sem contacto com as raízes das plantas foram detectadas bactérias degradadoras da MC-LR (*Sphingomonas*, *Novosphingobium* e *Shinella*) e bactérias oxidantes de Mn (*Hyphomicrobium*) potencialmente capazes de degradar CIN. As ZHC com *C. indica* apresentaram enriquecimento em microorganismos promotores de crescimento das plantas (*Leifsonia, Terrabacter, Altererythrobacter*) e múltiplos taxa associados à degradação da MC-LR por via não dependente dos genes *mlr* (*Sphingorhabdus* e *Porphyrobacter*).

No geral, os resultados demonstram que as ZHC de fluxo vertical sub-superficial que incorporam carvão vegetal, LECA e *C. indica* podem tratar de forma eficiente, e adaptativa, águas contaminadas com cianotoxinas, com potencial aplicação na gestão descentralizada da água. Os resultados fornecem uma base para estudos em escala piloto sobre resiliência do sistema, evapotranspiração e segurança da reutilização de efluentes, promovendo a integração das SBN nas estratégias de proteção de águas superficiais.

Palavras-chave: Cianotoxinas, Cianobactérias, Soluções baseadas na natureza, Ecotecnologias, Zonas húmidas construídas, Adsorventes de baixo custo

Abstract

The research reported in this thesis aims to give knowledge and steps for improving NBS through the valorisation of local resources, testing of different plant species, their efficacy in cyanotoxin removal, and the mechanisms involved in cyanotoxin removal. Cyanobacterial harmful algal blooms are increasing worldwide, intensifying pressure on freshwater resources. The associated cyanotoxins pose significant ecological and public health risks, yet current treatment technologies often fail to provide sustainable and integrated remediation, particularly in decentralised contexts. Nature-based solutions (NBS), such as treatment wetlands (TWs), offer promising alternatives but remain underutilised for cyanotoxin removal.

This thesis investigated vertical subsurface flow TWs, at lab scale, for the removal of two prevalent cyanotoxins- microcystin-LR (MC-LR) and cylindrospermopsin (CYN), evaluating substrates and plant species roles, and associated microbial processes. Initially, it also evaluated a variety of agro-waste materials and benchmark NBS substrates such as biochar and light expanded clay aggregates (LECA) for sorption capability, with the most suitable being selected for the TWs.

To assess the cyanotoxin sorption of the relevant agro-waste materials and benchmark NBS substrates, the sorbent materials were tested in batch studies, beginning with an initial screening of MC-LR and CYN sorption with two contact periods. Here the sorption percentages observed were higher for biochar (>86% MC-LR and >98% CYN) and LECA (78% MC-LR and 80% CYN), lower for rice husk (<10% in MC-LR and CYN), cork (<10% in MC-LR and CYN), and sand (<26% in MC-LR and CYN). Consequently, the two best performing sorbents (biochar and LECA) were further characterized for their sorption kinetics with seven contact periods (1, 2, 4, 8, 16, 24, and 48 h) and using the pseudo first-order and second-order kinetic models. Sorption isotherms were also determined with three different cyanotoxin concentrations (0.1, 1, and 10 mg/L) and three different sorbent masses (0.2, 0.5, and 1 g) at the equilibrium period of 24 h. The experimental data was fitted with the relevant sorption isotherm models- Langmuir, Freundlich, Dubinin–Radushkevich (D-R), and Temkin, to understand the favourability of MC-LR and CYN sorption onto biochar and LECA. The pseudo second-order kinetic model fit the sorption of both cyanotoxins onto biochar and LECA (R^2: 0.94 – 0.99), suggesting that the sorption rate was limited by chemisorption. The Freundlich and D-R model fit the cyanotoxins' sorption onto biochar and LECA, owing their superior sorption capacity to significant surface heterogeneity, porosity (which was also confirmed by microscopic

surface analysis using SEM/EDS) and multilayer sorption behaviour. The sorption capacity was observed to be higher for biochar (Kf: MC-LR = 0.05, CYN = 0.16) than LECA (Kf: MC-LR = 0.02, CYN = 0.01).

The TW study was carried out by setting up nine lab-scale TW microcosms with layers of gravel, LECA, biochar, and sand. In the sand, *Canna indica*, *Sparganium erectum*, and a polyculture of both were transplanted (adult plants). Spring water spiked with environmentally relevant concentrations of MC-LR and CYN (2-15 µg /L) was treated in the TW for one week, over a course of 10-week cycles. Water physicochemical parameters (pH, chemical oxygen demand (COD), oxidative-reductive potential (ORP), and nutrients) and plant status were monitored over time, showing systems were working properly, simulating a TW, even in the presence of cyanotoxins. Cyanotoxin water removal rates, cyanotoxins substrate matrices' retention and plant accumulation in the different treatment conditions were determined. The microbiome of TW substrates was also investigated. Across all systems, MC-LR water removal exceeded 96 %, while CYN removal varied between 70- 98% over the experimental period, being higher in the presence of *C. indica*. No cyanotoxin accumulation was observed in plant tissues, although toxins degradation within plants cannot be ruled out. Besides, *C. indica* in polyculture TWs exhibited stress adaptation. Substrate retention was low (< 8 %), pointing to potential toxins biodegradation. Over the period of the experiment, there was a marked shift in microbial communities present in the substrate, especially in the plant rhizosphere (substrate in contact with plant roots). While genes from the known microcystin degrading mlr pathway were not detected in TW substrate, the bulk substrate (not in contact with plant roots) hosted MC-LR–degrading taxa (*Sphingomonas*, *Novosphingobium* and *Shinella*) and Mn-oxidising bacteria (*Hyphomicrobium*) potentially capable of CYN degradation. TWs with *C. indica* was enriched in plant-growth promoting microbes (*Leifsonia, Terrabacter, Altererythrobacter*) and multiple taxa linked to mlr-independent degradation (*Sphingorhabdus* and *Porphyrobacter*).

Overall, these findings demonstrate that vertical subsurface flow TWs (VSSF-TWs) incorporating biochar, LECA, and *C. indica* can efficiently and adaptively treat cyanotoxin-contaminated waters, with potential application in decentralised water management. The results provide a foundation for pilot-scale studies on system resilience, evapotranspiration, and effluent reuse safety, advancing the integration of NBS into freshwater protection strategies.

Keywords: Cyanotoxins, Cyanobacteria, Nature-based Solutions, Eco-technologies, Treatment Wetlands, Low-cost sorbents

சுருக்கம் (Abstract):

இந்த ஆய்வறிக்கையில் விவரிக்கப்படும் ஆராய்ச்சி, உள்ளூர் வளங்களை மதிப்பூட்டல் (valorisation), தாவர இனங்களின் பரிசோதனை, அவற்றின் நச்சு நீக்க திறன் மற்றும் நீலப்பாசி நச்சு நீக்கத்தில் ஈடுபடும் செயல்முறைகள் ஆகியவற்றை அடிப்படையாகக் கொண்டு இயற்கை அடிப்படையிலான தீர்வுகளை (Nature-based Solutions – NBS) மேம்படுத்த அறிவு மற்றும் நடைமுறைகளை வழங்குவதை நோக்கமாகக் கொண்டது. உலகளவில் நீலப்பாசி சார்ந்த தீங்கு விளைவிக்கும் பாசிப்பூக்கள் (Harmful Algal Blooms) அதிகரித்து, குடிநீர் வளங்களுக்கு பெரும் அழுத்தத்தை ஏற்படுத்துகின்றன. இவற்றுடன் தொடர்புடைய சியானோடாக்ஸின்கள் (cyanotoxins) முக்கியமான சூழலியல் மற்றும் பொதுச் சுகாதார ஆபத்துகளை உருவாக்குகின்றன. ஆனால், தற்போதைய சிகிச்சை நுட்பங்கள் நிலையான மற்றும் ஒருங்கிணைந்த சீரமைப்பை, குறிப்பாக மையப்படுத்தப்படாத சூழல்களில், வழங்க முடியாமல் தவறுகின்றன. செயற்கை ஈரநிலங்கள் (Treatment Wetlands – TWs) போன்ற NBS தொழில்நுட்பங்கள் நம்பிக்கைக்குரிய மாற்றாகக் கருதப்படுகின்றன; இருப்பினும் சியானோடாக்ஸின் நீக்கத்தில் அவை போதுமான முறையில் பயன்படுத்தப்படவில்லை.

இத்தீர்வுக் கட்டுரையில், இரு பொதுவான சியானோடாக்ஸின்கள்—மைக்ரோசிஸ்டின்-LR (MC-LR) மற்றும் சிலிண்ட்ரோஸ்பெர்மோப்சின் (CYN)—இவற்றின் நீக்கத்திற்காக ஆய்வக அளவிலான செங்குத்துச் சிதறல் ஓட்ட செயற்கை ஈரநிலங்கள் (Vertical Subsurface Flow TWs – VSSF-TWs) ஆய்வு செய்யப்பட்டது. இதில், அடித்தளம் (substrates), தாவர இனங்கள் மற்றும் தொடர்புடைய நுண்ணுயிர் செயல்முறைகள் ஆகியவற்றின் பங்கு மதிப்பீடு செய்யப்பட்டது. தொடக்கத்தில், வேளாண்மை கழிவுப் பொருட்கள் மற்றும் உயிர்த்தூள் (biochar), LECA (Light Expanded Clay Aggregates) போன்ற தரச் சான்று பெற்ற NBS அடித்தளங்கள் சோர்ப்ஷன் திறன் (sorption capacity) பரிசோதிக்கப்பட்டன. அதில் சிறந்த செயல்திறன் கொண்ட உயிர்த்தூள் மற்றும் LECA தேர்ந்தெடுக்கப்பட்டன.

பகுதி-அளவிலான சோர்ப்ஷன் ஆய்வுகளில், உயிர்த்தூள் மற்றும் LECA அதிக திறன் காட்டின (>86% MC-LR, >98% CYN; LECA: 78% MC-LR, 80% CYN). அதேசமயம் நெல் தோல், கார்க், மணல் போன்றவை மிகக் குறைவான திறன் (<26%) காட்டின. சோர்ப்ஷன் கினெடிக்ஸ் (sorption kinetics), சோர்ப்ஷன் ஐசோதெர்ம்கள் (Langmuir, Freundlich, Dubinin–Radushkevich, Temkin) ஆகியவைகளின் மூலம் தரவுகள் பகுப்பாய்வு செய்யப்பட்டன. உயிர்த்தூள் அதிக சோர்ப்ஷன் திறனை (Freundlich Kf: MC-LR = 0.05, CYN = 0.16) கொண்டிருந்தது, LECA (MC-LR = 0.02, CYN = 0.01) விட.

ஆய்வக அளவிலான 9 TW மைக்ரோகாஸ்ம்கள், பல அடுக்கு வடிவமைப்பில் (கற்கள், LECA, உயிர்த்தூள், மணல்) அமைக்கப்பட்டன. மணலில் *Canna indica*, *Sparganium erectum* மற்றும் இரண்டின் கலவையும் நட்டனர். சுற்றுச்சூழலுக்கு தொடர்புடைய அளவிலான MC-LR மற்றும் CYN கலந்த நீர், 10 வார சுழற்சிகளில் சிகிச்சை செய்யப்பட்டது. நீரின் இயற்பொருள்-வேதியியல் அளவுகள் மற்றும் தாவர நிலை தொடர்ந்து கண்காணிக்கப்பட்டது. அனைத்து முறைகளிலும் MC-LR நீக்க விகிதம் >96% ஆக இருந்தது; CYN நீக்கம் 70–98% வரை மாறுபட்டது, குறிப்பாக *Canna indica* இருந்தபோது அதிகமாக இருந்தது. தாவர திசுக்களில் நச்சு சேமிப்பு காணப்படவில்லை; ஆனால் தாவர உள் சிதைவின் வாய்ப்பு மறுக்கப்படவில்லை.

நுண்ணுயிர் சமூகங்களின் (microbial communities) பகுப்பாய்வில், MC-LR சிதைவிற்கு உதவும் *Sphingomonas, Novosphingobium, Shinella* போன்றவை, மேலும் CYN சிதைவிற்கு வாய்ப்புள்ள Mn-ஆக்ஸிடைசிங் *Hyphomicrobium* கண்டறியப்பட்டன. *Canna indica* கொண்ட TW-களில் தாவர வளர்ச்சியை ஊக்குவிக்கும் நுண்ணுயிர்கள் (*Leifsonia, Terrabacter, Altererythrobacter*) மற்றும் mlr-சார்பற்ற சிதைவிற்கு தொடர்புடைய பல இனங்கள் (*Sphingorhabdus, Porphyrobacter*) வளமாகக் காணப்பட்டன.

மொத்தத்தில், உயிர்த்தூள், LECA மற்றும் Canna indica ஆகியவற்றை உள்ளடக்கிய செங்குத்துச் சிதறல் ஓட்ட TW-கள், சியானோடாக்ஸின் கலந்த நீரைச் சிறப்பாகவும் தகுந்த விதமாகவும் சிகிச்சை செய்யும் திறன் கொண்டன. இத்தீர்வுகள், மையப்படுத்தப்படாத நீர்

மேலாண்மையில் (decentralised water management) பயன்படக்கூடியவை என்பதை உறுதிப்படுத்துகின்றன. இந்த முடிவுகள், பெரிய அளவிலான பைலட் ஆய்வுகளுக்கான அடித்தளத்தை வழங்குகின்றன; குறிப்பாக, முறைமைகளின் உறுதித்தன்மை, ஆவியாவுதல், மற்றும் சிகிச்சைக்குப் பிந்தைய நீர் பாதுகாப்பு ஆகியவற்றை மதிப்பீடு செய்யும் ஆய்வுகளுக்கு வழிவகுக்கின்றன. இதன் மூலம் NBS முறைகளை குடிநீர் பாதுகாப்புத் திட்டங்களில் ஒருங்கிணைக்க முன்னேற்றம் ஏற்படும்.

முக்கிய சொற்கள்: சியானோடாக்ஸின்கள், நீலப்பாசி, இயற்கை அடிப்படையிலான தீர்வுகள், சூழல் நுட்பங்கள், செயற்கை ஈரநிலங்கள், குறைந்த செலவிலான சோர்பண்டுகள்

Table of Contents

List of Tables

List of Figures

List of Abbreviations

AC	ACTIVATED CARBON
AD	ANAEROBIC DIGESTOR
CyanoHAB	CYANOBACTERIAL HARMFUL ALGAL BLOOM
CYN	CYLINDROSPERMOPSIN
COD	CHEMICAL OXYGEN DEMAND
CG	CONTROL WITH CYANOTOXIN CONTAMINATED DEIONIZED WATER AND GRAVEL
Chl	CHLOROPHYLL
D-R	DUBININ-RADUSHKEVICH
EDI	ESTIMATED DAILY INTAKE
ET	EVAPOTRANSPIRATION
FM	FRESH MASS
FWS	FREE WATER SURFACE
GV	GUIDELINE VALUE
HAB	HARMFUL ALGAL BLOOM
HRT	HYDRAULIC RETENTION TIME
HSSF	HORIZONTAL SUBSURFACE FLOW
LC-MS/MS	LIQUID CHROMATOGRAPHY COUPLED WITH TANDEM MASS SPECTROMETRY
LDA	LINEAR DISCRIMINANT ANALYSIS
LECA	LIGHT EXPANDED CLAY AGGREGATE
LEfSe	LINEAR DISCRIMINANT EFFECT SIZE
LOD	LIMIT OF DETECTION
LOQ	LIMIT OF QUANTIFICATION
MC	MICROCYSTIN
MC-LR	MICROCYSTIN-LR
MOB	Mn-OXIDISING BACTERIA
MSL	MULTI-SOIL LAYERING
NBS	NATURE-BASED SOLUTION
NMDS	NON-METRIC MULTIDIMENSIONAL SCALING
OP	OLIVE PULP POMACE PELLETS
ORP	OXIDATIVE REDUCTIVE POTENTIAL
PP	PROTEIN PHOSPHATASE
PSII	PHOTOSYSTEM II

QTOF	QUADRUPOLE-TIME-OF-FLIGHT
RM	REPEATED MEASURES
SPE	SOLID-PHASE EXTRACTION
TDI	TOLERABLE DAILY INTAKE
TSS	TOTAL SUSPENDED SOLIDS
TW	TREATMENT WETLAND
VSSF	VERTICAL SUBSURFACE FLOW
WEF	WATER-ENERGY-FOOD NEXUS
WHO	WORLD HEALTH ORGANIZATION

Chapter 1. Introduction

1.1. Background

1.1.1. Harmful Algal Blooms and Cyanotoxins as an Emerging Pollutant of Concern

Although harmful algal blooms (HABs) might seem like a modern issue, the group of organisms responsible for these blooms and their associated toxins are naturally found in the environment. Historical records indicate that occurrences of HABs and related food poisoning have been documented for centuries [1]. However, the recent increase in HAB occurrences is linked to the combined pressures of an increasing global population, food production, urban development, agriculture and aquaculture expansion. Specific actions contributing to the increase in HABs are the nutrient inputs to water bodies (leading to eutrophication), construction of dams and storage of water in artificial lentic systems that promote cyanobacteria growth. Cyanobacterial harmful algal blooms (CyanoHABs), specifically, have significantly escalated in recent decades and are expected to persist in this trend. A primary concern about CyanoHABs is the production of harmful secondary metabolites, known as cyanotoxins, which can lead to fatal hepatic, gastrointestinal, and neurological disorders, in addition to causing dermal disease upon exposure [2]. In addition to rising temperatures and eutrophication, rising atmospheric CO2 has also shown to stimulate bloom forming cyanobacteria [3]. This results in a global rise in the occurrence, duration and intensity of cyanotoxins in freshwater bodies and reservoirs [4]. A recent study using predictive freshwater modelling found earlier onset and prolongation of CyanoHABs even in the temperate climatic zones under climate change conditions [5]. Another study evaluating the algal bloom frequency in lakes world-wide from 2003 to 2022, observed a marked increase in bloom frequency in more than half of the affected lakes of the sub-tropical region, specifically after the year 2015 [6]. This emphasises how important it is to acknowledge and deal with this expanding environmental issue considering climate change.

Microcystis aeruginosa is the predominant bloom-forming species among harmful cyanobacteria, and microcystin-LR (MC-LR), mostly produced by it, is the most toxic chemical congener of microcystins (MCs) [7]. MCs are common cyanotoxins produced by several cyanobacterial genera including *Microcystis, Anabaena, Planktothrix (Oscillatoria), Nostoc, Hapalosiphon, Anabaenopsis*; but *Microcystis* occurs in most climatic zones [8]. MCs are present in multiple congeners that have a similar cyclic

structure composed by seven amino acid residues, cyclo-(D-Ala[1])-X[2]-(D-MeAsp[3])-Z[4]-Adda[5]-(D-Glu[6])-Mdha[7]. Figure 1 shows the chemical structure of MC-LR. The X[2] and Z[4] amino acid residues were used to name MC congeners because these two amino acids of the MCs at position 2 and 4 are the most variable by substitution and account for the diversity in MCs. More than 300 microcystin derivatives have been identified to date, with the degree of methylation, peptide sequence, and toxicity varying in natural environments, the most prevalent amino acids are MC-Leucine[2]-Arginine[4](MC-LR), MC-Arginine[2]-Arginine[4](MC-RR), MC-Leucine[2]-Phenylalanine[4](MC-LF), and MC-Leucine[2]-Alanine[4](MC-LA). Among these prevalent MCs, MC-LR is commonly utilised as a model for MC investigations because of its widespread distribution and high toxicity [9]. The primary cytotoxicity of MCs is due to the inhibition of Protein Phosphatases 1 and 2a (PP1 and 2a), leading to several harmful effects. MC toxicity can have both acute and chronic effects on mammals. Acute MC intoxication above levels of 10 µg/L in mammals can cause hepatocyte necrosis and haemorrhage, with serious cases resulting in death. Long-term exposure to low microcystin concentrations has also been linked to tumour promotion [10], [11], [12].

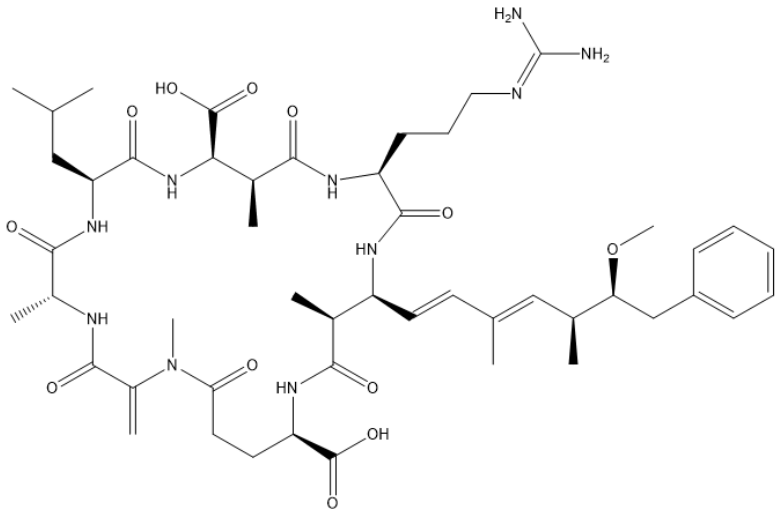
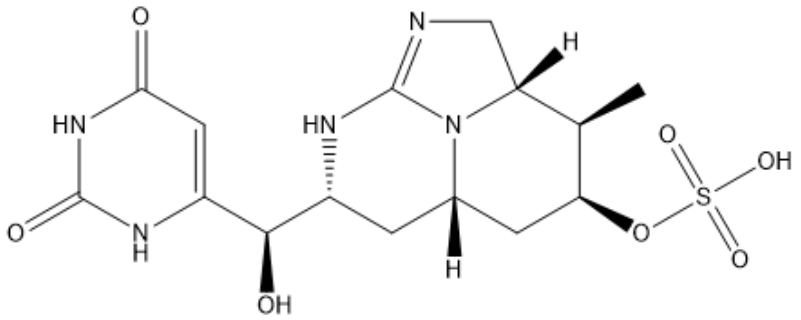


Figure 1.The chemical structure of MC-LR was generated using ChemDraw Professional 20.0.0.41.

Nonetheless, another cyanotoxin called cylindrospermopsin (CYN) has been identified as a cause for concern due to the invasive nature of its main producers, *Cylindrospermopsis raciborskii* and *Aphanizomenon ovalisporum* (now known as *Chrysosporum ovalisporum*), which creates favourable conditions for these cyanobacteria to proliferate in water supplies. Previously thought to proliferate only in

more tropical regions of the world, CYN-producing strains are now being detected in more temperate climates [13], indicating that these toxin-producing cyanobacteria are highly adaptable, which has important global concern. CYN is resistant to high temperatures, sunlight, and extreme pH. CYN, unlike MCs, is frequently released from cells into the surrounding water. It bioaccumulates, notably in species at the bottom of the food chain such as gastropods, bivalves, and crustaceans [14]. Furthermore, the world's changing climate will create more favourable conditions for these cyanobacteria to proliferate in freshwater systems used for agriculture and production of drinking water. Figure 2 shows the chemical structure of CYN. CYN is a highly biologically active alkaloid, consisting of a tricyclic guanidine and hydroxymethyluracil. It is a very water-soluble zwitterionic compound. There are two structural variations of CYN reported: deoxycylindrospermopsin, and 7-epicylindrospermopsin. It has hepatotoxic, general cytotoxic, and neurotoxic properties and is regarded as a possible carcinogen [14]. The toxicity of cylindrospermopsin is facilitated by the suppression of glutathione, protein synthesis, and cytochrome P450, with the uracil moiety and the hydroxyl group at C7 being essential for its toxic effects. CYN poisoning in mammals can result in damage to the liver, kidneys, thymus, and heart [14], [15], [16].



[(4S,5R,6S,8S,10R)-10-[(R)-(2,4-dioxo-1H-pyrimidin-6-yl)-hydroxymethyl]-5-methyl-2,11,12-triazatricyclo[6.3.1.04,12]dodec-1-en-6-yl] hydrogen sulfate

Figure 2. The chemical structure of CYN was generated using ChemDraw Professional 20.0.0.41.

With increasing CyanoHABs and rise in water scarcity, there is a serious and increasing public health threat, with both acute and chronic health effects. Exposure occurs through multiple pathways, with vulnerable groups (children, elderly, immunocompromised and individuals with frequent or occupational exposure) at heightened risk. Exposure routes include ingestion, dermal contact and inhalation (aerosols) through contaminated water (recreational/ drinking), and food [17]. While most documented cases of human sickness resulting from recreational exposure to cyanobacteria are minor and self-resolving,

symptoms can be wide ranging from gastrointestinal or respiratory symptoms to irritant effects, fever, headache, and fatigue [18]. However, depending on the cyanotoxin, exposure rate, dose and body-weight, health consequences can also be severe and potentially fatal; demonstrating the diverse chemical structures and toxic routes of cyanotoxins [2]. Animals, on the other hand, are more likely to be fatally poisoned, not necessarily because they are more susceptible to cyanotoxins, but rather because they may lack access to treated water or have limited ability to obtain alternative drinking water supplies when their primary source is contaminated [19]. Furthermore, cyanotoxins may potentially reach humans via the food chain through bio-accumulation in aquatic organisms and food crops [20], [21]. For instance, a recent study in Egypt revealed the presence of microcystins (MCs) in irrigation water sources and in vegetables cultivated with this water, with estimated daily intake (EDI) levels above the WHO guideline for the toxin [22]. The most recent WHO guideline values (GVs) state that the GV for chronic exposure (lifetime) of drinking water is 1 µg/L for MC-LR and 0.7 µg/L for CYN, the GV for short-term drinking water is 12 µg/L for MC-LR and 3 µg/L for CYN, and the GV for recreational waters is 24 µg/L for MC-LR and 6 µg/L for CYN. There is still no provided GV for MC-LR or CYN in irrigation waters, however the tolerable daily intake (TDI) for chronic MC-LR exposure is 0.04 µg/kg/day and CYN is 0.03 µg/kg/day. It is to be noted that these values are provisional, as there are insufficient data available to derive a GV for MC variants except MC-LR, and for CYN there are limited studies, lack of in-vivo data and unclear role of metabolites, especially related to potential genotoxicity [23], [24]. While there are other equally potent neurotoxic cyanotoxins such as anatoxin-a and saxitoxins, they are less frequent. Currently, the most frequently detected in the environment are microcystins (MCs) and cylindrospermopsin (CYN) [14]. With the escalation of unsustainable agricultural practices, lack of irrigation water in regions facing water stresses, the and the lack of irrigation water guidelines, the mandatory use of surface waters contaminated with cyanotoxins for agricultural irrigation is very likely [25], [26]. This subsequently increases the risk of cyanotoxin contaminated crops and agricultural products, posing a direct threat to public health [25]. Moreover, with the globalisation of the food market and marked increase in the import of processed foods, it makes it a global health concern [27]. So, prevention or removal of cyanotoxins from surface water is needed, ultimately protecting human health.

1.1.2. Need for Nature- based Solutions (NBS) as De-centralized Water Treatment Option for treating Surface Waters Contaminated with Cyanotoxins

The UN-Water SDG 6 Indicator Report (2024) states that agriculture was responsible for 72% of global freshwater withdrawals in 2021, followed by industry (15%), and the service sector (13%) [27]. It also expresses the interlinkage between the level of water stress and food security, with high levels of water stress restricting access to clean water and adequate sanitation, which in turn undermines the nutritional outcomes and quality of food production. So, water shortage not only translates to thirst, but also a threat to public health and hunger. Coping with these water strategies in unsustainable manners has led to worse environmental catastrophes. For instance, diversion of the main rivers that flowed into the Aral Sea for purposes of irrigation led to eventually drying up of the sea [28]. Excessive drilling of borewells and extraction of groundwater has led to rapid decrease in the water column [29].

In light of the growing water management challenges and the public health risk concerns with regards to cyanotoxins mentioned in section 1.1, sustainable and decentralized treatment options are increasingly critical. The current water-energy-food nexus (WEF) signifies the strong interdependencies between water, energy and food sectors; emphasizing the need for their integrated management to ensure sustainable resource use and avoid unintended consequences. Moreover, the adoption of centralizsed water, and expensive technologies has demonstrated ineffective in tackling the intricate water-related issues resulting from increased urbanization in developing countries [30].

In this context, communities and cities are adopting Nature-Based Solutions (NBS) and de-centralized water treatment options to tackle the WEF nexus. NBS leverages natural ecosystem functions and services to address complex environmental and societal challenges sustainably. They are executed by various units or initiatives customised to local settings, including- habitat/ ecosystem protection and restoration (for e.g., forest conservation, wetland rehabilitation), urban green infrastructure (for e.g., green roofs), hybrid or engineered solutions (for e.g., treatment wetlands (TWs),, bioswales), sustainable management, and habitat implementation (for e.g., agroforestry, native habitat creation in cities) [31], [32]. NBS offers advantages in ecological restoration, climate change adaptation and mitigation, social well-being, and economic savings, rendering them essential tools for tackling urbanisation and climate concerns globally. For example, treatment wetlands TWs, an eco-technology used to reduce nutrient and pollutant loads in water, improves flood management by increasing natural water storage

and infiltration capacity, reduces green-house gas emissions; as well as enhances local landscapes through green spaces that provide recreational and aesthetic value. They also provide cost savings in construction, maintenance and implementation compared to conventional water treatment infrastructure. Their appeal mainly lies in these extensive range of services they provide and their affordability; moreover, they play a crucial role in the regeneration of water for environmental reintegration and can also provide social benefits [33], [34].

De-centralized water treatment options refer to systems that treat contaminated waters (including wastewaters)or wastewater close to the point of use, rather than centralized treatment options that rely on extensive networks of pipes and pumps to transport water over long distances. This approach offers several advantages including reduced infrastructure, flexibility and adaptability in terms of capacity and treatment methods, sustainability and cost-effectiveness.

Given the nature of CyanoHABs, de-centralized water treatment options would be an apt approach for minimizing their risks and harmful outcomes. The most ideal solution would be to limit the discharge of nutrients into waterbodies, that would prevent algae blooms, but the current regulations in place are insufficient. With the advent of increasing biological data sets, AI, machine learning tools, and the construction of comprehensive prediction models, understanding the occurrence of cyanobacterial HABs (CyanoHABs) and preventing them should become easier. However, there is still a long way to go in this regard, considering the limitations related to disparate data sets, lack of data standardization, and major gaps in our understanding of the conditions that trigger and sustain cyanoHABs over time [35]. While we wait for new models to be tailored to expedite HAB monitoring and forecasting broadly to enhance the security of freshwater systems and protect public health, strategies need to be formed for the remediation of cyanoHABs in surface waters.

Current advanced treatment options for cyanotoxin removal from water include membrane filtration, advanced oxidation processes, activated carbon adsorption, chemical disinfection and chemical oxidation [36] (USEPA, 2025). These treatment techniques are expensive and are more suitable in centralized water treatment stations such as drinking water-treatment plants, and do not consider factors such as the cyanobacterial biomass, dissolved and suspended-particulate cyanotoxins. Dissolved air flotation is effective in removing intracellular cyanotoxins without disrupting the cyanobacterial cells, but are only effective for certain cyanotoxins (buoyant

cyanobacteria), and can be energy intensive [37]. Alternatively, cost effective methods such as mechanical harvesting, chemical flocculants and algaecides (copper sulphate, potassium permanganate and chlorine) hydrogen-peroxide, can be moderate to high in effectiveness but in the long run disrupts the ecosystem or harms non-targets. Moreover, mechanical mixing and aeration of large water bodies can also be expensive [38]. In this regard, NBS as decentralized water treatment options seems to have a great potential to deal with the complexities of treating surface waters contaminated with cyanotoxins and cyanobacteria. NBS including TWs, reed beds, and soil infiltration systems, are becoming recognised as efficient decentralised water treatment alternatives [39]. They harness natural processes, renewable energy, or innovative materials to achieve water treatment with minimal ecological disruption.

TWs (also known as constructed wetlands) are a well-established NBS for water treatment that are, although considered near-natural systems, engineered to increase efficiency by different system designs [40]. Some other emerging NBS for water treatment include biofilters, slow sand filters, bio-coagulants, photocatalytic treatment, and solar disinfection. Among these, the most cost-effective and relevant options with regards to the effective removal of cyanobacteria and cyanotoxins from water bodies include TWs [41], [42], multi-soil layering system (MSL) [43], enhanced floating-type biological treatment system [44], and plant-based coagulants [45]. Additionally, researchers have been focussing on the advancement of hybrid NBS that integrate the operational benefits of advanced technologies or physico-chemical treatment systems to address the complex pollution challenges as well as to minimize ecological disruption. However, more research on NBS cyanotoxins removal capacity is still needed [46].

1.2. Knowledge Gaps Addressed in the Thesis

Given that advanced solutions are not accessible in remote or rural areas, and cost-effective decentralized approaches for the removal of cyanotoxins for remote areas are needed, NBS are of increasing interest. NBS needs to be designed to be locally adapted, resource-efficient, and incorporate more and more diversified environmental and natural characteristics into the landscape [47]. Their objective is to effectively and adaptively handle socio-economic and environmental concerns, while offering human well-being, ecosystem services, resilience, and biodiversity advantages. Some of the major limitations of their wide-spread application include significant space requirements,

climatic sensitivity, consistency of treatment efficiency and the lack of standardized policies and regulations. In this thesis, we focus on TWs as the model NBS.

TWs are well established NBS, that have demonstrated efficiency in the treatment of various wastewaters and contaminated waters, including for the removal of micropollutants. They are mainly categorized on their hydrological modes and system configuration, the most common being - free water surface (FWS) wetlands, horizontal subsurface flow (HSSF) wetlands, and vertical subsurface flow (VSSF) wetlands [48], [49].

TWs primary components are substrates, plants, and microbial communities (both in plants' rhizosphere (the area surrounding plant roots) and in the substrate itself). These components work synergistically to remove contaminants from diverse wastewater sources [50].

In terms of cyanotoxin removal, there is limited research on the types of substrates used, preventing deeper comparisons, as well as on the role of different plant species in cyanotoxin removal [38]. Moreover, geographic locations influence the availability of substrate types and plant species to be incorporated in NBS, which makes it particularly difficult to determine plant adaptability to different climatic conditions. For this, it is important to test more substrate types and other native plant species and their performance in TWs with regards to cyanotoxins removal.

1.2.1. Low-Cost Sorbents or Media used in NBS for Cyanotoxin Removal

The integration of sorbents to intensify the performance of water treatment are of increased interest in several categories of NBS [46] Furthermore, the integration of sorbents and biofilters in TWs offers greater benefits than their standalone application where additional issues such as the disposal of spent sorbents arise.

So far, many conventional sorbents such as activated carbons (ACs), zeolites, and clays have been studied for the sorption of cyanotoxins [51], [52]. Their sorption capacity depends on their physico-chemical properties, and generally ACs demonstrated higher sorption capacity of multiple cyanotoxins [52] Other chemically modified sorbents that have been studied for the sorption of cyanotoxins include graphene, iron, and silica-based sorbents [53], [54], [55], [56]**.** However, these cannot be considered for incorporation into NBS as they involve complex and expensive production. Also, most studies have been limited to MC sorption, and information on other cyanotoxins, like CYN, is still lacking.

Nowadays, low-cost sorbents are gaining popularity, namely, new materials, such as carbons from waste or waste by-products from agricultural, household, and industrial sectors [57]**.** Moreover, raw agro-based waste materials without chemical modifications remain unexplored as sorbents. Incorporating sorbents to NBS should be considered not only for enhanced performance, but also for the circularity of resources. This was explored in the thesis, and is detailed more in the introduction of Chapter 2.

1.2.2. Use of Wetland vs Ornamental Species in TWs Treating Cyanotoxins

The selection of plant species in TWs is influenced by several factors, including but not restricted to the geographical availability of plants, their adaptability to climatic conditions, pollutant removal efficiency, the rate of establishment and development in wetland environments, and their competitive capacity against weeds. Most current studies indicate that macrophytes' indirect roles in TWs may far outweigh their direct role (uptake of pollutants) in removal processes [58] It has been established so far that TWs treating cyanotoxins demonstrated better removal efficiencies in the presence of plants than unplanted, by promoting better plant associated microbial population [42], [59]. However, the direct role of plants in the cyanotoxin removal process in the TWs have not been well studied. So far, the following plant species have been studied in TWs treating cyanotoxins- *Arundo donax, Iris pseudacorus, Phragmites australis,* and *Juncus effusus* [38]. While removal rates of cyanotoxins were evaluated in these studies, the role of plants in the TW removal mechanisms have not been explored. Cyanotoxin uptake by aquatic or wetland plants in hydroponic experiments have been demonstrated, and although there seems to be an effect on cyanotoxin removal by different plant species [60], [61], [62] they have been done in water systems and not water-soil systems. Cyanotoxin uptake has been more extensively studied in edible plants and agricultural crops than in wetland plants [63] primarily due to the direct implications of food security and safety. Understanding plant roles in cyanotoxin removal in TWs is crucial, not only to increase TW performance but also for managing water quality, assessing ecological risks, and developing potential remediation strategies if necessary. Both monoculture and polyculture systems can provide pollution removal efficiency in TWs; nevertheless, their effectiveness is contingent upon several factors, including plant growth rates and the levels of intra- and inter-specific competition among species [64]. Recent studies have also shown that plant diversity in TWs increases their resilience and functional stability under weather variations, supporting more consistent performance year- round than monoculture systems [65], [66], [67]. However, other studies show no significant effect of polycultures in TW performance, and it is still unclear if polycultures can improve

TWs or not [68]. Moreover, the use of polyculture TWs for cyanotoxin removals remains unexplored. This was further explored in this thesis in Chapter 3.

Wetland species are the emergent macrophytes that are the common vegetation found in natural wetland ecosystems. Ornamental species are flowering plants that have some similar physiological characteristics to natural wetland species, that can also stimulate the removal of pollutants in wastewater treatment systems such as TWs [69]**.** Natural wetland plants, including *Phragmites australis*, *Typha* spp., and *Scirpus* spp., are frequently included in TWs because they are widely distributed wetland plants found in many regions of the world. They also exhibit strong adaptability to a broad range of climates and environmental conditions, with high efficacy in eliminating various pollutants [70]. Ornamental plants such as *Canna spp., Iris spp., Heliconia spp.,* and *Zantedeschia spp.,* are increasingly considered for use in TWs, especially in public or urban settings where aesthetics is important [71]. Studies indicate that ornamental plants can perform comparably to the natural wetland species in removing pollutants, including chemical oxygen demand (COD), nitrogen, phosphorus, fluoride, and chloride [72], [73]. For instance, *C. indica* has shown encouraging results for contaminant removal and is recommended for both monoculture and mixed plantings [71]. While ornamental plants show promise, more research is needed regarding their removal of emerging contaminants and their interactions with microbial communities. Moreover, considering that plant-microbiome interactions play an important role in pollutant removal processes, it is important to study their interactions with microbial communities in monoculture and mixed planting [74].

Sparganium erectum, a natural wetland species, represents a valuable candidate in the plant palette available for TW design due to its distinct physiological and remediation characteristics. Its demonstrated effectiveness in nutrient and pollutant removal, particularly in combination with other macrophytes, makes it a viable option for water treatment applications [75]. The species shows particular promise for metal remediation in neutral to alkaline conditions and for systems where depth zonation can accommodate its growth requirements [76], [77], [78]. Beyond water treatment, the presence of *S. erectum* in wetland systems can contribute to habitat heterogeneity, potentially increasing β-diversity (variation in species composition between sites) [79]. This represents an added ecological benefit beyond the primary water treatment function of TWs aligning with approaches that seek to maximize ecosystem services from these systems. *S.erectum* has a widespread presence across Europe, being most prevalent in the Baltic region [80] and with notable documentation in the United Kingdom particularly

in the lochs of Scotland [81]. Recent research has also documented it to be distributed in the Mediterranean regions [82]. *S.erectum* has mostly been studied in surface flow TWs, and only one other study has used *S.erectum* in VSF-TW design [78], [83]. To our knowledge, *S.erectum* has not been studied for the removal of cyanotoxins.

Canna indica is increasingly used in TWs for wastewater treatment due to its robust growth, aesthetic appeal, and phytoremediation capabilities [71], [84]. Although native to tropical and sub-tropical regions of the America, over time, *C. indica* has been widely introduced and naturalized in many other parts of the world, including Africa, Asia, Europe and Oceania. Its distribution in Europe is mostly in southern countries (Portugal, Spain, Italy, France, and Albania) [85]. While *C. indica* is effective for many contaminants, more research is needed on its ability to remove emerging pollutants including cyanotoxins, and on the potential toxicity of these compounds to the plant itself in different climatic conditions. Research gaps with *C.indica* planted TWs include plant-microbe interactions and their influence on TWs performance, potential pollutant effects on them including bioaccumulation in their tissues, and performance efficiency when mixed with other plants as polycultures. While *C.indica* has been previously studied for treating hyper-eutrophic surface water containing cyanobacterial blooms, however removal rates of relevant cyanotoxins were not quantified [38], [86].

So, more research on plant species, either as a monoculture or polyculture, should be explored for cyanotoxins removal by TWs, including potential plants cyanotoxin bioaccumulation, which was done in this thesis in Chapter 3.

1.2.3. Microbial Communities' Role in the Removal of Cyanotoxins

Research into the removal mechanisms of cyanotoxins in TWs is expanding, but several critical gaps remain that hinder a comprehensive understanding of how these systems mitigate harmful Cyano-HABs byproducts like MCs and CYN. Although it is established that microorganisms, particularly in planted systems, are crucial for the degradation of MCs [42], ambiguity about the specific microbial populations involved and the environmental conditions that maximise their activity remains. Microbial communities are central to the functioning and efficiency of TWs- acting as a key pollutant degradation mechanism as well as nutrient cycling for the overall performance of the TWs [87]. Therefore, further research of how different system types and operational parameters (e.g., plant species, substrate type, hydraulic retention time) influence microbial community, and removal performance for various cyanotoxins is important. Bacterial species, including *Sphingomonas*, *Pseudomonas, Bacillus*, *Burkholderia, Arthrobacter,* and *Novosphingobium* have been recognised as MC degraders, with the mlr gene cluster

playing a key role. Moreover, a few fungi were identified as MC degraders, such as *Trametes versicolor*, *Phanerochaete chrysosporium*, and *Aspergillus niger* [88]. Another study demonstrated a positive correlation between the fungus *Rhizophagus* and *Myrmecridium* and the removal of cyanotoxins [89]. However, it is highly likely that there are many unidentified cyanotoxin degrading microorganisms, especially present in TWs.

Besides not only the potential identification of cyanotoxins degraders, but also the characterisation of microbial communities in TWs can be relevant to fully understand cyanotoxins removal processes as well as to increase those processes, enhancing TW performance, a topic explored in Chapter 3 of this thesis.

Finally, while the potential for using modified adsorbents in engineered systems are discussed [52], the integration of such materials into TWs remains under-reported.

1.3. Research Aim and Objectives

To address the knowledge gaps stated in the previous section, the following research objectives were formulated for this thesis:

a) Evaluating agro-based waste materials (rice husks, olive pulp pomace (OP) pellets, cork granules) and the benchmark NBS substrates (biochar, light expanded clay aggregates (LECA), and sand) for (i) their MC-LR and CYN sorption potential, and (ii) the sorption kinetics and sorption mechanism of the two best sorbent materials aiming for their future incorporation into eco-technologies.
b) Comparing the performance and resilience of a wetland (*S. erectum*) and an ornamental (*C. indica*) species in the removal of cyanotoxins in a planted vertical subsurface flow (VSSF) TW design, both in monoculture and mixed plant treatment conditions.
c) Understanding the removal mechanisms of MC-LR and CYN in the studied TW design both by: (i) evaluating bioaccumulation/ retention of MC-LR and CYN in the different plant tissues and substrates matrices (which also integrated two of the best low-cost sorbent materials); (ii) investigating the influence of the different plant treatment conditions, substrate type, and cyanotoxin exposure on the microbial community.

1.4. Thesis Outline

The thesis consists of four chapters, with the present part (chapter 1) serving as an introduction to the topic. The subsequent two chapters contain the research findings that contribute to a comprehensive investigation of the research's aim and objectives. Chapter 2 "Evaluating Agro-Based Waste Materials for Cyanotoxin Sorption for Future Incorporation in Nature-Based Solutions (NBS)" covers the first objective of the thesis, assessing unmodified agro-based waste materials (rice husks, olive pulp pomace (OP) pellets, cork granules) and the benchmark NBS substrates (biochar, light expanded clay aggregates (LECA), and sand) for their MC-LR and CYN sorption potential, and the sorption kinetics and sorption mechanism of the two best sorbent materials aiming for their future incorporation into NBS. Chapter 3 "Vertical Subsurface Flow Treatment Wetlands for Cyanotoxin Remediation: Plant Composition, Microbial Dynamics, and Removal Efficiency" covers the second and third objectives of the thesis, where two plant species (*S. erectum* and *C. indica*) were compared in a hybrid VSSF TW, alone and in combination, for the removal efficiency of cyanotoxins. The cyanotoxin removal rates, substrates' retention rates, plant accumulation and status, and the TW microbiome were explored in this chapter, to deepen the understanding of the removal mechanisms of MC-LR and CYN in the studied design with different plant compositions, providing insight into further scale-up and piloting of the TW systems**.**

Finally, the knowledge gained from these chapters were summarized, providing insight into further scale-up and piloting of the TW systems briefed, and future research perspectives were discussed in Chapter 4.

References

[1] W. v. Koenigswald, A. Braun, and T. Pfeiffer, “Cyanobacteria and seasonal death: A new taphonomic model for the Eocene Messel lake,” *Palaontol Z*, vol. 78, no. 2, pp. 417–424, Oct. 2004, doi: 10.1007/bf03009232.

[2] S. Merel, D. Walker, R. Chicana, S. Snyder, E. Baurès, and O. Thomas, “State of knowledge and concerns on cyanobacterial blooms and cyanotoxins,” 2013, *Elsevier Ltd*. doi: 10.1016/j.envint.2013.06.013.

[3] P. M. Visser *et al.*, “How rising CO2 and global warming may stimulate harmful cyanobacterial blooms,” 2016, *Elsevier B.V.* doi: 10.1016/j.hal.2015.12.006.

[4] L. Song *et al.*, “Annual Review of Environment and Resources Harmful Cyanobacterial Blooms: Biological Traits, Mechanisms, Risks, and Control Strategies,” vol. 48, p. 52, 2025, doi: 10.1146/annurev-environ-112320.

[5] H. G. Kim, Y. K. Cha, and K. H. Cho, “Projected climate change impact on cyanobacterial bloom phenology in temperate rivers based on temperature dependency,” *Water Res*, vol. 249, Feb. 2024, doi: 10.1016/j.watres.2023.120928.

[6] Y. Wang *et al.*, “Global elevation of algal bloom frequency in large lakes over the past two decades,” *Natl Sci Rev*, vol. 12, no. 3, Mar. 2025, doi: 10.1093/nsr/nwaf011.

[7] M. A. D. Mowe, S. M. Mitrovic, R. P. Lim, A. Furey, and D. C. J. Yeo, “Tropical cyanobacterial blooms: A review of prevalence, problem taxa, toxins and influencing environmental factors,” Dec. 30, 2015, *Page Press Publications*. doi: 10.4081/jlimnol.2014.1005.

[8] W. W. Carmichael, “The toxins of cyanobacteria.,” 1994. doi: 10.1038/scientificamerican0194-78.

[9] A. Krishnan and X. Mou, “A brief review of the structure, cytotoxicity, synthesis, and biodegradation of microcystins,” Aug. 02, 2021, *MDPI AG*. doi: 10.3390/w13162147.

[10] C. Mackintoshl, K. A. Beatti, S. Klumpp3, P. Cohen1, and G. A. Codd2, “Cyanobacterial microcystin-LR is a potent and specific inhibitor of protein phosphatases 1 and 2A from both mammals and higher plants,” 1990.

[11] K. A. Cottrill *et al.*, “Identification of novel microcystins in algal extracts by a liquid chromatography–high-resolution mass spectrometry data analysis pipeline,” *Harmful Algae*, vol. 139, Nov. 2024, doi: 10.1016/j.hal.2024.102739.

[12] N. L. McLellan and R. A. Manderville, “Toxic mechanisms of microcystins in mammals,” *Toxicol Res (Camb)*, vol. 6, no. 4, pp. 391–405, 2017, doi: 10.1039/c7tx00043j.

[13] L. Brient, M. Lengronne, M. Bormans, and J. Fastner, “First occurrence of cylindrospermopsin in freshwater in France,” *Environ Toxicol*, vol. 24, no. 4, pp. 415–420, 2009, doi: 10.1002/tox.20439.

[14] A. A. De La Cruz *et al.*, “A review on cylindrospermopsin: The global occurrence, detection, toxicity and degradation of a potent cyanotoxin,” Nov. 2013. doi: 10.1039/c3em00353a.

[15] S. Pichardo, A. M. Cameán, and A. Jos, “In vitro toxicological assessment of cylindrospermopsin: A review,” Dec. 16, 2017, *MDPI AG*. doi: 10.3390/toxins9120402.

[16] L. Pearson, T. Mihali, M. Moffitt, R. Kellmann, and B. Neilan, “On the chemistry, toxicology and genetics of the cyanobacterial toxins, microcystin, nodularin, saxitoxin and cylindrospermopsin,” 2010, *MDPI AG*. doi: 10.3390/md8051650.

[17] W. W. Carmichael and G. L. Boyer, “Health impacts from cyanobacteria harmful algae blooms: Implications for the North American Great Lakes,” 2016, *Elsevier B.V.* doi: 10.1016/j.hal.2016.02.002.

[18] J. O’keeffe, “Cyanobacteria in recreational freshwaters: Understanding exposures and health effects,” 2024.

[19] R. Wood, “Acute animal and human poisonings from cyanotoxin exposure - A review of the literature,” May 01, 2016, *Elsevier Ltd*. doi: 10.1016/j.envint.2016.02.026.

[20] S. Corbel, C. Mougin, and N. Bouaïcha, “Cyanobacterial toxins: Modes of actions, fate in aquatic and soil ecosystems, phytotoxicity and bioaccumulation in agricultural crops,” 2014, *Elsevier Ltd*. doi: 10.1016/j.chemosphere.2013.07.056.

[21] A. D. Chatziefthimiou, J. S. Metcalf, W. B. Glover, S. A. Banack, S. R. Dargham, and R. A. Richer, “Cyanobacteria and cyanotoxins are present in drinking water impoundments and groundwater wells in desert environments,” *Toxicon*, vol. 114, pp. 75–84, May 2016, doi: 10.1016/j.toxicon.2016.02.016.

[22] Z. A. Mohamed *et al.*, “Microcystin levels in irrigation water and field-vegetable plants, and food safety risk assessment: A case study from Egypt,” *Toxicon*, vol. 247, Aug. 2024, doi: 10.1016/j.toxicon.2024.107846.

[23] “Cyanobacterial toxins: microcystins Background document for development of WHO,” 2020. [Online]. Available: http://apps.who.int/bookorders.

[24] “Cyanobacterial toxins: cylindrospermopsins Background document for development of WHO,” 2020. [Online]. Available: http://apps.who.int/bookorders.

[25] P. Mehta *et al.*, “Half of twenty-first century global irrigation expansion has been in water-stressed regions,” *Nature Water*, vol. 2, no. 3, pp. 254–261, Mar. 2024, doi: 10.1038/s44221-024-00206-9.

[26] E. M. Redouane *et al.*, “Health risk assessment of lake water contaminated with microcystins for fruit crop irrigation and farm animal drinking,” *Environmental Science and Pollution Research*, vol. 30, no. 33, pp. 80234–80244, Jul. 2023, doi: 10.1007/s11356-023-27914-1.

[27] *Progress on the level of water stress*. FAO, 2024. doi: 10.4060/cd2179en.

[28] P. Micklin, “The future Aral Sea: hope and despair,” *Environ Earth Sci*, vol. 75, no. 9, May 2016, doi: 10.1007/s12665-016-5614-5.

[29] S. M. Khair, S. Mushtaq, K. Reardon-Smith, and J. Ostini, “Diverse drivers of unsustainable groundwater extraction behaviour operate in an unregulated water scarce region,” *J Environ Manage*, vol. 236, pp. 340–350, Apr. 2019, doi: 10.1016/j.jenvman.2018.12.077.

[30] M. A. S. Islam, T. Saeed, and N. Majed, “Role of constructed wetlands in mitigating the challenges of industrial growth and climate change impacts in the context of developing countries,” Nov. 21, 2022, *Frontiers Media S.A.* doi: 10.3389/fenvs.2022.1065555.

[31] M. Dremel, B. Goličnik Marušić, and I. Zelnik, “Defining Natural Habitat Types as Nature-Based Solutions in Urban Planning,” Sep. 01, 2023. doi: 10.20944/preprints202309.0054.v1.

[32] L. Ramakreshnan and N. Aghamohammadi, “The Application of Nature-Based Solutions for Urban Heat Island Mitigation in Asia: Progress, Challenges, and Recommendations,” Mar. 01, 2024, *Springer Science and Business Media Deutschland GmbH*. doi: 10.1007/s40572-023-00427-2.

[33] A. I. Stefanakis, C. S. C. Calheiros, and I. Nikolaou, “Nature-Based Solutions as a Tool in the New Circular Economic Model for Climate Change Adaptation,” *Circular Economy*

and Sustainability, vol. 1, no. 1, pp. 303–318, Jun. 2021, doi: 10.1007/s43615-021-00022-3.

[34] I. Schiavi, “PhD Programme in Innovation for the Circular Economy-XXXIV Doctorate Cycle,” 2023.

[35] B. L. Marrone, S. Banerjee, A. Talapatra, C. R. Gonzalez-Esquer, and G. Pilania, “Toward a Predictive Understanding of Cyanobacterial Harmful Algal Blooms through AI Integration of Physical, Chemical, and Biological Data,” Mar. 08, 2024, *American Chemical Society*. doi: 10.1021/acsestwater.3c00369.

[36] P. K. Kulabhusan and K. Campbell, “Physico-chemical treatments for the removal of cyanotoxins from drinking water: Current challenges and future trends,” Mar. 20, 2024, *Elsevier B.V.* doi: 10.1016/j.scitotenv.2024.170078.

[37] M. R. Teixeira and M. J. Rosa, “Comparing dissolved air flotation and conventional sedimentation to remove cyanobacterial cells of Microcystis aeruginosa. Part I: The key operating conditions,” *Sep Purif Technol*, vol. 52, no. 1, pp. 84–94, Nov. 2006, doi: 10.1016/j.seppur.2006.03.017.

[38] A. Martinez i Quer, Y. Larsson, A. Johansen, C. A. Arias, and P. N. Carvalho, “Cyanobacterial blooms in surface waters – Nature-based solutions, cyanotoxins and their biotransformation products,” Mar. 01, 2024, *Elsevier Ltd*. doi: 10.1016/j.watres.2024.121122.

[39] A. G. Capodaglio, S. Bolognesi, and D. Cecconet, “Sustainable, decentralized sanitation and reuse with hybrid nature-based systems,” Jun. 01, 2021, *MDPI AG*. doi: 10.3390/w13111583.

[40] P. N. Carvalho, C. A. Arias, and H. Brix, “Constructed wetlands for water treatment: New developments,” Jun. 02, 2017, *MDPI AG*. doi: 10.3390/w9060397.

[41] G. Bavithra *et al.*, “Assessment of constructedwetlands’ potential for the removal of cyanobacteria and microcystins (MC-LR),” *Water (Switzerland)*, vol. 12, no. 1, 2020, doi: 10.3390/w12010010.

[42] A. Martinez i Quer *et al.*, “Saturated constructed wetlands for the remediation of cylindrospermopsin and microcystin-LR: Plants, microbes, and biodegradation pathways,” *Science of the Total Environment*, vol. 948, Oct. 2024, doi: 10.1016/j.scitotenv.2024.174745.

[43] R. P. Aba *et al.*, “First report on cyanotoxin (Mc-lr) removal from surface water by multi-soil-layering (msl) eco-technology: Preliminary results,” *Water (Switzerland)*, vol. 13, no. 10, 2021, doi: 10.3390/w13101403.

[44] Y. Wang, X. Yin, Y. Cai, and Z. Yang, “An enhanced system with macrophytes and polyurethane sponge as an eco-technology for restoring eutrophic water: A pilot test,” *Water (Switzerland)*, vol. 11, no. 9, 2019, doi: 10.3390/w11091828.

[45] W. El Bouaidi *et al.*, “A review of plant-based coagulants for turbidity and cyanobacteria blooms removal,” Jun. 01, 2022, *Springer Science and Business Media Deutschland GmbH*. doi: 10.1007/s11356-022-20036-0.

[46] E. R. Rene, L. Shu, and V. Jegatheesan, “Editorial: Sustainable eco-technologies for water and wastewater treatment,” 2019, *IWA Publishing*. doi: 10.2166/aqua.2019.100.

[47] T. Dunlop *et al.*, “The evolution and future of research on Nature-based Solutions to address societal challenges,” *Commun Earth Environ*, vol. 5, no. 1, Dec. 2024, doi: 10.1038/s43247-024-01308-8.

[48] R. H. Kadlec and S. Wallace, “TREATMENT WETLANDS 2nd Ed,” 2008.

[49] J. Vymazal, “Constructed wetlands for wastewater treatment: Five decades of experience,” *Environ Sci Technol*, vol. 45, no. 1, pp. 61–69, Jan. 2011, doi: 10.1021/es101403q.

[50] S. Kataki, S. Chatterjee, M. G. Vairale, S. K. Dwivedi, and D. K. Gupta, “Constructed wetland, an eco-technology for wastewater treatment: A review on types of wastewater treated and components of the technology (macrophyte, biolfilm and substrate),” *J Environ Manage*, vol. 283, Apr. 2021, doi: 10.1016/j.jenvman.2021.111986.

[51] R. S. P. Couto, A. Guarino, W. S. Branco, C. W. C. Palermo, and E. F. A. Azero, “Application of Clay Minerals and Polymeric Resins to Remove Dissolved Microcystin-LR from Water,” *Int. J. Environ. Res*, vol. 7, no. 2, pp. 435–442, 2013.

[52] T. Abbas *et al.*, “Recent advancements in the removal of cyanotoxins from water using conventional and modified adsorbents—a contemporary review,” Oct. 01, 2020, *MDPI AG*. doi: 10.3390/w12102756.

[53] S. Pavagadhi, A. L. L. Tang, M. Sathishkumar, K. P. Loh, and R. Balasubramanian, “Removal of microcystin-LR and microcystin-RR by graphene oxide: Adsorption and

kinetic experiments," *Water Res*, vol. 47, no. 13, pp. 4621–4629, Sep. 2013, doi: 10.1016/j.watres.2013.04.033.

[54] Y. Gao, N. Gao, Y. Deng, J. Gu, Y. Shen, and S. Wang, "Adsorption of Microcystin-LR from Water with Iron Oxide Nanoparticles," *Water Environment Research*, vol. 84, no. 7, pp. 562–568, Jul. 2012, doi: 10.2175/106143012x13373575830674.

[55] J. Lee and H. W. Walker, "Adsorption of microcystin-Lr onto iron oxide nanoparticles," *Colloids Surf A Physicochem Eng Asp*, vol. 373, no. 1–3, pp. 94–100, Jan. 2011, doi: 10.1016/j.colsurfa.2010.10.032.

[56] J. A. Park *et al.*, "Investigating Microcystin-LR adsorption mechanisms on mesoporous carbon, mesoporous silica, and their amino-functionalized form: Surface chemistry, pore structures, and molecular characteristics," *Chemosphere*, vol. 247, May 2020, doi: 10.1016/j.chemosphere.2020.125811.

[57] S. De Gisi, G. Lofrano, M. Grassi, and M. Notarnicola, "Characteristics and adsorption capacities of low-cost sorbents for wastewater treatment: A review," Sep. 01, 2016, *Elsevier B.V.* doi: 10.1016/j.susmat.2016.06.002.

[58] N. M. Kulshreshtha, V. Verma, A. Soti, U. Brighu, and A. B. Gupta, "Exploring the contribution of plant species in the performance of constructed wetlands for domestic wastewater treatment," Jun. 01, 2022, *Elsevier Ltd*. doi: 10.1016/j.biteb.2022.101038.

[59] L. A. Thyssen, A. Martinez i Quer, C. A. Arias, L. Ellegaard-Jensen, P. N. Carvalho, and A. Johansen, "Constructed wetland mesocosms improve the biodegradation of microcystin-LR and cylindrospermopsin by indigenous bacterial consortia," *Harmful Algae*, vol. 131, Jan. 2024, doi: 10.1016/j.hal.2023.102549.

[60] M. Kucała, M. Saładyga, and A. Kaminski, "Phytoremediation of cyn, mc-lr and antx-a from water by the submerged macrophyte lemna trisulca," *Cells*, vol. 10, no. 3, pp. 1–18, Mar. 2021, doi: 10.3390/cells10030699.

[61] S. Pflugmacher, K. S. Kwon, S. Baik, S. Kim, S. Kühn, and M. Esterhuizen-Londt, "Physiological responses of Cladophora glomerata to cyanotoxins: a potential new phytoremediation species for the Green Liver Systems," *Toxicol Environ Chem*, vol. 98, no. 2, pp. 241–259, Feb. 2016, doi: 10.1080/02772248.2015.1119835.

[62] A. Kaminski, B. Bober, E. Chrapusta, and J. Bialczyk, “Phytoremediation of anatoxin-a by aquatic macrophyte Lemna trisulca L.,” *Chemosphere*, vol. 112, pp. 305–310, 2014, doi: 10.1016/j.chemosphere.2014.04.064.

[63] C. Weralupitiya, R. P. Wanigatunge, D. Gunawardana, M. Vithanage, and D. Magana-Arachchi, “Cyanotoxins uptake and accumulation in crops: Phytotoxicity and implications on human health,” May 01, 2022, *Elsevier Ltd*. doi: 10.1016/j.toxicon.2022.03.003.

[64] M. Licata, M. C. Gennaro, T. Tuttolomondo, C. Leto, and S. la Bella, “Research focusing on plant performance in constructed wetlands and agronomic application of treated wastewater - A set of experimental studies in Sicily (Italy),” *PLoS One*, vol. 14, no. 7, Jul. 2019, doi: 10.1371/journal.pone.0219445.

[65] Y. Luo, Q. Chen, F. Liu, and C. Dai, “Both species richness and growth forms affect nutrient removal in constructed wetlands: A mesocosm experiment,” *Front Ecol Evol*, vol. 11, 2023, doi: 10.3389/fevo.2023.1139053.

[66] V. Carrillo, Y. Casas-Ledón, P. Neumann, and G. Vidal, “Environmental performance of constructed wetland planted with monocultures and polycultures for wastewater treatment,” *Ecol Eng*, vol. 193, Aug. 2023, doi: 10.1016/j.ecoleng.2023.107015.

[67] L. Y. Kochi, P. L. Freitas, L. T. Maranho, P. Juneau, and M. P. Gomes, “Aquatic macrophytes in constructed wetlands: A fight against water pollution,” Nov. 01, 2020, *MDPI*. doi: 10.3390/su12219202.

[68] A. M. Leiva, R. Núñez, G. Gómez, D. López, and G. Vidal, “Performance of ornamental plants in monoculture and polyculture horizontal subsurface flow constructed wetlands for treating wastewater,” *Ecol Eng*, vol. 120, pp. 116–125, Sep. 2018, doi: 10.1016/j.ecoleng.2018.05.023.

[69] L. Sandoval, S. A. Zamora-Castro, M. Vidal-Álvarez, and J. L. Marín-Muñiz, “Role of wetland plants and use of ornamental flowering plants in constructed wetlands for wastewater treatment: A review,” Feb. 17, 2019, *MDPI AG*. doi: 10.3390/app9040685.

[70] J. Vymazal, “The Historical Development of Constructed Wetlands for Wastewater Treatment,” Feb. 01, 2022, *MDPI*. doi: 10.3390/land11020174.

[71] P. N. Karungamye, “Potential of Canna indica in Constructed Wetlands for Wastewater Treatment: A Review,” Sep. 01, 2022, *Multidisciplinary Digital Publishing Institute (MDPI)*. doi: 10.3390/conservation2030034.

[72] G. Nani, M. Sandoval-Herazo, G. Martínez-Reséndiz, O. Marín-Peña, F. Zurita, and L. C. Sandoval Herazo, “Influence of Bed Depth on the Development of Tropical Ornamental Plants in Subsurface Flow Treatment Wetlands for Municipal Wastewater Treatment: A Pilot-Scale Case,” *Plants*, vol. 13, no. 14, Jul. 2024, doi: 10.3390/plants13141958.

[73] J. L. Marín-Muñiz, M. E. Hernández, and S. Zamora Castro, “Ornamental Plant Growth in Different Culture Conditions and Fluoride and Chloride Removals with Constructed Wetlands,” *Hydrology*, vol. 11, no. 11, Nov. 2024, doi: 10.3390/hydrology11110182.

[74] V. Riva *et al.*, “Plant species influences the composition of root system microbiome and its antibiotic resistance profile in a constructed wetland receiving primary treated wastewater,” *Front Microbiol*, vol. 15, 2024, doi: 10.3389/fmicb.2024.1436122.

[75] F. Karam, R. Haddad, N. Amacha, W. Charanek, and J. Harmand, “Assessment of the Impacts of Phyto-Remediation on Water Quality of the Litani River by Means of Two Wetland Plants (Sparganium erectum and Phragmites australis),” *Water (Switzerland)*, vol. 15, no. 1, Jan. 2023, doi: 10.3390/w15010004.

[76] S. H. Kim, J. M. Nam, and J. G. Kim, “Establishment strategy of a rare wetland species sparganium erectum in Korea,” *J Ecol Environ*, vol. 41, no. 1, Aug. 2017, doi: 10.1186/s41610-017-0045-0.

[77] M. Senze, M. Kowalska-Góralska, and K. Czyż, “Emergent (branched bur-reed—Sparganium erectum L.) and submergent (river water-crowfoot—Ranunculus fluitans Wimm., 1841) aquatic plants as metal biosorbents under varying water pH conditions in laboratory conditions,” *Environmental Science and Pollution Research*, vol. 30, no. 40, pp. 92053–92067, Aug. 2023, doi: 10.1007/s11356-023-28752-x.

[78] P. Porras-Socias *et al.*, “Removal of metals and emergent contaminants from liquid digestates in constructed wetlands for agricultural reuse,” *Front Microbiol*, vol. 15, 2024, doi: 10.3389/fmicb.2024.1388895.

[79] I. W. Bashinskiy, “Beaver-created microhabitats in a small water body and their impact on flora and fauna (the Khoper River floodplain, Russia),” *International Journal of Limnology*, vol. 58, pp. 480–776, 2022, doi: 10.1051/limn/2022016.

[80] A. Bot, “ANNALI DI BOTANICA,” 2011.

[81] D. H. N. Spence and E. D. Allen, “The macrophytic vegetation of Loch Urigill and other lochs of the Ullapool area,” *Transactions of the Botanical Society of Edinburgh*, vol. 43, no. 2, pp. 131–144, Nov. 1979, doi: 10.1080/03746607908685346.

[82] L. Lastrucci, V. Gambirasio, F. Prosser, and D. Viciani, “First record of Sparganium oocarpum in Italy and new regional distribution data for Sparganium erectum species complex,” *Plant Biosyst*, vol. 158, no. 4, pp. 595–600, 2024, doi: 10.1080/11263504.2024.2347851.

[83] J. Vymazal, “Emergent plants used in free water surface constructed wetlands: A review,” *Ecol Eng*, vol. 61, pp. 582–592, Dec. 2013, doi: 10.1016/j.ecoleng.2013.06.023.

[84] K. Ghezali, N. Bentahar, N. Barsan, V. Nedeff, and E. Moșneguțu, “Potential of Canna indica in Vertical Flow Constructed Wetlands for Heavy Metals and Nitrogen Removal from Algiers Refinery Wastewater,” *Sustainability (Switzerland)*, vol. 14, no. 8, Apr. 2022, doi: 10.3390/su14084394.

[85] M. Taylor, “Canna indica (canna lilly),” 2024.

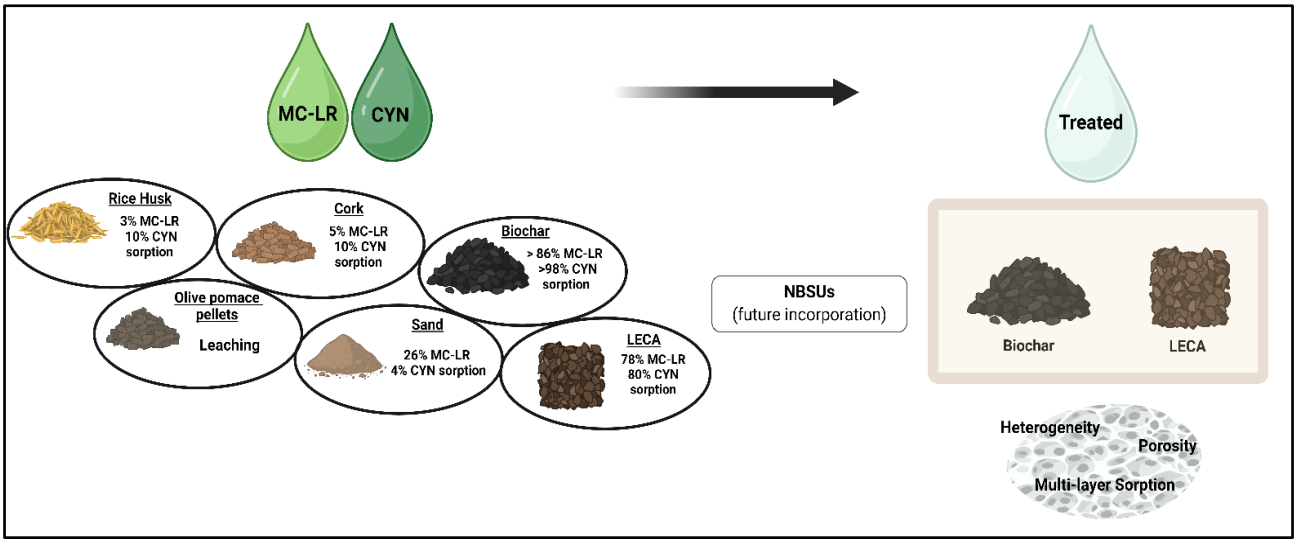
[86] F. Zhong *et al.*, “The use of vertical flow constructed wetlands for the treatment of hyper-eutrophic water bodies with dense cyanobacterial blooms,” *Water Science and Technology*, vol. 77, no. 5, pp. 1186–1195, Mar. 2018, doi: 10.2166/wst.2017.635.

[87] J. Wang *et al.*, “A Review on Microorganisms in Constructed Wetlands for Typical Pollutant Removal: Species, Function, and Diversity,” Apr. 05, 2022, *Frontiers Media S.A.* doi: 10.3389/fmicb.2022.845725.

[88] Z. A. Mohamed, M. Hashem, S. Alamri, A. Campos, and V. Vasconcelos, “Fungal biodegradation and removal of cyanobacteria and microcystins: potential applications and research needs,” May 2021, doi: 10.1007/s11356-021-14623-w/Published.

[89] Á. González Álvarez, A. Martinez i Quer, L. Ellegaard-Jensen, R. Sapkota, P. N. Carvalho, and A. Johansen, “Fungal removal of cyanotoxins in constructed wetlands: The forgotten degraders,” *Science of the Total Environment*, vol. 929, Jun. 2024, doi: 10.1016/j.scitotenv.2024.172590.

Chapter 2. Evaluating Agro-Based Waste Materials for Cyanotoxin Sorption for Future Incorporation in Nature-Based Solutions



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Abstract

Toxic cyanobacterial blooms are a growing environmental problem, persisting in freshwater bodies globally, and potentially hazardous to populations that rely on surface freshwater supplies. Nature-based solutions (NBS) are effective and sustainable approaches for water treatment, with sorption being an important process. The purpose of this study was to evaluate unmodified agro-based waste materials (rice husks, olive pulp pomace pellets (OP), cork granules) and the benchmark NBS substrates (biochar, light expanded clay aggregate (LECA), and sand) for their microcystin-LR (MC-LR) and cylindrospermopsin (CYN) sorption potential. The kinetics and sorption mechanism of the two best sorbent materials were studied for future incorporation into NBS. Pre-screening of the sorbents showed highest sorption with biochar (>86% MC-LR and >98% CYN) and LECA (78% MC-LR and 80% CYN) and lower sorption with rice husk (<10%), cork (<10%), and sand (<26%). Leaching from OP made them unsuitable for further use. The sorption of both the cyanotoxins onto biochar was rapid (8 h), whereas onto LECA it was steadier (requiring 48 h for equilibrium). The pseudo-second-order kinetic model fit the sorption of both cyanotoxins onto biochar and LECA (R2: 0.94–0.99), suggesting that the sorption rate is limited by chemisorption. The sorption of MC-LR and CYN to biochar and LECA fit the Freundlich and D–R models better, suggesting multilayer sorption, high heterogeneity, and porosity in the sorbents (which was also confirmed by SEM/EDS). The sorption capacity was observed to be higher for biochar (Kf: MC-LR = 0.05, CYN = 0.16) than LECA (Kf: MC-LR = 0.02, CYN = 0.01).

Keywords: microcystin-LR; cylindrospermopsin; low-cost sorbents; treatment wetlands; biofilters

2.1. Introduction

Cyanobacterial blooms are widely spread and are a global concern. The rising frequency of CyanoHABs and cyanotoxins in freshwater systems is an overlooked issue. With urban expansions and increased anthropogenic activities, nutrient inlet and greenhouse gas emissions have been favouring this phenomenon. Cyanotoxins are secondary metabolites produced by certain cyanobacterial species [1]. They can be released into the water directly, or through cell lysis. Cyanotoxins persist in freshwater bodies and are stable for lengthy periods of time, even through extreme conditions. This is frequently a threat to human and animal health by direct or indirect exposure [2]. Humans can be exposed directly through drinking water or indirectly through ingestion of contaminated food items. The exposure to cyanotoxins through food is primarily through aquatic animals, livestock, and agricultural crop consumption [3]. Among cyanotoxins, microcystins (MCs) and cylindrospermopsin (CYN) have been identified as the ones of major concern due to the invasive nature of their producers, widespread distribution, and high toxicity. More than 250 variants of MCs have been identified so far, with MC-LR being the model for MC investigations due to its prevalence and toxicity [4]. Previously thought to proliferate only in more tropical regions of the world, CYN-producing strains are now being detected in more temperate climates [5], indicating that these toxin-producing cyanobacteria are highly adaptable, which has significant implications for water authorities worldwide.

Treatment technologies are required for the effective removal of dissolved cyanotoxins from water bodies. When advanced solutions are not accessible in remote or rural areas, cost-effective decentralized approaches for the removal of cyanotoxins for remote areas are needed. Among such water treatment options, nature-based solutions or eco-technologies are of increasing interest. NBS focus on the implementation at a local level. They are designed to be locally adapted, resource-efficient, and incorporating more and more diversified environmental and natural characteristics into the landscape [6]. They are designed to effectively and adaptively handle social, economic, and environmental concerns, while offering human well-being, ecosystem services, resilience, and biodiversity advantages [6]. TWs, biofilters, vegetated swales, and rain-gardens are all examples of NBS used for water treatment [7], removing different types of pollutants [8]. A limiting factor of the said NBS is the requirement for space for effective treatment; sometimes a vast area of land being needed, which limits their wide-spread application, especially in urban areas. However, that is normally not an issue in rural areas. One way

to enhance the performance of NBS towards specific pollutant removal is to incorporate sorbent materials to enhance their effectiveness. Nowadays, low-cost sorbents are gaining popularity, namely, new materials, such as carbons from waste or waste by-products from agricultural, household, and industrial sectors [9]. Incorporating sorbents into NBS should be considered for enhanced performance, as well as circularity of resources.

To date, many conventional sorbents such as activated carbons (ACs), zeolites, and clays have been studied for the sorption of cyanotoxins [10–12]. Their sorption capacity depends on their physico-chemical properties. ACs generally have high sorption capacity of multiple cyanotoxins [10], but it includes major drawbacks such as complex and expensive production and difficulty in regeneration and disposal. On the other hand, biochar (considered a precursor of ACs) is obtained simply by biomass pyrolysis and does not undergo any physical or chemical modification [13], making it potentially more cost-effective. Biochar has been a promising sorbent of pollutants such as metals, microplastics, and nutrients [14,15]. Although limited, they have also been investigated for the sorption of cyanotoxins, namely, MC-LR [16–21]. Based on previous studies, biochar generated from various waste biomasses can be used as a prospective sorbent of MC-LR. Wei and Lu [16] investigated the sorption of MC-LR by biochars derived from rice straw obtained at different pyrolysis temperatures. Their study showed that sorption capacity increased with higher pyrolysis temperature of the rice straw. Another study conducted by Song et al. [18] compared biochars derived from Kentucky bluegrass (*Poa pratensis* L.), microalgae (*Spirulina* sp.), grape pomace, and coffee residues, where the maximum MC-LR sorption capacity was observed in biochar derived from Kentucky bluegrass. Li et al. [21] reported that biochar derived from wood chips could also effectively adsorb MC-LR. Among these studies, the sorption capacities ranged from 0.14 to 42.4 mg/g MC-LR; the highest sorption capacity being in giant reed biochar pyrolyzed at 600 ° C [19], and the lowest by giant reed biochar pyrolyzed at 300 ° C [19]. Biochar generated from woody residues has also been proven to have great potential for the sorption of MC-LR [21], as well as to remove organic micropollutants from real wastewaters [22]. However, there have been no studies so far on the sorption of CYN by biochar.

With natural clays and sediments, the sorption depends not only on sediment/clay physico-chemical properties, but also on contact period, organic matter content, and the toxin structure [11,12,23]. One study by Wu et al. [12] showed a direct correlation between organic matter and MC sorption, with higher organic matter content resulting in

higher MC sorption due to the interaction between MCs and adsorbed organic matter. Clays can also be chemically modified to enhance their sorption capacity. A study comparing the sorption of MC-LR by natural and chemically modified clay (smectite) showed that the modified clay had better sorption efficiency with different MC-LR concentrations [11].

Light expanded clay aggregate (LECA), sand, and biochar have already been widely incorporated in NBS [24–26], especially TWs. However, the sorption of MC-LR and CYN by LECA and sand has not been studied. Although natural sandy sediments from water bodies have been tested for cyanotoxin sorption [23], and sand from ripened sand filter systems have been tested for CYN sorption [27], the sorption capacity of pure filter sand with no organic matter has not been studied.

Other chemically modified sorbents that have been studied for the sorption of cyanotoxins include graphene, iron, and silica-based sorbents [28–31]. Most of these chemically modified sorbents are easily regenerable with no loss of their sorption capacity. However, modified sorbents are more expensive to synthesize, and most research has been restricted to lab scale [32]. Moreover, extensive chemical modification required for these sorbents can lead to increased environmental impact due to the use of chemicals and potential waste generation. Additionally, there is a growing focus on sustainable methods for the disposal of used sorbents, ensuring that they do not harm the environment further.

Another option for sorbents is agro-based materials. Palagama et al. [33] tested raw and treated rice husks for the sorption of MCs, and concluded that acid and heat treatment of rice husks improved the sorption of MCs. More than 50% overall removal of MCs could be obtained from raw rice husks, but sorption of CYN was not tested. To the best of our knowledge, agro-based waste materials without any chemical modifications, such as dried olive pulp pomace (OP) pellets and cork granules, have not been tested for the sorption of cyanotoxins. So, more research on new sorbents for toxins removal is needed, extending also the research on different toxins, other than MC.

Hence, the objectives of this study were to test agro-based waste materials (rice husks, OP pellets, cork granules) and the benchmark NBS substrates (biochar, LECA, and sand) for (a) their MC-LR and CYN sorption potential, and (b) the kinetics and sorption mechanism of the two best sorbent materials aiming for their future incorporation into NBS.

2.2. Materials and Methods

2.2.1. Preparation of Sorbent Materials

Rice husk (from *Oryza sativa*) was obtained from Valente Marques S.A. (Portugal), an agricultural firm that processes rice and other grains. Biochar was obtained from the company Swiss Biochar, Switzerland. The biochar was generated by slow pyrolysis (620 °C) of residues from wood chip production. More details on its characteristics can be found in Prodana, M., et al. [34]. Cork was obtained from a cork processing company in Portugal (name not disclosed as requested by the company). Dried olive pomace pellets were obtained from the Union of Agricultural Cooperatives of the South (UCASUL), Alentejo, Portugal. LECA (SIRO HYDROTON) and sand (AXTON SILICE) were obtained from Leroy Merlin retailer in Matosinhos, Portugal.

All of the sorbent materials underwent pre-treatment to ensure similar particle size for further testing. The materials, initially supplied in different particle size ranges, were first sieved using a Retsch vibratory sieve shaker (AS 200) at 1.50 amplitude mm/g for 10 min. Sorbent materials at the size range 2–4 mm were collected for further use. In the case of LECA, the material was first broken down with the help of a mortar and pestle to get smaller bits comparable to the other sorbents, and then sieved. All materials of particle size 2–4 mm were then rinsed with deionized water, left to air-dry in the dark, and then stored in glass bottles for use in sorption experiments. In this work, all sorbent materials were used directly without chemical or thermal processing to make the method simple, eco-friendly, and cost-efficient.

2.2.2. Cyanotoxin Preparation

The cyanobacterial strains *Microcystis aeruginosa* (LEGE 91094) and *Chrysosporum ovalisporum* (LEGE X-001) were used for the production of MC-LR and CYN, respectively. They were obtained from the Blue Biotechnology and Ecotoxicology Culture Collection (LEGE-CC) at CIIMAR, Matosinhos, Portugal (http://lege.ciimar.up.pt/; accessed on 2 February 2022), and grown to the exponential phase in Z8 medium [35, 36].

After the culture was grown to the exponential phase, the biomass was harvested. *M. aeruginosa* cells were harvested by centrifugation (20 min, 4 ° C, 4495 × *g*), frozen at − 20 ° C for three days, and then lyophilized. *C. ovalisporum* cells were gathered by filtration with an 11 µm Kunstoff-Analysensieb filtration unit (LINKER Industrie-Technik, Germany), frozen at - 20 ° C for three days, and then lyophilized. The lyophilized biomass

was stored at room temperature in a dark dry space. MC-LR was extracted from *M. aeruginosa,* and CYN was extracted from *C. ovalisporum* biomass following a modified version of the method described by Welker et al. [37] and Ramanan et al. [38], respectively. Initially, 10 mg of the lyophilized biomass was taken in 5 mL deionized water and homogenized by sonicating it in a water bath at room temperature for 5 min, three times. It was then left in the fridge at 4 ° C for 2 h and subsequently sonicated again in a water bath at room temperature for 5 min, three times. Afterwards, the homogenized biomass solution was centrifuged at 3000 rpm for 5 min, and the supernatant was vacuum-filtered through a microfiber filter paper of 0.22 μm and 47 mm diameter. Then, the supernatant was analyzed (see Section 2.4) and the MC-LR and CYN concentration in the respective biomass was determined.

To perform the different sorption experiments, proportional amounts of cyanobacteria biomass were routinely extracted in 1 L deionized water following the same protocol. Each batch was sub-sampled for determination of cyanotoxins concentration, and the remaining solution was stored at - 20 ° C until further use in sorption studies.

2.2.3. Sorption Studies

2.2.3.1. Screening of MC-LR and CYN Sorption by Selected Sorbents-

The screening of MC-LR and CYN sorption by rice husk, OP, cork, biochar, LECA, and sand was done at room temperature, with an average of 22.4 °C during the day and 17.5 °C at night, by putting the selected sorbents in contact with cyanotoxin contaminated water. Sorbent amounts were pre-determined by filling 100 mL beakers with the materials till the 20 mL mark and weighing them. The volume of cyanotoxin contaminated water used for exposure was 30 mL. This was done to simulate the substrate to water ratio of 2:3 (*v*:*v*) in TW mesocosms previously used by Bavithra et al. [39]. The average weights of the materials in g were as follows: 2.5, 2.5, 8.5, 3.5, 12.5, and 35 for rice husk, cork, LECA, biochar, OP, and sand, respectively. Inert gravel stones were placed on top of the sorbents to prevent them from floating above the water level and to ensure equal exposure to the cyanotoxins. Cyanotoxin-contaminated water was prepared by adding previously prepared cyanotoxin extract (as described in Section 2.2) to deionized water. The concentration of the cyanotoxins was selected based on the relevant environmental concentration (100 μg/L) that is within the most likely concentration range observed in dissolved form in surface waters [40]. For each material, triplicates were prepared. The beakers were covered with parafilm to prevent loss of water. However, the weight of the beakers was also checked before and after the contact periods to calculate the loss of water through evaporation. From the loss of weight of the beakers before and after the

contact periods, it was concluded that there was negligible loss of water due to evaporation during the tests (below 0.04% after 24 h, below 1.3% after 48 h). Therefore, volume loss was not considered for calculating removal percentages. The beakers were shaken on an automatic shaker (J.P. Selecta Unitronic series, Spain) at 80 rpm, for 24 h and 48 h.

The preliminary sorption studies were carried out in two sets, according to the capacity of the automatic shaker. In the first set, the materials tested were rice husk, cork, and LECA. In the second set, the materials tested were biochar, OP, and sand. Two controls were also prepared: one control with just the cyanotoxin contaminated deionized water, and another control with cyanotoxin-contaminated deionized water with the gravel stones (CGs).

After the contact period, the samples and controls were immediately taken off the shaker and the solutions were vacuum-filtered with a microfiber filter paper of 0.2 µm and 47 mm diameter. From that, 5 mL was stored at −20 ° C until cyanotoxin analysis. Controls showed negligible loss of cyanotoxins, indicating that cyanotoxins were not retained in the filter or degraded during the contact period.

2.2.3.2. Kinetic Studies-

Following the screening of the sorbent materials, the two materials with the highest sorption capacities were studied for their sorption kinetics, determining also the equilibrium time for the sorption of MC-LR and CYN. The experimental setup was the same as the screening test (Section 3.3.1). The contact periods were 1, 2, 4, 8, 16, 24, and 48 h.

2.2.3.3. Sorption Isotherm Studies-

Sorption isotherms were also determined for the two materials with the highest sorption capacity, using a similar experimental setup and testing 3 different cyanotoxin concentrations (0.1, 1, and 10 mg/L) of MC-LR and CYN at the equilibrium period of 24 h (as determined in kinetic studies). These three orders of concentrations were selected keeping in mind the environmental relevance, as well as practicality of the study (maximum produced by the cyanobacterial strains and extractable from the biomass). Moreover, due to the inherent variability of cyanotoxin in biomass, achieving accurate initial cyanotoxin concentrations was not possible. For that reason, the sorbent weights were lowered to achieve saturation capacities at equilibrium (q_e) of varying magnitudes. For this, sorbent weights of the two sorbents were lowered to 0.2, 0.5, and 1 g, and studied with an initial cyanotoxin concentration of 1 mg/L. Then, the data were treated for fitting different isotherm models using the equations given in Table 1.

Table 1. Equations used to analyze the experimental data of the sorption pre-screening, kinetics, and isotherm studies ([41] [42][43][44])

EQUATION	ISOTHERM MODEL	NON-LINEARIZED FORM	LINEARIZED FORM
1	N/A	N/A	$\% \, Sorption = \left(\frac{C_0 - C_e}{C_0}\right) \times 100$
2	N/A	N/A	$q_e = (C_0 - C_e)\, V/m$
3	N/A	N/A	$\ln (q_e - q_t) = \ln q_e - K_1 t$
4	N/A	N/A	$t/q_t = t/q_e + 1/q_e^2 K_2$
5	Langmuir	$q_e = q_{max} \cdot \frac{bc_e}{1 + bc_e}$	$\frac{c_e}{q_e} = \frac{1}{q_{max} b} + \frac{c_e}{q_{max}}$
6	Freundlich	$q_e = K_f \cdot C_e^{1/n}$	$ln\, q_e = ln\, k_f + \frac{1}{n} ln\, C_e$
7	D-R	$q_e = Q_{max} \cdot e^{-k_{DR}\varepsilon^2}$	$log\, e = ln\, Q_{max} - k_{DR}\varepsilon^2$
8	Temkin	$q_e = \frac{RT}{B_T} ln\, A_T C_e$	$q_e = B_T ln\, A_T + B_T ln\, C_e$
9	N/A	$\varepsilon = RT ln \left(1 + \frac{1}{C_e}\right)$	N/A

2.2.3.3. Sorption Isotherm Studies-

Table 1 gives information on the equations used to treat the data. Equations (1) and (2) were used to calculate the percentage sorption and sorption capacity (q) of the sorbents. Here, C_0 and C_e are the starting and equilibrium cyanotoxin concentrations (mg/L), respectively, and V denotes the volume (L) and m denotes the weight of the sorbents (g). Equations (3) and (4) describe the pseudo-first-order and pseudo-second-order kinetic models, respectively, which were used to assess the sorption kinetics more comprehensively. Here, (q_t), the sorption capacity of the cyanotoxins onto each sorbent at time t (h) was calculated using Equation (2), where C_t, the concentration at each sampling time, is substituted for C_e. The parameters K_1 (1/ h) and K_2 (g/ mg/ h) are the

rate constants associated with the pseudo-first-order and pseudo-second-order kinetic reactions, respectively.

The Langmuir (5), Freundlich (6), Dubinin–Radushkevich (D–R) (7), and Temkin (8) models were also used. The Langmuir constant is denoted by b. By plotting C_e vs. C_e/q_e, the q_{max} and b can be determined from the slope and intercept. The Freundlich constant, denoted by K_f, indicates sorption capacity, n indicates sorption intensity, and 1/n denotes the heterogeneity factor [16]. Plotting $\ln q_e$ vs. $\ln C_e$ in Equation (6) revealed the values of K_f and n. K_{DR} (kJ/mol) is the D–R constant linked to sorption free energy (ε is the Polanyi potential), and Q_{max} (mg/g) is the sorption capacity in relation to the micropore volume [45,46]. The values of K_{DR} and Q_{max} were found by plotting $\ln q_e$ vs. ε^2 using Equation (7). B_T (J/mol) and A_T (L/mg) are Temkin isotherm constants, where B_T is a Temkin constant linked to heat of sorption, analogous to the sorption intensity, while A_T is the equilibrium binding constant, analogous to the sorption capacity. Equation (9) can be used to compute the Polanyi potential, where R is the universal gas constant (8.313 J mol^{-1} K^{-1}) and T is temperature (K). The values of A_T and B_T were obtained by plotting q_e against $\ln C_e$ using Equation (8).

Although non-linear isotherm model parameter values fit the experimental data better, are more representative, and dependable, the linearized least-square technique is generally favoured because of its simplicity and ease of use [47]. As a result, linearized models were used in the current investigation. Computation and data fitting were done on Microsoft Excel (2019).

2.2.4. Cyanotoxin Analysis

Cyanotoxins in all solutions were determined by liquid chromatography with tandem mass spectrometry (LC-MS/MS). For that, 5 mL of the solutions stored at −20°C was thawed and subsequently homogenized by shaking. Then, 1 mL was filtered by Pall ACRODISC (New York, USA) 13 mm PTFE filters with 0.2 µm, and transferred into 2 mL HPLC vials for LC-MS/MS analysis. These filters were previously tested internally in our lab for cyanotoxin retention, with full recovery (work not published). This was applied to all solutions except those obtained from the sorption tests with OP, as it showed strong matrix interference in direct LC-MS/MS analysis.

Solid phase extraction (SPE) was carried out to eliminate the matrix from OP samples. For MC-LR, a modified protocol adapted from Lawton et al. [48] and Ramanan et al. [38] was used; for CYN, the protocol was carried out as described in Guzmán-Guillén R et

al. [49]. Recovery tests were made with samples doped with a known amount of MC-LR toxin to evaluate SPE recoveries, which were 84 ± 3%. However, CYN could not be recovered with this method due to a persisting strong matrix interference with the LC-MS/MS even after the SPE, preventing quantification of CYN in solutions obtained from the sorption tests with OP.

The LC-MS/MS analytical method follows indications from Pekar et al. [50] and Zervou et al. [51], with some modifications. The instrument used to quantify MC-LR and CYN was a Waters Alliance e2695 LC system, coupled with a triple quadrupole MS (Micromass® Quattro micro TM API) with electrospray (ESI) interface. The program used for data acquisition and processing was Mass Lynx version 4.1.

Separation was achieved on C18 Hypersil Gold column (100 × 4.6 mm I.D., 5 µm, Thermo Scientific, Waltham, MA, USA) kept at 30 °C, with a flow rate of 0.35 mL/min and injected volume of 10 µL. A gradient elution was used with mobile phase A, methanol, and B ultrapure water both acidified with 0.1% formic acid (2% A and 98% B for 3 min, 40% A and 60% B at 4 min for 1 min, increasing to 60% A at 7 min for 2 min, increasing to 80% A for 2 min, and returning to initial conditions at 20 min and equilibrating for 5 min).

The MS was operated in positive mode with multiple reaction monitoring (MRM). The capillary voltage was maintained at 3.5 kV; cone at 30 V; extractor at 3 V; and Lens at 0.2 V. The source temperature was held at 120 °C and desolvation at 350 °C and 500 L/h. Nitrogen was used as a sheath and auxiliary gas and Argon as a collision gas at a pressure of 0.5 bar. MRM conditions were optimized with standard solutions injected in positive polarity mode, in full scan (30–1500 m/z), and later in MRM mode. Transitions, cone, and collision energy voltages for each cyanotoxin are present in Table A1. The cyanotoxin standard solution and sample solutions were injected in duplicate in the LC-MS/MS equipment, and at each set of 10 samples, a blank and two standard mix solutions of different cyanotoxin concentrations were introduced for QA/QC. Quantification was performed by external calibration curve. All of the standard cyanotoxin solutions of MC-LR and CYN were injected in the LCMS/MS individually, with a concentration range from 1 µg/L to 1000 µg/L. The limits of detection were 28.7 µg/L and 12.5 for MC-LR and CYN, respectively, and the limits of quantification were 86.9 µg/L and 37.8 µg/L for MC-LR and CYN, respectively. The MC-LR (CRM-00-MC-LR, Lot 19-001, 96% purity) and CYN (CRM-03-CYN, Lot 16-001, 99% purity) standard solutions were supplied by Cifga (Lugo, Spain).

2.2.5. Characterization of Sorbent Materials

The sorbent materials were characterized for comparing the surface areas and the element compositions among the sorbent materials; specifically, to understand their morphologies, degrees of porosity, and composition variances. This was done using Scanning Electron Microscopy/Energy Dispersive Spectroscopy (SEM/EDS). Since the goals were to observe degrees of porosity and composition variance among the different materials, and not the pore size itself, a 100× magnification was used for all of the sorbent materials, as it gave an apt field of view to observe this. Care was taken during sampling to provide a more representative sample of each sorbent, in order to obtain conclusions on the materials' surface characteristics. For each sorbent, each visually different surface was analyzed by EDS (Supplementary Materials Section A1, Figures A5–A10), where each of the surfaces were labelled as different zones of the material (Z1, Z2, Z3. . .). Moreover, EDS data were also seen in conjunction with the backscattered electron diffraction mode of SEM.

The SEM/EDS analysis was performed using a high-resolution (Schottky) Environmental Scanning Electron Microscope with X-Ray Microanalysis and Electron Backscattered Diffraction analysis: FEI Quanta 400 FEG ESEM/EDAX Genesis X4M. For sand, samples were characterized using low-vacuum mode because it is a bad conductor. The scanning electron mode was used to give information on the topography of the materials. The backscattered electron mode was used to give information on the distribution of elements on the surface of the materials (brighter areas denoting higher atomic number elements and darker areas denoting lower atomic number elements), and the X-ray detector was used to give information on the elemental composition. All SEM/EDS analyses were performed at Centro de Materiais da Universidade do Porto (CEMUP).

2.2.6. Data Treatment

Statistical analyses were performed in Sigmaplot v.14, Systat Software Inc. (San Jose, CA, USA), and Microsoft Excel (2019). Statistical differences between the two controls (C and CG) were initially tested by performing a two-way ANOVA followed by a Tukey test. The significance levels of all the tests were $p < 0.05$. No significant difference was found between the two controls for any of the experiments, therefore, CG was considered as the control for the subsequent analyses. For the pre-screening of the sorbents, one-way ANOVA was performed to test sorption differences between the sorbents at contact periods 24 h and 48 h. For statistical purposes, when values were <LOD, it was

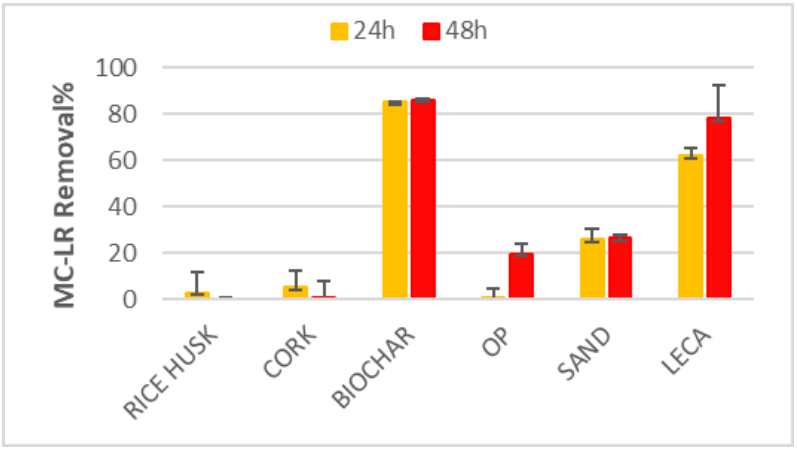
considered as half of the LOD. All graphs and regression statistical analysis for the kinetic studies were done in Microsoft Excel (2019).

2.3. Results

2.3.1. Pre-screening of Sorbents

Biochar and LECA showed the highest sorption potential of both MC-LR (100 µg/L) and CYN (300 µg/L) (Figure 3.). Highest MC-LR sorption rates were > 86% by biochar, with MC-LR levels < LOD; and an average 62 % and 78 % by LECA at 24 h and 48 h, respectively (Figure 3a). The highest CYN sorption rates were> 98% by biochar, with CYN levels < LOD; and an average 68% and 80% by LECA at 24h and 48h, respectively (Figure 3b). Controls showed no sorption of both compounds, and for simplification not included in Figure 3. Tables including data and statistics are provided in appendix A (Tables A2 and A3).

a)



b)

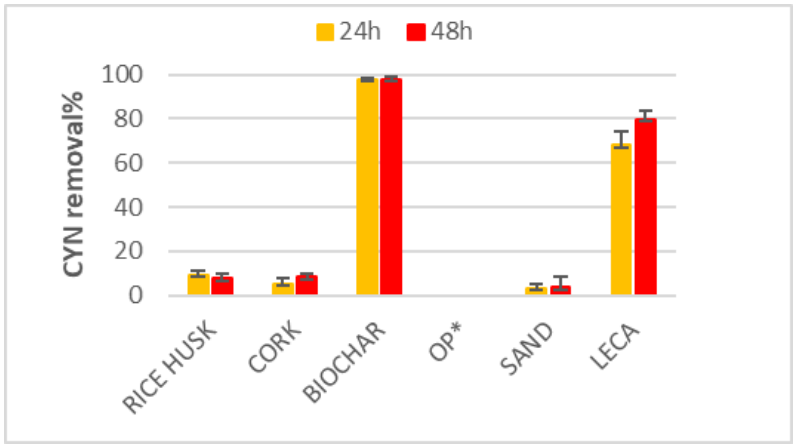


Figure 3.a) MC-LR sorption rates by different sorbents at 24h and 48h during the pre-screening (mean and standard deviation, n=3), b) CYN sorption rates by different sorbents at 24 h and 48 h during the pre-screening (OP*- matrix not suitable for CYN detection)

One-way ANOVA analysis for these pre-screening results showed significant difference between the control and the sorbents at 24h and 48h for both MC-LR and CYN ($p < 0.05$). The Tukey post-hoc tests revealed that for MC-LR sorption there was no significant difference between the control and rice husk and cork at 24 and 48 h. However, at 48 h OP showed significant difference from the control ($p<0.05$). For CYN sorption there was no significant difference from the control and sand at 24 and 48 h. Rice husk had low MC-LR sorption rate of 3 % at 24 h and no sorption at 48 h, as well as low CYN sorption rates of 10 % and 9 % at 24 h and 48 h respectively. Cork also had low MC-LR sorption rates of 5 % and 0.6 % at 24 h and 48 h respectively, and low CYN sorption rates of 6% and 10% at 24 h and 48 h respectively. OP gave problems with the LC-MS/MS analysis due to matrix effects, even after a tentative clean-up step with SPE. Although there was MC-LR sorption of 1% and 20% at 24 h and 48 h respectively, by OP, this material seems not feasible to be incorporated in NBS due to the leaching observed. Filter sand had an average 26 % MC-LR sorption at 24 and 48 h, while negligible CYN sorption of 3% and 4% at 24 h and 48 h respectively.

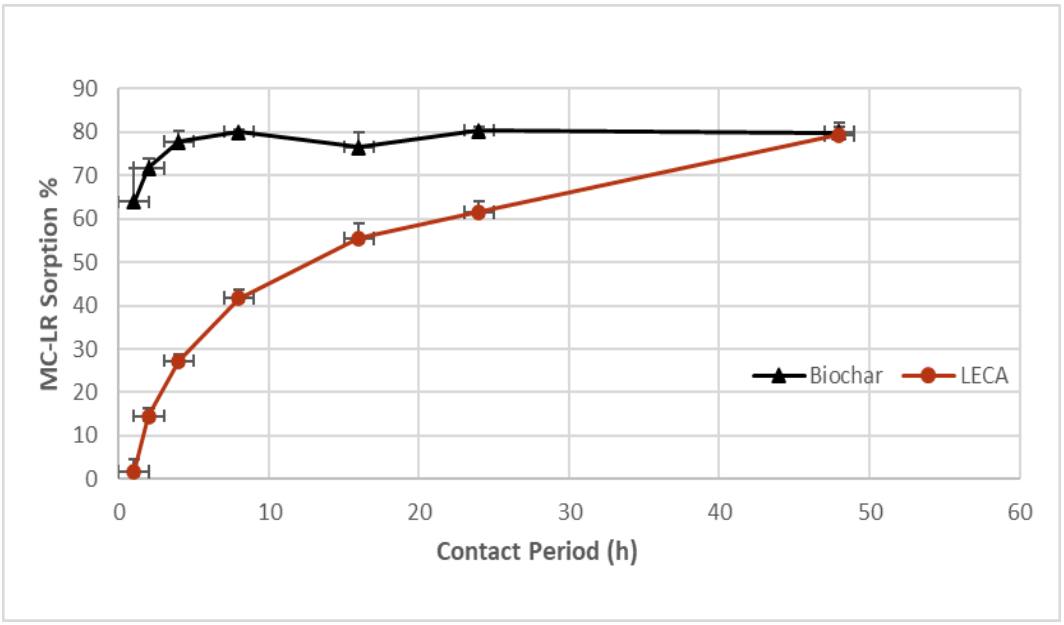
All in all, biochar and LECA were further studied for kinetics and different sorption isotherm models due to their high sorption capacity.

2.3.2. Kinetic Studies

Figure 4 shows the sorption of the cyanotoxins MC-LR and CYN by biochar and LECA at different contact periods up to 48 h. Results show that the sorption of MC-LR and CYN by biochar is rapid, occurring within the first 8 h and reaching equilibrium thereafter. By 8 h, biochar reached an average sorption of 80 % for MC-LR and 90 % for CYN. There

was no significant increase in the sorption rates from 8 h to 48 h with biochar. However, there was a significant increase in the sorption rates from 8 h to 48 h with LECA. Sorption by LECA increased more steadily before reaching equilibrium at 48 h. Biochar sorbed less MC-LR (80 %) than CYN (95 %) from 16 h. LECA sorbed equally MC-LR (83 %) and CYN (80 %).

a)



b)

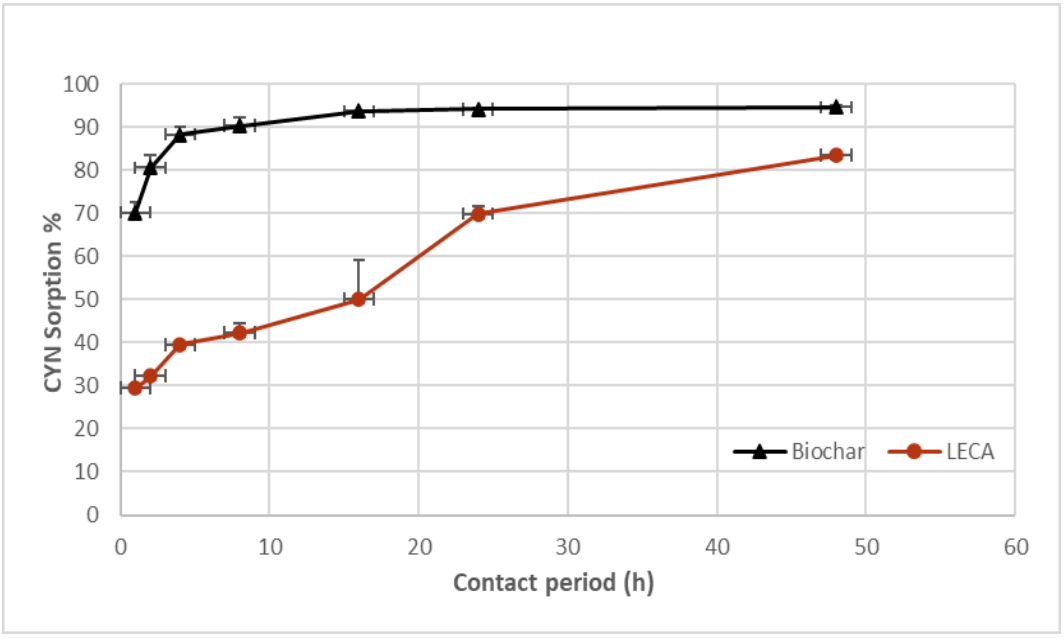


Figure 4.Sorption kinetics of MC-LR (a), and CYN (b) on to biochar and LECA (mean and standard deviation, n=3)

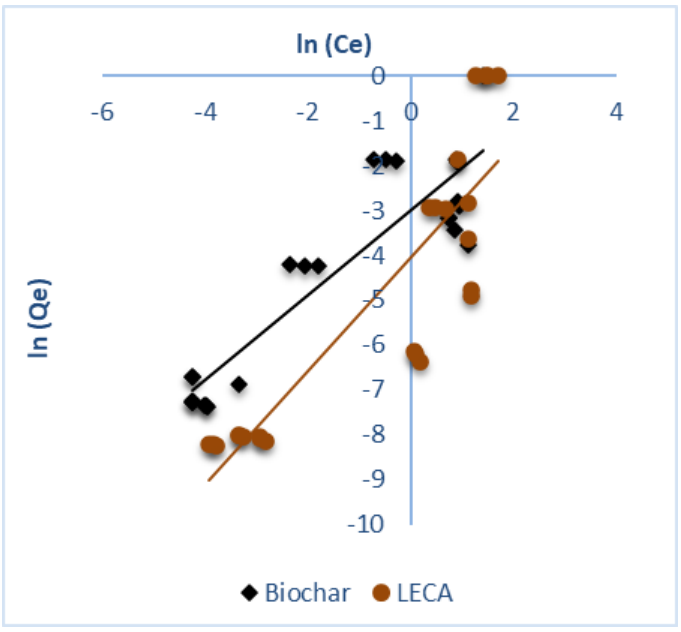
The pseudo-first order and pseudo-second-order sorption kinetics [equations (8) and (9), Table 1] resulted in the parameter fitting summarized in Table 2, while graphics are shown in Figure4a and b. It can be deduced that the pseudo-second-order kinetic model fits the data better than the pseudo-first order kinetics model, giving R^2 between 0.94-0.99 (appendix A- figures A1 and A2).

Table 2. Kinetic parameters of MC-LR and CYN onto biochar and LECA

Sorbent		**Pseudo-First-Order Kinetic Model**				**Pseudo-Second-Order Kinetic Model**		
		q_e (mg/g)	**q_e(cal) (mg/g)**	**k_1 (1/ h)**	**R^2**	**q_e(cal) (mg/g)**	**k_2 (g/ mg/ h)**	**R^2**
Biochar	**MC-LR**	0.0007	0.0001	0.0010	0.33	0.0006	6736	0.99
	CYN	0.0034	0.0007	0.0018	0.95	0.0035	497	0.99
LECA	**MC-LR**	0.0004	0.0004	0.0013	0.95	0.0005	210	0.98

CYN	0.0012	0.0012	0.0022	0.92	0.0012	134	0.94

a)



b)

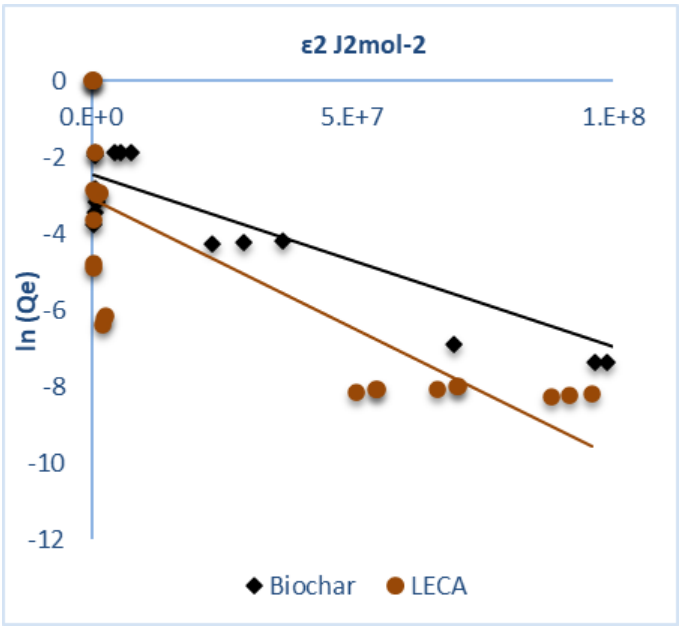
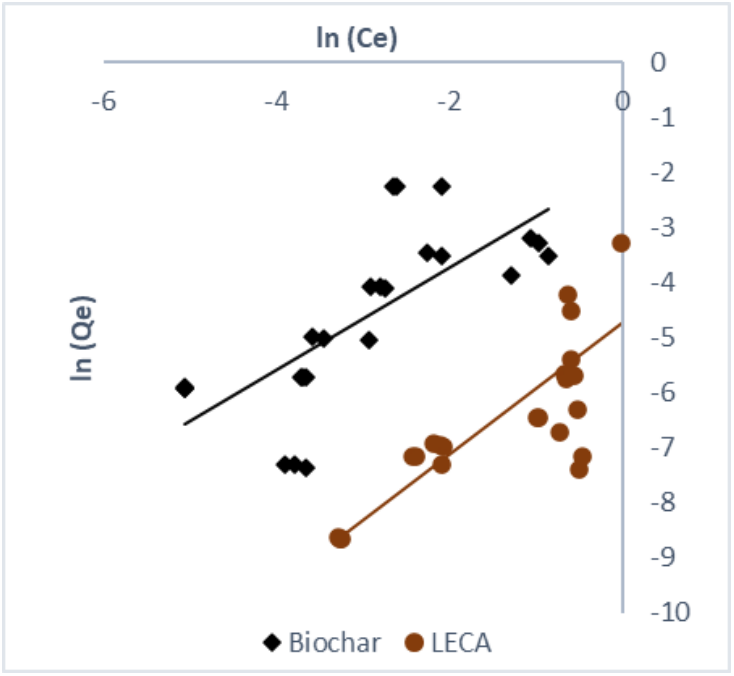


Figure 5.Sorption isotherm plots of MC-LR onto LECA and biochar: a) Freundlich, b) D-R

a)



b)

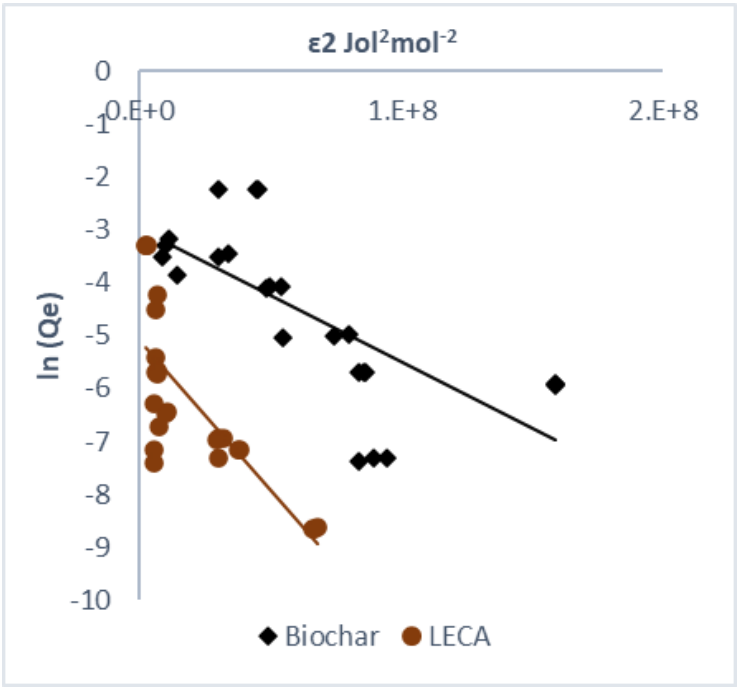


Figure 6. Sorption isotherm plots of CYN onto LECA and biochar: a) Freundlich, b) D-R

2.3.3. Sorption Isotherms

Four sorption models (Langmuir, Freundlich, D-R, and Temkin) were applied to the experimental data to understand the favorability of MC-LR and CYN sorption onto biochar and LECA, and potential processes involved. Detailed modelling results of

sorbents biochar and LECA are given in Table 3. The sorption isotherm plots that had the best fit for the sorption of MC-LR by biochar and LECA, Freundlich and Dubinin-Radushkevich (D-R), are shown in Figure 5 and of CYN by biochar and LECA are shown in Figure 6 The isotherm plots that did not fit the data well are in the supplementary information (Figure S3 and S4). The sorption capacity, Kf, were estimated to be 0.05 and 0.16 L/g for MC-LR and CYN for biochar and 0.02 and 0.01 L/g for MC-LR and CYN for LECA; and the Q_{max} were estimated to be 0.09 and 0.05 mg/g for MC-LR and CYN for biochar and 0.05 and 0.01 mg/g for MC-LR and CYN for LECA.

Table 3. Isotherm modelling results for MC-LR and CYN sorption by biochar and LECA

Isotherm Model	Sorbent	Cyanotoxin	R^2	q_{max} (mg/g)	b	R_L
Langmuir	Biochar	MC-LR	0.06	0.164	0.276	0.162
		CYN	$2e^{-04}$	0.802	0.141	0.359
	LECA	MC-LR	0.09	0.024	0.193	0.238
		CYN	$1e^{-05}$	0.469	0.009	0.897
			R^2	**K_f (L/g)**	**n**	**1/n**
Freundlich	Biochar	MC-LR	0.83	0.050	1.056	0.95
		CYN	0.53	0.155	1.076	0.93
	LECA	MC-LR	0.77	0.017	0.793	1.26
		CYN	0.67	0.009	0.840	1.19
			R^2	**Q_{max} (mg/g)**	**K_{DR}**	
Dubinin-Radushkevich (D-R)	Biochar	MC-LR	0.87	0.092	$5e^{-08}$	
		CYN	0.51	0.051	$3e^{-08}$	
	LECA	MC-LR	0.62	0.045	$7e^{-08}$	
		CYN	0.58	0.006	$6e^{-08}$	
			R^2	**A_T (L/g)**	**B_T**	
Temkin	Biochar	MC-LR	0.01	116.5	4.758	
		CYN	0.23	129.6	4.865	
	LECA	MC-LR	0.01	298.9	5.700	
		CYN	0.34	9.897	2.292	

2.3.4. SEM/EDS of Sorbents

SEM/EDS characterization was done to compare the surface morphology and surface elemental composition among the sorbent materials. The results show the distribution of the elemental composition on the different surfaces of each of the materials. The different surfaces of each sorbent material are marked as Z (1,2, 3…) on the SEM images. This section focuses on the SEM/EDS results of biochar and LECA. The SEM/EDS results of rice husk, cork, OP, sand, and the EDS results of biochar and LECA, can be found in the appendix A (Table A1).

2.3.4.1. Biochar-

Two perspectives of the biochar could be observed in the SEM image (Figure 7). The horizontal part of the biochar represented the smooth part of the wood, or the surface (Z2), and the vertical part represented the longitudinal part of the wood that has channels (Z1). The latter part had more pores than the former. Differences in the element composition can be observed in both parts as well (Figure A10 a-b).

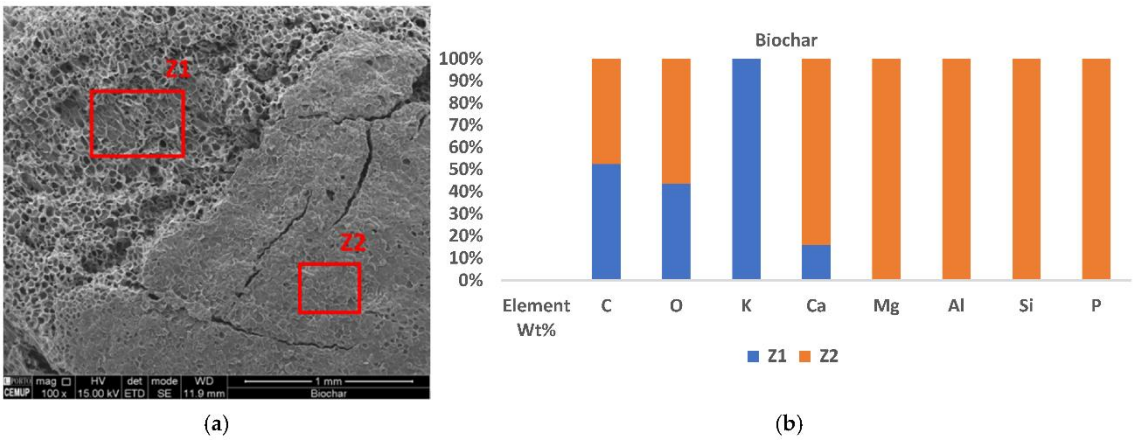


Figure 7. (a) SEM image of biochar and (b) elemental composition of Z1 and Z2 of biochar.

2.3.4.2. LECA-

Since LECA was broken down, there were differences in surface morphologies and element composition in the outer (brown) (Figure 8a) and inner (grey) (Figure 8b) parts. The difference in element composition on the outer and inner surfaces can be seen in the backscattered electron diffraction mode SEM images (Figure 8a, b and c) and the EDS graphs (Figure A9 a-d). The brighter spots on the SEM image (Z1 and Z3) denote the concentration of elements of higher atomic number, and the darker spots on the SEM image denote the concentration of elements of lower atomic number, in the respective areas.

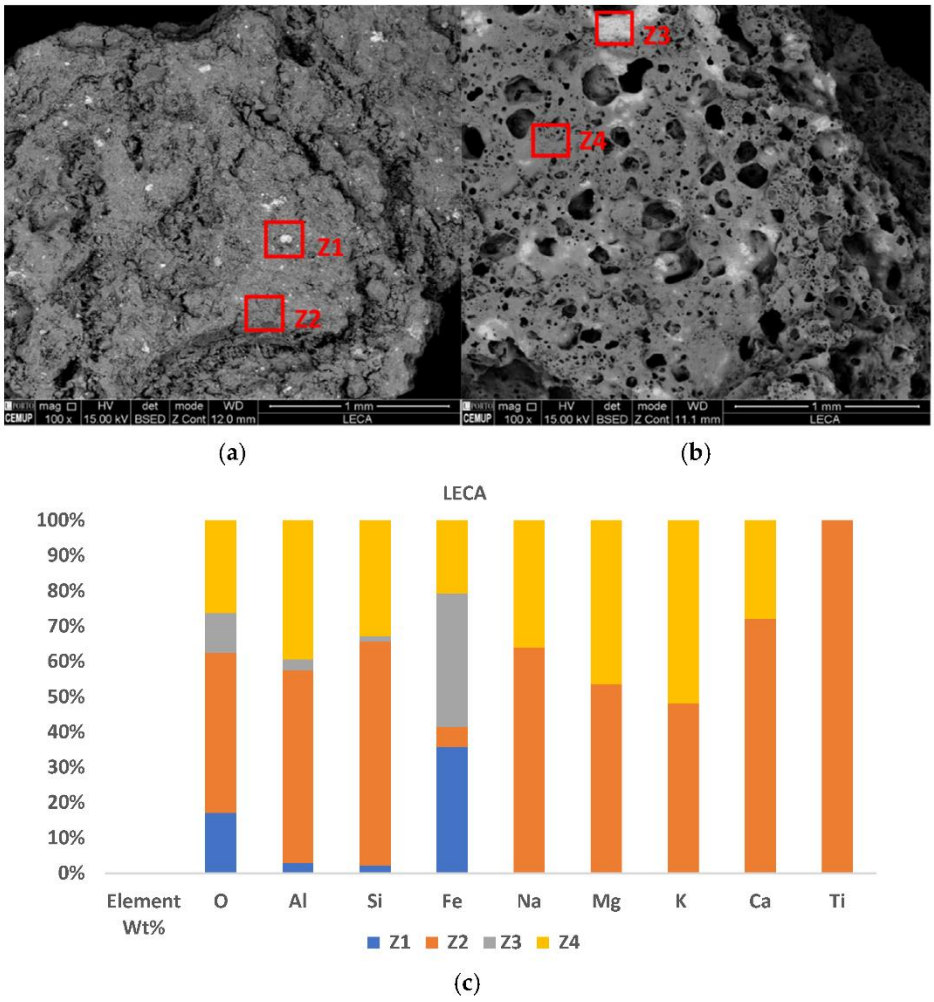


Figure 8. a) SEM images of outer part of LECA; b) SEM image of inner part of LECA; c) elemental composition of Z1, Z2, Z3, and Z4 of LECA

2.4. Discussion

2.4.1. Pre-screening of Sorbents

The pre-screening reports the sorption potential of cyanotoxins in agro-based waste materials that can be incorporated as substrates materials in NBS (Figure 3). To the best of our knowledge, this is the first report on the sorption capacity of MC-LR and CYN by cork granules, and OP; as well as to benchmark them against NBS common substrates such as LECA and sand.

In contrast to the study by Palagama et.al.,[33] which had >50 % sorption of MC-LR, the present study had lower sorption of MC-LR by rice husk (4 %). However, Palagama

et al. [33] also showed a significant improvement in MC-LR sorption in treated rice husks over raw rice husks. The MC concentrations and sorbent amounts used also varied highly between the studies. CYN sorption by rice husks was presently studied for the first time, showing limited sorption capacity (below 10%).

In general, sorption of MC-LR is governed by mesopore filling, hydrogen bonding, π–π interactions, hydrophobic interactions (induced by carbon materials), electrostatic attraction, and dispersive interactions [31]. According to the literature, due to the molecular size of MC-LR (ranging from 1.3 to 2.94 nm in diameter), mesoporous sorbents (diameter between 2 and 50 nm) have been verified to preferentially adsorb MC-LR, rather than micro- or macropores [52]. In this study, materials such as rice husk, OP, and sand lacked porosity (Figures A5a, A7a and A8a), cork was macroporous (Figure A6a), and biochar and LECA had an uneven distribution of smooth areas, macropores, and mesopores (Figure 7 and Figure 8). Regarding CYN sorption, it is considered to be weaker in comparison to other cyanotoxins due to its high water-solubility and zwitterionic properties. CYN sorption is primarily driven by interactions with organic carbon. Moreover, for CYN, the literature indicates that carbon-based materials with a greater volume of mesopores is better suited for sorption of the molecule [53]. Thus, the reason why agro-waste materials such as rice husk, OP, and cork did not show significant sorption potential could be because they were not activated by chemical/thermal modification; this significantly improves sorption capacity by increasing surface area (porosity), accessible active sites, removal of pre-adsorbed substances (thermal activation), and activation for better selectivity [54]. However, doing so would increase the costs and complexity of production, hence decreasing the interest in potentially incorporating them in NBSUs. While there have been several studies on the sorption of cyanotoxins by chemically modified sorbents [10], they are less attractive for application in pilot or full-scale NBSUs due to their high manufacturing costs and energy consumption. Therefore, agro-waste materials could potentially be interesting for direct usage in NBSUs if there would be potential without activation, but that was not the case in the present study for the selected materials.

On the other hand, biochar and LECA showed good sorption of MC-LR and CYN. Biochar derived from various waste biomasses, including rice husk [55] and wood residues [21], have seen sorption of MCs and, in some cases, nodularin [55]. The sorption of CYN has not been studied in biochar and is reported here for the first time. As for LECA, the MC-LR and CYN sorption was reported for the first time in the present

study. Sand showed low sorption of MC-LR (26%) and CYN (1%) in the present study, which was comparable to previous MC-LR and CYN sorption studies with sand. Kumar et al. [56] showed that sand used as filter media could only remove <20% MC-LR during the sorption phase. In a study with natural sandy sediments [23], there was little to no sorption of CYN. Another study that evaluated sand from a ripened slow sand filter [27] showed that sorption by sand was not part of the removal process.

Due to their good sorption capacity, biochar and LECA were further studied. While their sorptive properties might change when being incorporated into mesocosm- or full-scale NBSUs, it is highly relevant to characterize their sorption potential and understand the kinetics and mechanisms of the sorption of cyanotoxins.

2.4.2. Kinetics

The kinetic studies were done to assess the effects of contact time and sorption rates of MC-LR and CYN. Biochar showed quicker sorption of both MC-LR and CYN than LECA. Data fitted the pseudo-second-order kinetic model better than the pseudo-first-order kinetic model, as revealed by the higher regression constant (R^2) (Table 2.). This is in accordance with the sorption kinetics of previous works of MC-LR sorption by biochar [16,17,33]. The pseudo-second-order model views chemisorption as the process's rate-limiting mechanism, whereas the pseudo-first-order model considers that physisorption controls the rate at which particles adhere to the sorbent [41]. The calculated q_e of the pseudo-second-order kinetic model was also closer to the experimental q_e (Table 2.). From this it is apparent that the sorption rate of both cyanotoxins onto biochar and LECA was limited by chemisorption. In some studies [17,18], MC-LR sorption onto biochar fitted well with the Elovich kinetic model, which is also a model that describes activated chemisorption processes and valid for heterogenous surfaces [57].

It should be noted that the present study only focuses on the sorption kinetics of cyanotoxins onto biochar and LECA. In a practical setting there would be other pollutants present in the water to be treated which would influence the sorption of cyanotoxins. Moreover, when incorporated in an NBS, components of the system other than the substrate could also influence the sorption kinetics. This includes vegetation, oxygen transfer, pollutant loading, flow direction, area, temperature, etc. Despite this point of argument, the information on cyanotoxin sorption kinetics and rate coefficients of biochar and LECA is valuable for the design configurations of NBS for water treatment, including just sorption. This includes determining factors such as the hydraulic retention time.

2.4.3. Sorption Isotherms

From the R^2 values in Table 3, the experimental data for MC-LR and CYN sorption by biochar and LECA fit the Freundlich and D-R isotherm models better than the Langmuir or Temkin model. This is explained by the heterogenous surfaces of biochar and LECA, which results in variable bond energies on the surface. The fundamental assumption of the Langmuir model implies that sorption follows a monolayer pattern and that there are a limited number of binding sites on the sorbent surface, hence, the solid surface eventually approaches saturation. The Langmuir model also implies that the sorbate molecules adsorbed on the surface do not interact laterally [43,44] and it does not take into consideration surface roughness or multilayer sorption. To overcome these constraints, models such as the Freundlich, D-R and Temkin were applied. The Temkin isotherm model assumes that as the sorbent surface is covered, the sorption heat drops linearly. Sorption heat is the amount of heat released when one unit of sorbate gets sorbed on the sorbent surface. It also assumes that the sorption is characterized by a uniform distribution of binding energies, up to a maximum binding energy. Although the Temkin isotherm model is used to describe multilayer sorption and is commonly used for the sorption of water contaminants; its dimensional inconsistency, approximate nature, poor fitting capacity, and widespread misquotation are known major drawbacks when using it [63].

Freundlich isotherm is commonly used to model sorption processes in environmental applications as it describes sorption onto heterogeneous surfaces with a non-uniform distribution of sorption sites [64]. It is the first known model that describes non-ideal and reversible sorption that is not limited to the formation of monolayers. According to the Freundlich isotherm, the amount sorbed is the total sorption on all sites (each with its own bond energy), with the stronger binding sites occupied first, until sorption energy is exponentially diminished at completion of the sorption process [64]. It also describes a physical type of sorption in which sorption occurs in several layers with weak links (multilayer). Presently, the Freundlich isotherm represented the experimental data quite well, as evidenced by R^2 values equal or greater than 0.7 in all cases except for CYN sorption by biochar, in Table 3. This is similar with previous studies of MC-LR sorption onto biochar where they were also well described with the Freundlich model [16, 17, 18, 19, 20]. The value of n indicates the magnitude of the sorption and the sorbent site energy distribution. From Table 3., it can be seen that for biochar the n values were 1.08 (MC-LR) and 1.07 (CYN) while for LECA the n values were 0.79 (MC-LR) and 0.84 (CYN), reflecting that the sorption intensity was higher in biochar. The 1/n values also

indicated that biochar has a slightly lower surface heterogeneity than LECA (in broken form), which is consistent with the SEM/EDS data (section 2.3.4). The sorption capacity constant Kf was observed to be higher for biochar (MC-LR=0.05, CYN=0.16) than LECA (MC-LR=0.02, CYN=0.01), consistent with the screening and kinetics data. Compared to the Kf values for MC-LR sorption by biochar in previous works the Kf value in this study is lower. However, not all studies had the similar isotherm model fits. Even among previous studies, the MC-LR sorption capacities and mechanisms differed with different feedstock and pyrolysis temperatures of the biochar. The biochars from these previous studies were generated from rice straw (Kf = 0.69-2.45) [16], iron activated biochar generated from Bermuda grass (Kf = 12.33) [17], kentucky bluegrass (Kf = 230.9) [18], pine sawdust (Kf = 1.52- 4.53), maize straw (Kf = 1.47-5.69), and chicken manure (Kf = 2.57-6.35) [20]. In one study with biochar generated from giant reeds, the Kf values (0.086) [21] were similar to this study.

The D-R model, like the Freundlich model, describes heterogeneous surfaces and can be useful at especially high-to-intermediate sorbate concentrations. Although D-R isotherm is used to differentiate between physical and chemical sorption of metal ions [45], it was considered for this study because it explains the sorption process on a porous sorbent or a heterogeneous surfaced sorbent and reflects the sorption free energy. It assumes that the sorption process is based on the filling of micropores. D-R isotherm fit the experimental data fairly well ($R^2 \geq 0.6$), except for CYN with biochar. K_{DR} is a constant linked to the free energy of sorption per unit of cyanotoxin when the ions migrate from infinity to the sorbent's surface [47]. Values of KDR < 1 on average suggest that the sorbent's surface has micropores. As seen in Table 3, K_{DR} values are less than unity which suggest that the micropores on the sorbent surfaces are high. This is in conformity with another work where MC-LR sorption by biochar fit the D-R model [17]. The Q_{max} values observed are theoretical saturation capacities at equilibrium in relation to the micropore volume of the sorbents [47].

It is important to note that the isotherm models have different underlying assumptions that govern them and therefore discrepancies between the isotherm constants and maximum sorption capacities (q_{max} for Langmuir, K_f, for Freundlich, Q_{max} for D-R, A_T for Temkin). Furthermore, the heterogeneity of both biochar and LECA, the impracticality of attaining precise initial cyanotoxin concentrations, as well as the lack of complete temperature control contributed to the low R^2 values obtained in the current work. Moreover, there were practical constraints in terms of achieving higher cyanotoxin concentrations than 10 mg/L for the sorption studies, due to the amount of biomass

available for this study. This prevented us from determining the maximal sorption capacities from the experimental data to determine the saturating concentration for the materials.

Having said the above, the heterogeneity of biochar and LECA is what makes them favourable sorbent materials that can be incorporated in NBSUs. The multilayer sorption is a characteristic that would be beneficial with the frequent variability in the nutrients as well as cyanotoxin concentrations due to the on/off cycles of eutrophication and toxic algal blooms in surface waters. Moreover, NBSUs also have other components contributing to the degradation or removal of cyanotoxins. Some examples of the other component mechanisms are microbial degradation, phytodegradation, phytoextraction, filtration, and sedimentation [61]. The sorption capacity, as well as the loose availability for biotic degradation, are ideal characteristics that biochar and LECA present in NBSUs.

2.4.4. Influence of Surface Element Composition and Morphology of Sorbents on Sorption Potential

From the SEM/EDS data, it can be observed that all the sorbent materials had its own characteristic surface morphology and elemental compositions. Characterization of the surface morphology was important because it plays an important role in impacting sorption characteristics, especially the surface roughness, texture and porosity [65]. The heterogenous surface morphology of biochar and LECA (Figure 7 and 8) confirms that the Freundlich and D-R isotherm represented the experimental data well. Moreover, porosity was observed in both biochar and LECA which is characteristic to the D-R model. Despite not having done any chemical modifications to the sorbents, the surface element composition was characterized to check the uniformity or distribution of elements on their surfaces. High variability in surface element composition even within a sorbent further indicated its heterogeneity. It also presented the differences in surface element composition between the sorbent materials. However, the surface elemental compositions between the materials were too different to make any correlations with sorption capacity.

Although the effect of pH on sorption of the cyanotoxins was not within the scope of this study, the pH of the sorbate (cyanotoxin solutions) was maintained at pH 7. At pH 7, MC-LR has a net negative charge and CYN is zwitterionic. While negatively charged MC-LR is more available to positively charged surfaces such as those of clays with positively charged elements, CYN is a more stable cyanotoxin at pH 7. This results in more MC-LR adsorption onto Ca-saturated clays than for instance, Na-saturated clays [66]. From

the surface elemental composition of biochar and LECA, it can be seen there is a higher presence of positively charged elements (Figure A9 and A10).

However, whether the surface element composition would have an influence on the sorption is not within the scope of this study. Most studies using agro-waste biomasses as sorbents, chemically modify it to improve its sorption capacity [33,43]. But doing so would go against the objective of the study which is to help sustainably manage agro-waste, be locally adapted and resource efficient; thereby bringing more natural features into water-treatment technologies. Therefore, in this study there were no chemical modifications done on the materials to keep the NBS a clean technology.

2.5. Conclusion

This study explores the valorization of agro-waste materials as cyanotoxin sorbents in NBSUs, also testing other sorbents commonly used in NBSUs, like LECA. It also brings mechanistic insights on the sorptive properties of these materials for removing cyanotoxins from water. The most widely studied cyanotoxin for sorption is microcystin. Even with widely studied sorbents like biochar, sorption of cylindrospermopsin has not been studied [10]. LECA has not previously been separately studied for its sorptive properties of cyanotoxins. Therefore, this work fills the gaps in knowledge regarding research on new sorbents for cyanotoxin removal, also extending the research on different toxins other than MC. From the pre-screening, the highest sorption was seen in biochar (MC-LR > 86%, CYN > 99%,) and LECA (MC-LR = 78%, CYN = 80%). The sorption of both the cyanotoxins onto biochar was rapid (8 h), whereas onto LECA it was steadier (requiring 48 h for equilibrium). The pseudo-second-order kinetic model fit the sorption of both cyanotoxins onto biochar and LECA, suggesting that the sorption rate is limited by chemisorption. Freundlich and D–R models fit the suggested multilayer sorption, high heterogeneity, and porosity in the sorbents (which was also confirmed by SEM/EDS). These characteristics would make them ideal to be incorporated as sorbents in NBSUs, keeping the pollutants available for complete degradation by other biotic components of the systems. The cost-effectiveness of biochar and LECA would depend very much on how the materials are applied in practice and the technology it is implemented in, whether completely or partially being replaced as the media in NBSUs, or used separately as an extra column providing post-treatment purely focused on sorption. Such assessment goes beyond the scope of this work, which was a first

exploratory step for the potential of the different materials. Therefore, at the moment, it is difficult to make economic evaluations. The applicability of these materials in NBSUs should be further considered for mesocosm- and pilot-scale systems. Especially, the sorption potential, the life-span, and durability of these materials in NBSUs deserves to be further studied.

Author Contributions:

Conceptualization, G.B., A.C., C.M.R.A., and P.N.C.; methodology, G.B., A.C., C.M.R.A., and P.N.C.; validation, G.B., A.C., C.M.R.A., and P.N.C.; formal analysis, G.B., J.A., and P.N.C.; investigation, G.B.; resources, A.C., C.M.R.A., and P.N.C.; data curation, G.B.; writing— original draft preparation, G.B.; writing—review and editing, A.C., C.M.R.A., and P.N.C.; visualization, G.B.; supervision, A.C., C.M.R.A., and P.N.C.; funding acquisition, A.C. All authors have read and agreed to the published version of the manuscript.

References

[1] Haque, F., Banayan, S., Yee, J., & Chiang, Y. W. (2017). Extraction and applications of cyanotoxins and other cyanobacterial secondary metabolites. Chemosphere, 183, 164-175

[2] Mowe, M. A. D., Mitrovic, S. M., Lim, R. P., Furey, A., & Yeo, D. C. J. (2015). Tropical cyanobacterial blooms: A review of prevalence, problem taxa, toxins and influencing environmental factors. In Journal of Limnology (Vol. 74, Issue 2, pp. 205–224)

[3] Abdallah, M. F., Van Hassel, W. H. R., Andjelkovic, M., Wilmotte, A., & Rajkovic, A. (2021). Cyanotoxins and Food Con-tamination in Developing Countries: Review of Their Types, Toxicity, Analysis, Occurrence and Mitigation Strategies. Toxins, 13(11), 786. https://doi.org/10.3390/toxins13110786

[4] Cyanobacterial toxins: microcystins. Background document for development of WHO Guidelines for drinking-water quality and Guidelines for safe recreational water environments. Geneva: World Health Organization; 2020 (WHO/HEP/ECH/WSH/2020.6). Licence: CC BY-NCSA 3.0 IGO.

[5] Brient, L., Lengronne, M., Bormans, M., & Fastner, J. (2009). First occurrence of cylindrospermopsin in freshwater in France. Environmental Toxicology: An International Journal , 24 (4), 415-420

[6] Dunlop, T., Khojasteh, D., Cohen-Shacham, E., Glamore, W., Haghani, M., van den Bosch, M., Rizzi, D., Greve, P., & Felder, S. (2024). The evolution and future of research on Nature-based Solutions to address societal challenges. Communications Earth and Environment, 5(1). https://doi.org/10.1038/s43247-024-01308-8

[7] Nature-Based Solutions for Wastewater Treatment doi.org/10.2166/9781789062267

[8] Wang, J., Long, Y., Yu, G., Wang, G., Zhou, Z., Li, P., Zhang, Y., Yang, K., & Wang, S. (2022). A Review on Microorganisms in Constructed Wetlands for Typical Pollutant Removal: Species, Function, and Diversity. Frontiers in microbiology, 13, 845725. https://doi.org/10.3389/fmicb.2022.845725

[9] de Gisi, S., Lofrano, G., Grassi, M., & Notarnicola, M. (2016). Characteristics and adsorption capacities of low-cost sorbents for wastewater treatment: A review. In Sustainable Materials and Technologies (Vol. 9, pp. 10–40). Elsevier B.V. https://doi.org/10.1016/j.susmat.2016.06.002

[10] Abbas, T., Kajjumba, G. W., Ejjada, M., Masrura, S. U., Marti, E. J., Khan, E., & Jones-lepp, T. L. (2020). Recent advancements in the removal of cyanotoxins from water

using conventional and modified adsorbents—a contemporary review. In Water (Switzerland) (Vol. 12, Issue 10). MDPI AG. https://doi.org/10.3390/w12102756

[11] Couto, R. S. P., Guarino, A., Branco, W. S., Palermo, C. W. C., & Azero, E. F. A. (2013). Application of Clay Minerals and Polymeric Resins to Remove Dissolved Microcystin-LR from Water. Int. J. Environ. Res, 7(2), 435–442

[12] Wu, X., Xiao, B., Li, R., Wang, C., Huang, J., & Wang, Z. (2011). Mechanisms and factors affecting sorption of microcystins onto natural sediments. Environmental Science and Technology, 45(7), 2641–2647. https://doi.org/10.1021/es103729m

[13] Gęca, M., Wiśniewska, M., & Nowicki, P. (2022). Biochars and activated carbons as adsorbents of inorganic and organic compounds from multicomponent systems – A review. In Advances in Colloid and Interface Science (Vol. 305). Elsevier B.V. https://doi.org/10.1016/j.cis.2022.102687

[14] Jagadeesh, N. & Sundaram, B. Adsorption of pollutants from wastewater by biochar: A review. J. Hazard. Mater. Adv. 9, 100226. https://doi.org/10.1016/j.hazadv.2022.100226 (2023)

[15] Yaashikaa, P. R., Kumar, P. S., Varjani, S. & Saravanan, A. A critical review on the biochar production techniques, charac-terization, stability and applications for circular bioeconomy. Biotechnol. Rep. (Amst) 28, e00570. https://doi.org/10.1016/j.btre.2020.e00570 (2020)

[16] Wei, L. & Lu, J. Adsorption of microcystin-LR by rice straw biochars with different pyrolysis temperatures, (in en). Environ. Technol. Innov. 23, 101609. https://doi.org/10.1016/j.eti.2021.101609 (2021)

[17] Zeng, S. & Kan, E. Adsorption and regeneration on iron-activated biochar for removal of microcystin-LR, (in en). Chemosphere 273, 129649. https://doi.org/10.1016/j.chemosphere.2021.129649 (2021)

[18] Song, H. J. et al. Treatment of microcystin-LR cyanotoxin contaminated water using Kentucky bluegrass-derived biochar, (in en). J. Water Process Eng. 41, 102054. https://doi.org/10.1016/j.jwpe.2021.102054 (2021)

[19] Liu, G., Zheng, H., Zhai, X., & Wang, Z. (2018). Characteristics and mechanisms of microcystin-LR adsorption by giant reed-derived biochars: Role of minerals, pores, and functional groups. Journal of Cleaner Production, 176, 463–473. https://doi.org/10.1016/j.jclepro.2017.12.156

[20] Li, J., Cao, L., Yuan, Y., Wang, R., Wen, Y., & Man, J. (2018). Comparative study for microcystin-LR sorption onto biochars produced from various plant- and animal-wastes at different pyrolysis temperatures: Influencing mechanisms of biochar

properties. Bioresource Technology, 247, 794–803. https://doi.org/10.1016/j.biortech.2017.09.120

[21] Li, L., Qiu, Y., Huang, J., Li, F., & Sheng, G. D. (2014). Mechanisms and Factors Influencing Adsorption of Microcystin-LR on Biochars. Water, Air, and Soil Pollution, 225(12). https://doi.org/10.1007/s11270-014-2220-6

[22] Hagemann, N.; Schmidt, H.-P.; Kägi, R.; Böhler, M.; Sigmund, G.; Maccagnan, A.; McArdell, C.S.; Bucheli, T.D. Wood-based activated biochar to eliminate organic micropollutants from biologically treated wastewater. *Sci. Total Environ.* **2020**, *730*, 138417.

[23] Klitzke, S., Beusch, C., & Fastner, J. (2011). Sorption of the cyanobacterial toxins cylindrospermopsin and anatoxin-a to sediments. Water Research, 45(3), 1338–1346. https://doi.org/10.1016/j.watres.2010.10.019

[24] Kataki, S., Chatterjee, S., Vairale, M. G., Dwivedi, S. K., & Gupta, D. K. (2021). Constructed wetland, an eco-technology for wastewater treatment: A review on types of wastewater treated and components of the technology (macrophyte, biolfilm and substrate). Journal of Environmental Management, 283, 111986

[25] Mlih, R., Bydalek, F., Klumpp, E., Yaghi, N., Bol, R., & Wenk, J. (2020). Light-expanded clay aggregate (LECA) as a substrate in constructed wetlands – A review. In Ecological Engineering (Vol. 148). Elsevier B.V. https://doi.org/10.1016/j.ecoleng.2020.105783

[26] Deng, S., Chen, J., & Chang, J. (2021). Application of biochar as an innovative substrate in constructed wetlands/biofilters for wastewater treatment: Performance and ecological benefits. Journal of Cleaner Production, 293, 126156.

[27] Valencia-Cárdenas, D., Tavares, T. S., Silveira, R., Brandão, C. C. S., Soares, R. M., & Ginoris, Y. P. (2023). Evaluation of the Removal and Effects of Cylindrospermopsin on Ripened Slow Sand Filters. Toxins, 15(9). https://doi.org/10.3390/toxins15090543

[28] Pavagadhi, S., Tang, A. L. L., Sathishkumar, M., Loh, K. P., & Balasubramanian, R. (2013). Removal of microcystin-LR and microcystin-RR by graphene oxide: adsorption and kinetic experiments. Water research, 47(13), 4621-4629

[29] Gao, Y. Q., Gao, N. Y., Deng, Y., Gu, J. S., Shen, Y. C., & Wang, S. X. (2012). Adsorption of Microcystin-LR from Water with Iron Oxide Nanoparticles. Water environment research, 84(7), 562-568

[30] Lee, J., & Walker, H. W. (2011). Adsorption of microcystin-Lr onto iron oxide nanoparticles. Colloids and Surfaces A: Physicochemical and Engineering Aspects, 373(1-3), 94-100

[31] Park, J. A., Kang, J. K., Jung, S. M., Choi, J. W., Lee, S. H., Yargeau, V., & Kim, S. B. (2020). Investigating Microcystin-LR adsorption mechanisms on mesoporous carbon, mesoporous silica, and their amino-functionalized form: Surface chemistry, pore structures, and molecular characteristics. Chemosphere, 247, 125811

[32] Smith, S. C., & Rodrigues, D. F. (2015). Carbon-based nanomaterials for removal of chemical and biological contaminants from water: A review of mechanisms and applications. Carbon, 91, 122-143

[33] Palagama, D. S. W., Devasurendra, A. M., Baliu-Rodriguez, D., Kirchhoff, J. R., Isailovic, D. (2019). Treated rice husk as a recyclable sorbent for the removal of microcystins from water. Science of the Total Environment, 1292–1300. https://doi.org/10.1016/j.scitotenv.2019.02.042

[34] Prodana, M., Bastos, A. C., Amaro, A., Cardoso, D., Morgado, R., Machado, A. L., Verheijen, F. G. A., Keizer, J. J., & Loureiro, S. (2019). Biomonitoring tools for biochar and biochar-compost amended soil under viticulture: Looking at exposure and effects. Applied Soil Ecology, 137, 120–128. https://doi.org/10.1016/j.apsoil.2019.01.007

[35] Ramos, V.; Morais, J.; Castelo-Branco, R.; Pinheiro, Â.; Martins, J.; Regueiras, A.; Pereira, A.L.; Lopes, V.R.; Frazão, B.; Gomes, D.; et al. Cyanobacterial diversity held in microbial biological resource centers as a biotechnological asset: The case study of the newly established LEGE culture collection. *J. Appl. Phycol.* **2018**, *30*, 1437–1451.

[36] Kotai, J. (1972). Instructions for preparation of modified nutrient solution Z8 for algae. Norwegian Institute for Water Research, Oslo, 11(69), 5.

[37] Welker, M., Fastner, J., Erhard, M., & von Döhren, H. (2002). Applications of MALDI-TOF MS analysis in cyanotoxin re-search. Environmental Toxicology: An International Journal, 17(4), 367-374.

[38] Ramanan, S., Tang, J., & Velayudhan, A. (2000). Isolation and preparative purification of microcystin variants. Journal of Chromatography A, 883(1-2), 103-112.

[39] Bavithra, G., Azevedo, J., Oliveira, F., Morais, J., Pinto, E., Ferreira, I. M., ... & Almeida, C. M. R. (2019). Assessment of Constructed Wetlands' Potential for the Removal of Cyanobacteria and Microcystins (MC-LR). Water, 12(1), 10

[40] Machado, J., Campos, A., Vasconcelos, V., & Freitas, M. (2017). Effects of microcystin-LR and cylindrospermopsin on plant-soil systems: A review of their

relevance for agricultural plant quality and public health. Environmental research, 153, 191-204

[41] Yang, Q., Zhao, H., Peng, Q., Chen, G., Liu, J., Cao, X., Xiong, S., Li, G., & Liu, Q. (2024). Elimination of Pharmaceutical Compounds from Aqueous Solution through Novel Functionalized Pitch-Based Porous Adsorbents: Kinetic, Isotherm, Thermodynamic Studies and Mechanism Analysis. Molecules, 29(2). https://doi.org/10.3390/molecules29020463

[42] Rwiza, M. J., Oh, S. Y., Kim, K. W., & Kim, S. D. (2018). Comparative sorption isotherms and removal studies for Pb(II) by physical and thermochemical modification of low-cost agro-wastes from Tanzania. Chemosphere, 195, 135–145. https://doi.org/10.1016/j.chemosphere.2017.12.043

[43] Gupta, V. K., Mittal, A., Jain, R., Mathur, M., & Sikarwar, S. (2006). Adsorption of Safranin-T from wastewater using waste materials- activated carbon and activated rice husks. Journal of Colloid and Interface Science, 303(1), 80–86. https://doi.org/10.1016/j.jcis.2006.07.036

[44] Langmuir, I. (1918). The adsorption of gases on plane surfaces of glass, mica and platinum. Journal of the American Chemical society, 40(9), 1361-1403

[45] Ayawei, N., Ebelegi, A. N., & Wankasi, D. (2017). Modelling and interpretation of adsorption isotherms. Journal of chemistry, 2017

[46] Sheha, R. R., & El-Shazly, E. A. (2010). Kinetics and equilibrium modeling of Se (IV) removal from aqueous solutions using metal oxides. Chemical Engineering Journal, 160(1), 63-71

[47] Foo, K.Y., Hameed, B.H., 2010. Insights into the modeling of adsorption isotherm systems. Chem. Eng. J. 156, 2e10

[48] Lawton, L.A.; Edwards, C.; Codd, A. Extraction and high-performance liquid chromatographic method for the determination of micro-cystins in raw and treated waters. Analyst 1994, 119, 1525–1530

[49] Guzmán-Guillén R, Prieto AI, González AG, Soria-Díaz ME, Cameán AM. Cylindrospermopsin determination in water by LC-MS/MS: optimization and validation of the method and application to real samples. Environ Toxicol Chem. 2012 Oct;31(10):2233-8. doi: 10.1002/etc.1954. Epub 2012 Aug 19. PMID: 22825923

[50] Pekar, H., Westerberg, E., Bruno, O., Lääne, A., Persson, K. M., Sundström, L. F., & Thim, A. M. (2016). Fast, rugged and sensitive ultra high-pressure liquid chromatography tandem mass spectrometry method for analysis of cyanotoxins in raw water and drinking water—First findings of anatoxins, cylindrospermopsins and

microcystin variants in Swedish source waters and infiltration ponds. Journal of Chromatography A, 1429, 265-276.

[51] Zervou, S. K., Christophoridis, C., Kaloudis, T., Triantis, T. M., & Hiskia, A. (2017). New SPE-LC-MS/MS method for sim-ultaneous determination of multi-class cyanobacterial and algal toxins. Journal of hazardous materials, 323, 56-66.

[52] El Bouaidi, W.; Enaime, G.; Loudiki, M.; Yaacoubi, A.; Douma, M.; Ounas, A.; Lübken, M. Adsorbents Used for Microcystin Removal from Water Sources: Current Knowledge and Future Prospects. *Processes* **2022**, *10*, 1235.

[53] Antonieti, C.C.; Ginoris, Y.P. Removal of Cylindrospermopsin by Adsorption on Granular Activated Carbon, Selection of Carbons and Estimated Fixed-Bed Breakthrough. *Water* **2022**, *14*, 1630.

[54] Xie, R.; Jin, Y.; Chen, Y.; Jiang, W. The importance of surface functional groups in the adsorption of copper onto walnut shell derived activated carbon. *Water Sci. Technol.* **2017**, *76*, 3022–3034.

[55] Thenuwara, S. I., Kiridena, H. S., Kirchhoff, J. R., & Isailović, D. (2022). Evaluation of the mechanisms of adsorption of microcystins and nodularin-R onto rice husk-based biochar. Environmental Advances, 9. https://doi.org/10.1016/j.envadv.2022.100314

[56] Kumar, P., Pérez, J. A. E., Cledon, M., Brar, S. K., Duy, S. V., Sauvé, S., & Knystautas, É. (2020). Removal of microcystin-LR and other water pollutants using sand coated with bio-optimized carbon submicron particles: Graphene oxide and reduced graphene oxide. Chemical Engineering Journal, 397. https://doi.org/10.1016/j.cej.2020.125398

[57] Al-Harby, N. F., Albahly, E. F., & Mohamed, N. A. (2021). Kinetics, Isotherm and Thermodynamic Studiesfor Efficient Ad-sorption of Congo Red Dye from Aqueous Solution onto Novel Cyanoguanidine-Modified Chitosan Adsorbent. Polymers, 13(24), 4446. https://doi.org/10.3390/polym13244446

[58] Esfandiar, N.; Suri, R.; McKenzie, E.R. Competitive sorption of Cd, Cr, Cu, Ni, Pb and Zn from stormwater runoff by five low-cost sorbents; Effects of co-contaminants, humic acid, salinity and pH. *J. Hazard. Mater.* **2022**, *423*, 126938.

[59] Campinas, M.; Viegas, R.M.; Rosa, M.J. Modelling and understanding the competitive adsorption of microcystins and tannic acid. *Water Res.* **2013**, *47*, 5690–5699

[60] Orta-Ortiz, M.S.; Geneletti, D. What variables matter when designing nature-based solutions for stormwater management? A review of impacts on ecosystem services. *Environ. Impact Assess. Rev.* **2022**, *95*, 106802.

[61] Varma, M.; Gupta, A.K.; Ghosal, P.S.; Majumder, A. A review on performance of constructed wetlands in tropical and cold climate: Insights of mechanism, role of influencing factors, and system modification in low temperature. *Sci. Total Environ.* **2021**, *755*, 142540.

[62] Frazer-Williams, R.A. A Review of the Influence of Design Parameters on the Performance of Constructed Wetlands. *J. Chem. Eng.* **1970**, *25*, 29–42.

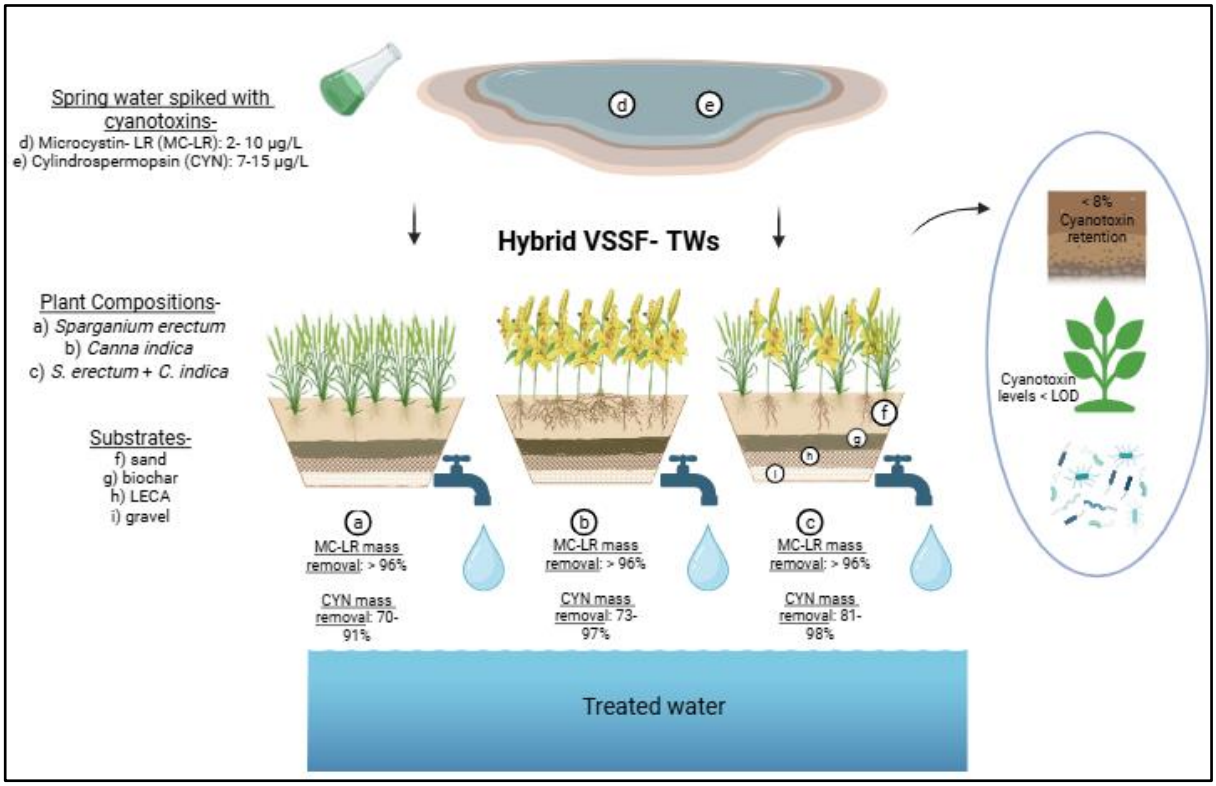
[63] Chu, K. H. (2021). Revisiting the Temkin Isotherm: Dimensional Inconsistency and Approximate Forms. Industrial and Engineering Chemistry Research, 60(35). https://doi.org/10.1021/acs.iecr.1c01788

[64] Vigdorowitsch, M., Pchelintsev, A., Tsygankova, L., & Tanygina, E. (2021). Freundlich isotherm: An adsorption model complete framework. In Applied Sciences (Switzerland) (Vol. 11, Issue 17). MDPI. https://doi.org/10.3390/app11178078

[65] Wang, F., Wu, P., Shu, L., Guo, Q., Huang, D., & Liu, H. (2022). Isotherm, kinetics, and adsorption mechanism studies of diethylenetriaminepentaacetic acid-modified banana/pomegranate peels as efficient adsorbents for removing Cd(II) and Ni(II) from aqueous solution. Environmental science and pollution research international, 29(2), 3051–3061. https://doi.org/10.1007/s11356-021-15766-6

[66] Liu, Y. L., Walker, H. W., & Lenhart, J. J. (2019). Adsorption of microcystin-LR onto kaolinite, illite and montmorillonite. Chemosphere, 220, 696–705. https://doi.org/10.1016/j.chemosphere.2018.12.137

Chapter 3. Hybrid Vertical Subsurface Flow Treatment Wetlands for Cyanotoxin Remediation: Plant Composition, Microbial Dynamics, and Removal Efficiency



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Guna Bavithra, Diogo A. M. Alexandrino, Maria de Fátima Carvalho, Rafael J. Mendes, Conceição Santos, Leticia Díez-Quijada, Ángeles Jos, Ana M. Cameán, Alexandre Campos, Pedro N. Carvalho, C. Marisa R. Almeida. “Hybrid Vertical Subsurface Flow Treatment Wetlands for Cyanotoxin Remediation: Plant Composition, Microbial Dynamics, and Removal Efficiency”.

Abstract

Harmful cyanobacterial blooms will become increasingly severe and frequent in the future, affecting vulnerable populations that depend primarily on surface-water supplies. Treatment wetlands are established as a low-cost decentralized treatment technology for the remediation of cyanotoxins. In this study, nine vertical subsurface flow treatment wetlands (VSSF-TW) microcosms were set-up with gravel, light expanded clay, biochar and sand, testing *Sparganium erectum* and *Canna indica* (as monocultures and polycultures) for the removal of MC-LR and CYN. Spring water spiked with environmentally relevant concentrations of MC-LR and CYN (2-15 µg /L) was treated over a course of 10-weeks. Cyanotoxin water removal rates and cyanotoxins substrate matrices' retention and plant accumulation of the different treatment conditions were determined. Water physicochemical parameters (pH, chemical oxygen demand (COD), oxidative-reductive potential (ORP), and nutrients) and plant status were monitored, and the microbiome of TW substrates was also investigated. TWs achieved high MC-LR mass removal rates in all different TWs (> 96 %). CYN mass removal rates were higher in TWs with *C. indica* (73- 98 %) than in TW with just *S. erectum* (70-91 %), especially over time. Water physico-chemical showed TWs were performing well, with no significant impact by cyanotoxins presence (parameters, in general, identical among TWs). Substrate retention of MC-LR and CYN were generally low (< 8 %), with *S. erectum* TW showing significantly higher retention than *C. indica* TW or the polyculture TW. *C. indica*, especially of the polyculture TWs, showed better resilience and plant stress adaptability to TW conditions treating cyanotoxins. However, no cyanotoxin was detected in the plant tissues (leaves and roots). The TW microbiome showed a marked shift over time, between the different substrate matrices of the TWs, and between the different plant compositions at the plant's rhizosphere level. The genes associated with the known mlr degradation pathway for MC-LR degradation were not found in the substrates, but known MC-LR degrading taxa (*Sphingomonas*, *Novosphingobium* and *Shinella*) and Mn-oxidizing bacteria (*Hyphomicrobium*) that have known potential to degrade CYN, were enriched in TW substrate. TWs with *C. indica* substrate also exhibited enriched plant-growth promoting taxa (*Leifsonia, Terrabacter, Altererythrobacter)*, further indicating their stress tolerance to the TW conditions treating cyanotoxins. Multiple taxa linked to mlr-independent cyanotoxin degradation, including *Sphingorhabdus* and *Porphyrobacter*, as well as taxa associated with harmful algal blooms (HAB) such as *Ilumatobacteraceae, Roseomonas,* and *Obscuribacteraceae*, were enriched in the systems following cyanotoxin exposure relative to pre-exposure levels, indicating

potential alternative unidentified cyanotoxin degrading mechanisms that warrant further investigation. VSSF-TWs planted with *C. indica* exhibited higher efficiency and adaptability in treating cyanotoxin-contaminated surface waters, warranting further pilot-scale investigations to evaluate evapotranspiration dynamics and effluent quality for safe water reuse.

3.1. Introduction

The rising frequency of eutrophication and harmful algal blooms (HAB) events in freshwater systems are closely linked to climate change and nutrient pollution, both of which are intensifying due to human activities. Several freshwater taxa among the numerous phyla composing the phytoplankton can form blooms; however, cyanobacteria are the most notorious bloom formers [1]. Cyanotoxins are secondary metabolites produced by certain cyanobacterial species, and can be released into the water directly, or through cell lysis [2]. Among the cyanotoxins found in CyanoHABs, the hepatotoxic group of MCs and cytotoxic CYN are the most frequently occurring [3]. Cyanotoxins persist in freshwater bodies and are stable for lengthy periods of time even through extreme conditions. This is frequently a threat to human and animal health by direct exposure through drinking water or recreational water, or indirect exposure through ingestion of contaminated food items from animal or plant origin subjected to cyanotoxin exposure [4], [5]. Bioaccumulation occurs in aquatic organisms or edible parts of fruits and vegetables through cyanotoxin contaminated surface waters and irrigation waters [6]. Therefore, it is important to monitor and treat surface and irrigation waters properly.

Current efficient treatment solutions for removing cyanotoxins from water are orientated towards drinking water producing plants. Membrane filtration, advanced oxidation techniques, activated carbon adsorption, and chemical oxidation are expensive and better suited to centralised water treatment facilities. [7] (USEPA, 2025). Alternatively, cost-effective techniques which are more suited to water bodies (e.g. lakes, reservoirs) such as mechanical harvesting and the use of chemical flocculants and algaecides (including copper sulphate, potassium permanganate, and chlorine) as well as hydrogen peroxide may exhibit moderate to high efficacy. However, they eventually disturb the ecosystem and harm non-target organisms. Moreover, residual flocs formed as a by-product of coagulation– flocculation can potentially be a secondary contamination source, if they are not removed or stabilized properly. Mechanical mixing and aeration of large water bodies can also be expensive [8]. To this end, cost-effective eco-technologies such as treatment wetlands (TWs) are considered for removing cyanotoxins from contaminated waters [9], [10], [11], [12].

TWs are engineered systems that replicate the capacity of natural wetlands by integrating plant, substrate, and the associated microorganisms' activities to eliminate pollutants by a combination of physical, chemical, and biological mechanisms [13], [14].

TWs can have different types and configurations, and they are mostly classified based on hydrology/flow direction and the type of macrophytes present [15]. TW systems are extensively utilised for a wide variety of water treatments and are prevalent technology for decentralised wastewater management [16]. Although TWs have been considered for the remediation of cyanotoxins from water, this approach remains relatively novel and is still under research. As recently reviewed by [8], the most studied cyanotoxin in TWs has been MC-LR, with only few recent studies for CYN [10], [17]. Moreover, knowledge on the specific processes occurring inside the system are still scarce, which is fundamental to improve their performance. So, more research on TW applications to remove different cyanotoxins and understand removal processes is still needed.

TW substrate choice is often based on their sorption capacity and support for macrophytes to grow in. Several types are commonly chosen and recently a combination of agro-based waste materials and benchmark TW substrates were studied for MC-LR and CYN sorption. The sorption properties of biochar and light expanded clay aggregates (LECA) exhibited multi-layer sorption, significant heterogeneity, and porosity, enabling the cyanotoxins' availability for complete degradation by other biotic components, therefore making them a good choice of substrates for incorporation into TWs [18].

In terms of TW plants, some of the main factors that control the choice of vegetation in TWs are the availability in the surrounding area, the ability to adapt and establish its growth to the climatic conditions, and the removal efficiency of the target pollutants. *S. erectum*, a known phyto-remediative wetland species, has been previously studied in TWs for nutrients, metals and antibiotics removal [19], [20], [21], [22]. However, its potential for cyanotoxin removal has not been explored yet. *C. indica* has also been widely studied in TWs of various designs and under different climatic conditions [23], [24], showing potential to remove metals and several emerging contaminants such as pesticides, pharmaceuticals and industrial chemicals [25]. Although being a highly effective ornamental plant commonly used in TWs, it has also not been explored for the removal of cyanotoxins. It has been established so far that TWs treating cyanotoxins demonstrated better removal efficiencies in the presence of plants than unplanted, by promoting better plant associated microbial population [10], [17]. However, most TW studies for cyanotoxin removal use monoculture vegetation. Polycultures can foster richer microbial communities, and their diversity can increase their ecosystem resilience, functional stability and aesthetic appeal [26]. However, while some studies have shown that polyculture vegetation in TWs can improve and stabilize the pollutant removal

efficiency even through seasonal weather variations and temperature extremes [27], [28], other studies show no significant effect of polycultures in TW performance [29]. It is still unclear if polycultures can improve TWs or not, and the use of polyculture TWs for cyanotoxin removals remains unexplored. Characterizing the microbial community within TWs is also important for understanding, optimizing, and sustaining the treatment performance and ecological function of these engineered systems. Several studies also demonstrated that the plant species, substrate/media, season and cyanotoxin presence influence microbial communities and performance of TWs [10], [30], [31]. Understanding the microbial composition and identifying key microbes can help enhance pollutant removal, and help in selecting appropriate substrate materials and plant species [32], [33], [34]. It also helps assess TW system resilience and monitor other disturbances and contaminants to provide risk management for minimizing environmental risks [35], [36], [37]. Therefore, it is important to characterize the microbial community in the context of different plant species and substrates for exploring possible ways of enhancing TW performance.

In this study we hypothesize that exposure of *S. erectum* and *C. indica* to cyanotoxins MC-LR and CYN will lead to multiple functional changes in TWs, and that these plants can be alone or in combination be capable of removing these toxins in TWs. To validate this, we assess i) the removal rates of MC-LR and CYN in TW planted with different plant species (monoculture or polyculture), as well as ii) the mechanisms of cyanotoxins removal in TWs, including cyanotoxin retention in the substrate (retention in this context includes not just sorption to the substrate, but also retention through filtration and biofilm uptake) and their potential plant uptake as well as plant health, and iii) the impact of plant and substrate composition on TW microbial communities and their contribution to cyanotoxin removal and TW performance. For that, a wetland and an ornamental species (*S. erectum* and *C. indica,* respectively), as monocultures and polycultures, were tested for the removal of MC-LR and CYN in VSSF TW microcosms. *S. erectum* was tested for the first time in TWs for the removal of cyanotoxins. While *C. indica* has been studied for the treatment of hyper-eutrophic water with cyanobacterial biomass, it has not been evaluated for cyanotoxin removal [38]. Moreover, substrates that were previously shown to sorb MC-LR and CYN in small batch studies were used to increase TW removal efficiency [18].

3.2. Materials and Methods

3.2.1. Cyanobacteria Culture and Biomass Production

Permission was granted by Blue Biotechnology and Ecotoxicology Culture Collection (LEGE-CC) at CIIMAR, Matosinhos, Portugal (http://lege.ciimar.up.pt/) to use the strains LEGE 91094 (*Microcystis aeruginosa*) and LEGE X-001 (*Chrysosporum ovalisporum*) that produce the cyanotoxins MC-LR and CYN, respectively. They were grown to exponential phase as described in [9]. Three 1000 mL aliquots of each of the cultures were frozen at -20 °C and later thawed for use in spiking the TW mesocosm experiment further described.

The cultures grown to an exponential phase were also harvested for their biomass. *M. aeruginosa* cells were harvested by centrifugation (20 min, 4 °C, 4495 g), frozen at -20 °C for three days and then lyophilized. *C. ovalisporum* cells were gathered by filtration with a 11 µm Kunstoff-Analysensieb filtration unit and frozen at -20 °C for three days and then lyophilized. The lyophilized biomass was stored at room temperature in a dark dry space. The biomasses were later used to do the recovery tests for the cyanotoxin extraction protocol in the substrate samples.

3.2.2. TW Microcosm Set-up and Acclimation

Nine TW microcosms were set up in triplicates, planted with *S. erectum* (TW- Sp), *C. indica* (TW- Ci), and a bi-culture of both species (TW- CS). The plants were collected a day before; *S. erectum* plants were collected from the Ribeira da Certagem, Lavra, Portugal (N 41°15′31.252″; W 8°43′24.924″) and *C. indica* were acquired from a horticulture facility in Arcozelo, Portugal. In the lab the plants' roots were separated from any soil attached and washed until all the sediments were removed, and then thoroughly rinsed in deionized water again. Plants were kept in Hoagland's nutrient solution for a day before being transplanted into the TW microcosms for the experiments. Multiple previous studies consistently show that the presence of plants improved the functioning of TWs [39]. Moreover, the main aim was to assess if plant composition in TWs had an effect on cyanotoxin removal and TW performance. Therefore, unplanted TWs were not used for comparison in this study.

The microcosms were set-up in plastic containers (37 cm x 25 cm x 27 cm), filled with a bottom layer of gravel (4cm; particle size 1-4 mm), then a layer of light expanded clay aggregates (LECA) (1 cm; particle size 8-13 mm) and a layer of biochar (1 cm; particle

size 1-4 mm), and a top layer of sand into which plants were transplanted (12 cm; particle size 0.4- 0.8 mm). The common TW substrates—sand, biochar, and LECA— were arranged in ascending particle size. Each system was transplanted with 6 plants (in the case of the polyculture, three *S. erectum* and three *C. indica* were transplanted). The containers were wrapped in aluminium foil to prevent exposure to sunlight, and the possibility of photo-degradation of the cyanotoxins. The microcosms were kept indoors in green-house like conditions, with natural day-light regime and temperatures fluctuating between 16 -28 ° C in July, 14-35 °C in August, 21 -34 °C in September, 20 - 33 °C in October and 13 -20°C in November (appendix B, Figure B1). Light intensities were measured once a week with a photometer when the chlorophyll fluorescence was measured for the plants' leaves (refer section 2.6.1). Ten readings were taken at mid-day (between 11:00- 13:00h), every 10 minutes (appendix B, Figure B1).

Prior to the experiments, the TW microcosms were acclimated for two weeks using a Hoagland nutrient solution (1.5 L every two days) to ensure suitable nutritional conditions for the plants. After that, the TW microcosms were acclimated with freshwater collected from a spring at the botanical garden of Porto, for two weeks (water characteristics in appendix B, Table B1). The water added was always maintained at a level just above the substrate surface, allowing saturation of the substrate, which was equated to be 1L for all the TWs. Water was added from above the TWs, allowing a vertical flow through the layers, with a tap at the bottom to drain the water, thus simulating a vertical subsurface flow treatment wetland system. Water was recirculated daily to prevent anoxic conditions, during which spring water was added to make up for the water loss due to evapotranspiration (ET).

3.2.3. TW Experiment and Sampling

After the acclimation period, the experiments were carried out for 10 weekly cycles between the months of September- November 2023. For the experiments, whole cell extracts of the *M. aeruginosa* and *C. ovalisporum* (freeze-thawed cultures) were used to artificially contaminate the spring water. The spiked waters reached toxin concentrations ranging between 2-10 µg/L (MC-LR) and 7-15 µg/L (CYN) during the experiment. Each system was treating 1 L of cyanotoxin contaminated water, which was recirculated daily for one week, adding uncontaminated spring water whenever necessary to make up for ET. This volume of uncontaminated spring water added daily was recorded to measure ET rates. After one week, the treated water was collected from each system and new freshly contaminated spring water was added to each system in the same conditions,

simulating the cumulative effect of full-scale TWs with a hydraulic retention time (HRT) of 1 week.

One litre of water samples of the inlet, outlet, and spring water were processed every week. For the outlets, at the end of each treatment cycle all the water was drained from the systems in 1L Schott bottles, and deionized water was added to make up the initial volume of 1 L to compensate for water loss by ET and to have sufficient effluent volume for all the analysis. The bottles were homogenized by shaking them well. 500 mL was filtered with a glass micro-fibre filter (1.2 µ, 47 mm diameter, PRAT DUMAS, France). The filters were used for measuring total suspended solids and the filtered water for nutrient analysis. The unfiltered 500 mL of the TWs outlet water were used as follows: pH and chemical oxygen demand (COD) were immediately analysed, duplicates of 1 mL were transferred to two HPLC vials and kept at -20° C freezer for cyanotoxin analyses.

The leaves of the plants were sampled prior to the experiment before cyanotoxins exposure (T0) (this was used as a baseline, as reference, and it reported to the day before adding the cyanotoxins, and will be used as a reference condition), at the fifth treatment cycle (middle) of the experiment (T-1/2), and at the end of the experiment (Tf). Triplicate leaf samples were collected from three separate plants per TW system, immediately put in liquid nitrogen and stored at -80 °C, until further analysis of cyanotoxins and plant physiological parameters. At Tf, the TWs were disassembled, and the plants' roots were sampled, washed with deionised water and kept at -20°C until analysis.

The bulk substrate (sand) was sampled along the root depth in three different sites of each of the TWs, at the reference T0 (before cyanotoxin exposure, baseline value) and Tf (at the end of the experiment), for chemical and microbial analysis. The samples collected from the three different sites were mixed to ensure high sample representativity. The samples collected were stored in aluminium foils for chemical analysis and in sterile bags for microbial analysis, labelled, and kept at -20°C until analysis. At Tf, the TWs were disassembled and the substrates at the rhizosphere (rhizo-substrate, in contact with plant roots) were sampled as described for sand and kept at -20°C until analysis. The rhizo-substrate samples comprised of a mix of biochar and sand. The sand and rhizo-substrate samples were considered two distinct substrate matrices. A schematic representation of the experimental set-up, and sampling points can be seen

below in Figure 9.

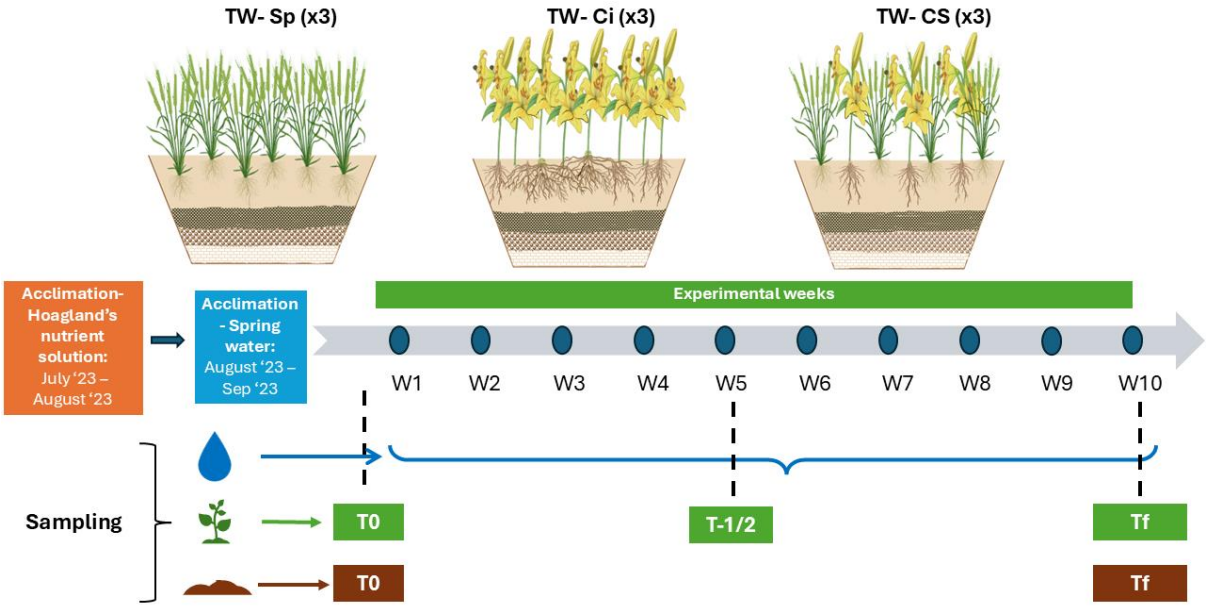


Figure 9. Diagram describing the TW experimental set-up and sampling. The three plant compositions were set-up in triplicates, with two acclimation stages, after which the experiment lasted for ten weeks. Sampling of the water, plant tissues, and substrate matrices were done at different time points (weekly, T0, T-1/2, and Tf). Created with BioRender.com.

3.2.4. MC-LR and CYN Extraction and Analysis

3.2.4.1. Water samples

The target analysis of MC-LR and CYN was conducted using high-performance liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) utilising a 1200 Series binary LC-gradient system (Agilent Technologies) linked to a Sciex 5500 QTRAP-MS. The method is detailed in [17]. For that, 10 µL of water samples were directly 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 and 0.1 µg/ L for MC-LR and CYN, respectively, and limits of detection (LOD) of 0.15 µg/ L and 0.03 µg/ L for MC-LR and CYN, respectively.

The removal efficiencies of MC-LR and CYN were calculated according to equation 1,

$$Removal\ \% = \frac{C\ in - C\ out}{Cin}\ x\ 100 \qquad (1)$$

where C_{in} and C_{out} are the target pollutants entering and leaving the individual TW system, respectively. To calculate the removal efficiency when the pollutant was not detected in the C_{out}, half the LOD value of the respective analytical methodology was considered [40]. The actual cyanotoxin concentrations in the outlets ($C_{effluent}$) were estimated taking into consideration the initial outlet volume ($V_{initial}$), before making up for ET (refer section 2.3.), using the formula in equation 2. The cyanotoxin volumetric removal rates were then calculated using $C_{effluent}$ in equation 1, instead of C_{out}.

$$C\ effluent = Cout * (1/V\ initial) \quad (2)$$

Suspect screening of the known MC-LR and CYN transformation products (TPs) were also screened in the inlet, and outlet water samples of week 1 and week 10, where 10 µL of the same samples (as prepared for the LC-MS/MS) were injected directly into an LC coupled to a 6600 quadrupole-time-of-flight (QTOF) equipment (SCIEX). Chromatographic separation was performed using an Acquity UPLC BEH Shield RP18 1.7 µm column (2.1 mm × 30 mm) from Waters Corporation. Eluents A and B were water and methanol, respectively, each containing 0.1 % formic acid. The chromatographic conditions, as well as the detailed parameters of the instrument, were similar to those used by [41]. The QTOF was operated in data dependent acquisition mode.

3.2.4.2. Substrate samples

Simultaneous MC-LR and CYN extraction from the sand and rhizo-substrates was done using a three-phase sequential extraction as described in [42] with slight modifications. For Tf sand samples, 2 g from each TW system were transferred into a 50 mL falcon and extracted with 3 mL solution of 90 % water and 10% methanol. All extraction solutions were vortexed followed by ultrasonication (15 minutes) and centrifugation (6000 RPM, 5 minutes). The supernatant was collected in a 12 mL HPLC vial. The pellet was further extracted in 2 mL of a 1:1 solution of water and methanol and the final extraction was performed using 1 mL of 100 % methanol. Rhizo-substrate samples were processed the same way as the sand samples, but 10 g of samples were extracted with 15 mL of methanol 10% (v/v) in water, and the pellet was further extracted in 10 mL of methanol 50% and the final extraction was performed using 5 mL of 100% methanol. Rhizo-substrate samples had a mix of sand and biochar and therefore a higher amount was taken to reduce the bias of sampling.

MC-LR and CYN analysis was done following the same method as described for the water samples (refer section 2.4.1.) The limits of detection for the substrate matrices were 0.27- 0.30 µg/ g and 0.08- 0.09 µg/ g for MC-LR and CYN, respectively.

Recovery tests were done for sand and rhizo-substrate samples at three concentration levels of MC-LR and CYN from extracts of *M. aeruginosa* and *C. ovalisporum* biomass as described in [17]. Since the concentrations of the cyanotoxin extracts could not be analysed before, recovery tests were done by adding extract volumes of 0.5 mL, 1 mL and 2 mL. T0 sand samples collected before the exposure to cyanotoxins were used for the recovery tests. For the recovery tests of the rhizo-substrate samples, unexposed biochar was mixed with T0 sand samples in the ratio 1:10 (biochar: sand). The recovery rates for sand were 36 % and 43 % for MC-LR and CYN, respectively, and the recovery rates for rhizo-substrate were 77 % and 88 % for MC-LR and CYN, respectively.

For further mass balance calculations, the MC-LR and CYN recovery rates were used to calculate total MC-LR and CYN accumulated in the substrate in regard to the total mass of sand and rhizo-substrate in the TW systems. For this, first the total cyanotoxin inlets were calculated by adding the cyanotoxin inlet concentrations of each of the 10 treatment cycles in terms of µg , divided by the total mass (in g) of the sand and the rhizo-substrate (sand + biochar; refer section 2.3), as shown in equation 3. The cyanotoxin concentrations in the substrate samples ($C_{\text{substrate sample}}$) were estimated in µg/g after considering the extraction volume, sample mass extracted, and the recovery rates. Then, the total cyanotoxin retention rate was estimated by dividing the cyanotoxin concentration in the substrate sample ($C_{\text{substrate sample}}$) by the total cyanotoxin inlet concentration for sand (T_{sand}) and for rhizo-substrate ($T_{\text{rhizo-substrate}}$), respectively, as shown in equation 4.

$$Total\ Inlet_{(sand/rhizo-substrate)} = \frac{Sum\ of\ C_{in}\ of\ the\ 10\ treatment\ cycles\ (\mu g)}{Total\ mass_{(sand/rhizo-substrate)}\ (g)} \quad (3)$$

$$Total\ Retention\ \% = \frac{C_{substrate\ sample}}{Total\ Inlet_{sand/rhizo-substrate}}\ x\ 100 \quad (4)$$

3.2.4.3. Plant Tissues

For estimating MC-LR and CYN in plant tissues, a different method was used that included solid phase extraction (SPE). Simultaneous MC-LR and CYN extraction, SPE and analysis from plant leaves and roots were done as described in [43]. *S. erectum* and *C. indica* leaves and roots were macerated with liquid nitrogen. For MC-LR and CYN

extraction, 50 ± 2 mg of each sample was extracted with 6 mL of 80 % methanol, homogenized in an ultraturrax (1 min), sonicated (15 min), and stirred in an orbital shaker (15 min). The mixture was centrifuged (3700 rpm, 15 min) and the supernatant collected for the clean-up. For the clean-up, SPE was done using a C_{18} cartridge (Oasis HLB 6cc, 500 mg, Massachusetts, USA) and a PGC cartridge BOND ELUT® (Agilent Technologies, Amstelveen, The Netherlands). The use of reverse phase C_{18} cartridges is suitable for the retention of moderately polar organic compounds, such as MCs, from aqueous matrices. However, due to the hydrophilic nature of CYN, it cannot be extracted by SPE with C_{18} cartridges, but PGC cartridges have been successfully used. Because CYN cannot be retained in the C_{18} cartridge, this was set at the top and PGC at the bottom, and the order was reversed (PGC on top and C_{18} on bottom) for elution to avoid MC-LR retention in the PGC column. The pH of the supernatant was adjusted to 11.0, and the following reagents were passed through the assembled cartridges: 6 mL dichloromethane, 6 mL 100 % methanol, 6 mL H_2O (pH 11.0), and sample (pH 11.0); then, cartridges were dried for 5 min and eluted with 10 mL dichloromethane/methanol (40/60) + 0.5 % formic acid. The purified fraction was evaporated to dryness in a SpeedVac vacuum concentrator and resuspended in 1 mL 20 % methanol for its analysis by LC-MS/MS. The details on the chromatographic conditions can be found in L. Díez-Quijada *et al.*, 2018 [43]. LOQs for this method were 0.16 ng/ g fresh mass (FM) for MC-LR and 0.19 ng/ g FM for CYN. The LODs for this method were 0.06 ng/g FM for MC-LR and 0.07 ng/ g FM for CYN. Matrix recovery was done on the *S. erectum* and *C. indica* plant tissues for both the cyanotoxins. In the case of *S. erectum*, both cyanotoxins had high recoveries of 98.7% for CYN and 94.64% for MC-LR. Negligible matrix effect was seen in CYN, and MC-LR had some matrix effect. In the case of *C. indica*, recoveries were above 90% and both MC-LR and CYN had stronger matrix effects.

3.2.5. Analysis of Water Quality and TW Parameters

3.2.5.1. Total Suspended Solids (TSS)

Initially, the glass microfibre filter of 47mm diameter and 1.2 µm retention was oven dried (100 °C) for 24 hours, weighed using a measuring scale (Kern ALJ, Germany) and kept in a desiccator. Then, 500 mL of the inlet water, spring water or outlet water were filtered. The filter was used to determine TSS, and the filtered water was used for nutrient analysis (see section 2.5.2). After filtration the filters were kept overnight in the oven (100 °C) until constant weight was achieved, cooled in the desiccator and then weighed again. The increase in weight was calculated and TSS was reported in mg/L.

3.2.5.2. COD and Nutrients Analysis

COD was measured using the kit HI93754B-25 (Hanna Instruments, Portugal), for mid-range COD from 0 to 1500 mg/L and following the provided protocol. Nitrate ion, ammonia ion and total phosphorus levels were measured using the kits HI93766-50, HI93764B-25 and HI93758C-50 (Hanna Instruments, Portugal), respectively, following the provided protocol. A multiparameter photometer (HI83399-02, Hanna Instruments, Portugal) was used to measure absorbances.

Since spring water was added during the experiment to make up for water loss by ET, the total inlet concentrations of all the water quality parameters were calculated taking into consideration the volume of spring water added over the week to each system, and the respective concentrations of the spring water parameters for each week. The removal rates of the above parameters were then calculated using equation 1 (section 2.4.1).

3.2.5.3. pH and Redox Potential

The pH of the TWs outlet water, inlet water and spring water were measured during each treatment cycle using a pH probe (CRISON microCM 2201), on the day of sampling. The redox potential of the TWs was measured weekly using a redox probe (ORP 30-1-BNC), and an ORP meter (Lutron YK-23RP) from SWAP Instruments, Netherlands, 10 cm deep along the plant-root bed substrate of the TWs. The redox potential was measured at three sites in each TW, and the average was calculated. This parameter was measured to monitor the aerobic conditions of the TWs over time.

3.2.6. Plant Physiological Parameters

3.2.6.1. Pigment Content and Fluorescence

Chlorophyll a (Chl a) fluorescence was measured in-situ using a pulse amplitude modulation system (Fluor-Pen FP 110, Photon Systems Instruments, Czech Republic) according to [44], on 3 plants per TW, on a weekly basis during the experiments. Before measurement leaves were kept for 30 minutes in the dark. To determine the maximal fluorescence (Fm), a saturating pulse of white light was used and the corresponding Fm' and the steady-state fluorescence (F') were obtained. The potential maximal quantum yield of photosystem II (PSII) [Fv/Fm = (Fm – F0)/Fm] and the effective quantum yield of PSII [ΦPSII = (Fm' – F')/Fm'] were subsequently determined.

Photosynthetic pigments were measured using the methodology of Sims and Gamon, (2002) [45]. Leaf samples frozen at -80 °C were macerated with liquid nitrogen under ice conditions, to form a frozen powder. Chl a, Chl b, carotenoids, and anthocyanins were extracted from 50 ± 2 mg of frozen leaf powders using an acetone: 50 mM Tris-HCl pH 7.8 buffer (80:20, v/v) solution. Absorbance was read at 470, 537, 647, and 663 nm using the Multiskan™ Microplate Spectrophotometer, Thermo Fisher Inc., MA, USA.

3.6.2.2. Total Soluble Sugars and Starch

Total soluble sugars and starch contents were quantified by the anthrone method as described by [46]. Absorbance at 625 nm was read at a Multiskan™ Microplate Spectrophotometer (Thermo Fisher Sci, MA, USA). Results are expressed as mg/g FM.

3.2.7. Microbiological analyses

3.2.7.1. DNA Extraction and Quantification

The sand samples collected for microbial analyses at T0, and the sand and rhizo-substrate samples collected at Tf, were later used for DNA extraction. The DNA extraction was done using the DNeasy PowerSoil Pro kit from QIAGEN, Inc., following the manufacturer's extraction protocol, except for the first step where 0.50 g of sample was used instead of 0.25 g. Each sample was extracted twice and then pooled, to increase the DNA yield. After the extractions, DNA was quantified fluorometrically using the Qubit dsDNA HS Assay (ThermoFisher Scientific, Waltham, USA).

3.2.7.2. Detection of mlrA Gene by PCR

PCR detections of the *mlrA* gene were performed in the sand and rhizo-substrate samples to investigate potential MC biotransformation via the intervention of *mlrA*-encoded microcystinases. For this end, a consensus primer pair of the *mlrA* gene (*mlrA*-Forward 5'-GACCCGATGTTCAAGATACT-3' and *mlrA*-Reverse 5'-CTCCTCCCACAAATCAGGAC-3') [47], acquired from Eurofins Genomics, (Ebersberg, Germany) were used for these PCR assays. PCR screenings were performed in a Veriti 96-well Thermal Cycler (Applied biosystems, Thermo Fisher Scientific) on a total volume of 10 µL as follows: 5 µl MyTaq DNA Polymerase (Bioline (Meridian), USA), 1 µl of each primer (0.4 mM) and genomic DNA at a concentration range of 0.1- 2.3 ng/µl. The PCR conditions consisted of an initial denaturation at 95 °C for 5 min; followed by 40 cycles of 10 s at 98 °C, annealing for 30 s at 56 °C, extension at 72 °C for 1 min; and a final elongation for 5 min at 72 °C. The PCR run included a positive control using genomic DNA of *Sphingosinicella microcystinivorans* 19791 (DSMZ, Leibniz Institute,

Braunschweig, Germany), a bacterial strain known to encode *mlrA* in its genome [48], [49] which had been previously isolated from pure cultures grown in R2A media using the Bacterial Genomic DNA prep mini-Spin kit (Cytiva, UK), following the manufacturer's instructions. A negative control was also contemplated in each PCR run, consisting of the same PCR mixture without genomic DNA.

The results of the PCR screenings of the *mlrA* gene were confirmed by electrophoresis, where amplification products were separated in a 1.5 % agarose gel containing SYBR® Safe (ThermoFisher Scientific, Massachusetts, USA). Amplicons were visualised under UV light in a BioRad Molecular Image® Gel Doc™ XR+ with Image Lab™ Software, and amplification bands with suitable molecular weights (of ca. 750 bp) were confirmed using a 100 bp ladder (GRS Ladder, GRISP Research Solutions, Porto, Portugal).

3.2.7.3. 16S rRNA Amplicon High-throughput Sequencing and Bioinformatics Analysis

To profile the prokaryotic microbial community of TWs substrate, a 16S rRNA metabarcoding approach was followed on the sand T0 and Tf samples and rhizo-substrate samples (Tf). High-throughput sequencing of the V3-V4 hypervariable region of the 16S rRNA gene was performed at Macrogen (Seoul, Korea). After confirming suitable DNA concentrations and quality in the samples, the sequencing library was prepared by using the Herculase II Fusion DNA Polymerase Nextera XT Index V2 kit, following the 16S Metagenomic Sequencing Library Preparation (Part # 15044223 Rev. B) protocol. Adapter-ligated fragments were then amplified by PCR with the bacterial primer pair Bakt_341F/ Bakt_805R which annealed at the end of each adapter. The library template underwent quality control and quantification process. High-throughput sequencing of the V3-V4 hypervariable region of the 16S rRNA gene was done on the Illumina MiSeq platform (300 PE (300 x 2bp),200k reads per library).

Sequencing data were quality filtered, trimmed, denoised and assembled in paired-end reads using the DADA2 pipeline [50], while taxonomical classifications were performed using the SILVA v138 database [51]. The phyloseq package in the R environment (version 3.6.1) was used to further curate the 16S rRNA dataset [52]. Firstly, ASVs taxonomically classified as "Eukaryota," "Chloroplast," and "Mitochondria" were specifically eliminated from the dataset. Then, relative abundances were estimated and analysed at all taxonomical levels, considering a 2 % abundance threshold on the whole dataset. When appropriate, subsets of the metabarcoding dataset were further created to enable finer taxonomical comparisons between experimentally relevant metadata (the

different substrate matrices at Tf, sand samples at T0 and Tf, and the rhizo-substrate samples). Alpha-diversity indices (Observed OTUs, Chao1 estimator, Shannon index) were also estimated using the *vegan* package in the R environment (version 3.6.1), and Bray-Curtis distances (β-diversity) were used to assess bacterial community divergences between the different plant compositions, substrate matrices and time.

Enrichment analysis was done using the *microbiomemarker* R package [53]. Specifically, a Linear Discriminant Effect Size (LEfSe) analysis was conducted to identify the ASVs enriched in the different conditions, considering only ASVs present in at least 2 replicates per condition.

3.2.8. Statistics

All statistical analysis for the chemical and biological parameters were performed using GraphPad Prism software v 10.4.1. Each TW condition was tested independently in three microcosm systems, and all the analyses were performed in triplicates. For plant photosynthetic and physiological parameters, biological (TW= 3) and technical replicates (3 plants per TW) were used (n= 6). A two-way repeated measures (RM) ANOVA was done on all the data (cyanotoxins in water and TW parameters) measured over the 10-weekly treatment cycles, and the Greenhouse-Geisser epsilon was used to adjust for the violation of sphericity in the data. For data measured at T0, T-1/2 and Tf (plant photosynthetic and physiological parameters), or at T0 and Tf (cyanotoxins in substrate matrices), a Shapiro–Wilk test with $p > 0.05$ was carried out to confirm the normality of the data. Then, ordinary two-way ANOVA was used. When normality was violated, Kruskal-Wallis' test was used with a Dunn's post-hoc test. A multiple comparison Tukey test was performed to determine the differences that were statistically significant between the treatments and the one-week TWs treatment cycles (or T0, T-1/2 and Tf) for a 95 % confidence interval.

Statistical analysis for the prokaryotic community structure was performed on R studio (Posit team, 2024). For the calculated alpha-diversity indexes, statistical validation was done for each index by first checking the normality of the data using Shapiro-wilk normality tests, followed by ANOVA and Kruskal-Wallis' test (when the data was not normal). Beta-diversity analyses were performed based on Bray-Curtis distances, which were statistically corroborated through PERMANOVA tests (999 permutations) to examine the dissimilarities between the prokaryotic microbial communities among experimental conditions. For LEfSe, a linear discriminant analysis (LDA) score

thresholds of 2.5, with only those exhibiting significance levels below 0.05 being deemed relevant, was used.

3.3. Results

3.3.1. MC-LR and CYN Removal

No MC-LR or CYN were detected in the spring water used for the TW experiments, and as so, the water was always spiked with cyanotoxin culture at the beginning of each one-week cycle.

MC-LR was below the LOD (0.15 µg/L) in the outlet waters the entire duration of the experiment, indicating > 96% mass removal rates in all the three tested TW conditions (no significant changes between systems) (appendix B- Table B2), despite varying MC-LR inlet concentrations (appendix B- Table B2). However, it is to be noted that due to the dilution of the effluent to have more volume for analysis, MC-LR may be below the LOD because of that. There were no significant differences between the three tested systems. CYN mass removal rates were more variable: 70-91 % in TW- Sp, 73-97 % in TW- Ci and 81-98 % in TW- CS (Figure 10). TW-Sp had a significant decline in mass removal rates with time in comparison to TW-CS and TW-Ci ($P < 0.05$). TW-Sp showed mass removal rates < 80 % after the 3rd week, with the lowest mass removal rates observed in the 7th week. On the other hand, TW- Ci and TW- CS systems had stable mass removal rates > 75%, which also increased with time. Additionally, there were no significant differences in removal efficiencies between TW-Ci and TW-CS systems. Two-way repeated measures ANOVA showed that the interaction effect was extremely significant (p value < 0.0001), indicating that the combination of the plant composition and the time influenced the mass removal rate. Details on the statistics can be seen in appendix B (Table B4).

CYN levels in the outlet were between 0.75- 2.88 µg/L (TW-Sp), 0.64- 1.42 µg/L (TW-Ci), 0.80- 1.13 µg/L (TW-CS) (appendix B- Table B2). This variation might be related with the varying inlet concentrations of CYN during the experiment (Figure 11a).

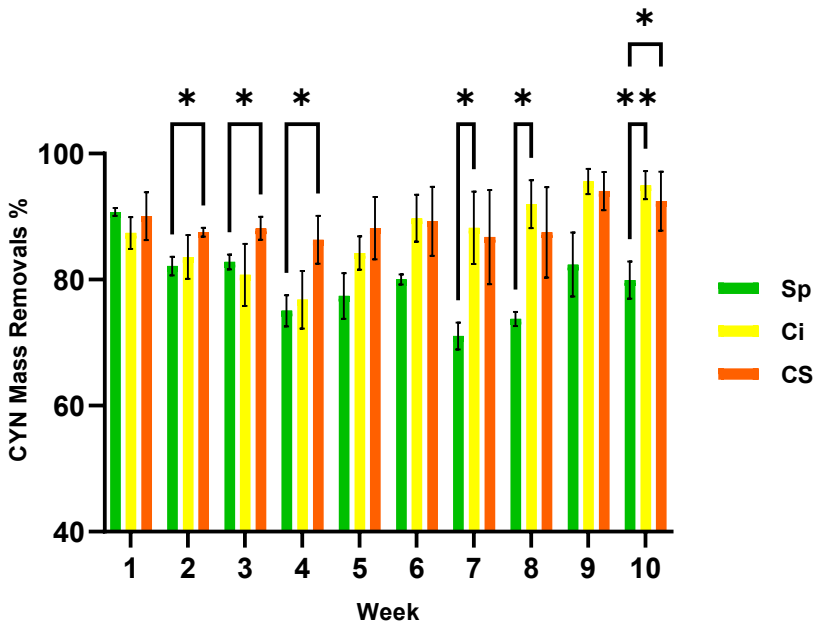


Figure 10. Average CYN mass removal rates in the different plant compositions throughout the experiment. Error bars represent standard deviation (n=3). The asterisk (*) and lines show statistical significance between treatment groups (* p ≤ 0.05 to 0.01, ** p ≤ 0.01 to 0.001) Sp indicates TW with S. erectum, Ci indicates TW with C. indica, and CS indicates TW with both S. erectum and C. indica

The actual CYN concentrations in the effluents (Figure 11a) were calculated using the original volumes of the outlets of the TWs. *S. erectum* had significantly higher ET rates (Figure 11 a and b), and hence the effluents were more concentrated (0.9 - 4 µg/L), being higher than the drinking water threshold value for short-term exposure (3 µg/L) after the 7th week [54]. The actual effluent concentrations from TW-Ci and TW-CS systems were 0.4- 2.3 µg/L and 0.3- 2.3 µg/L, respectively. Despite the effluent concentrations being much lower in the TW-Ci and Tw-CS systems, they were still above the drinking water threshold levels of chronic exposure (0.7 µg/L), set by the World Health Organization (WHO) [55].

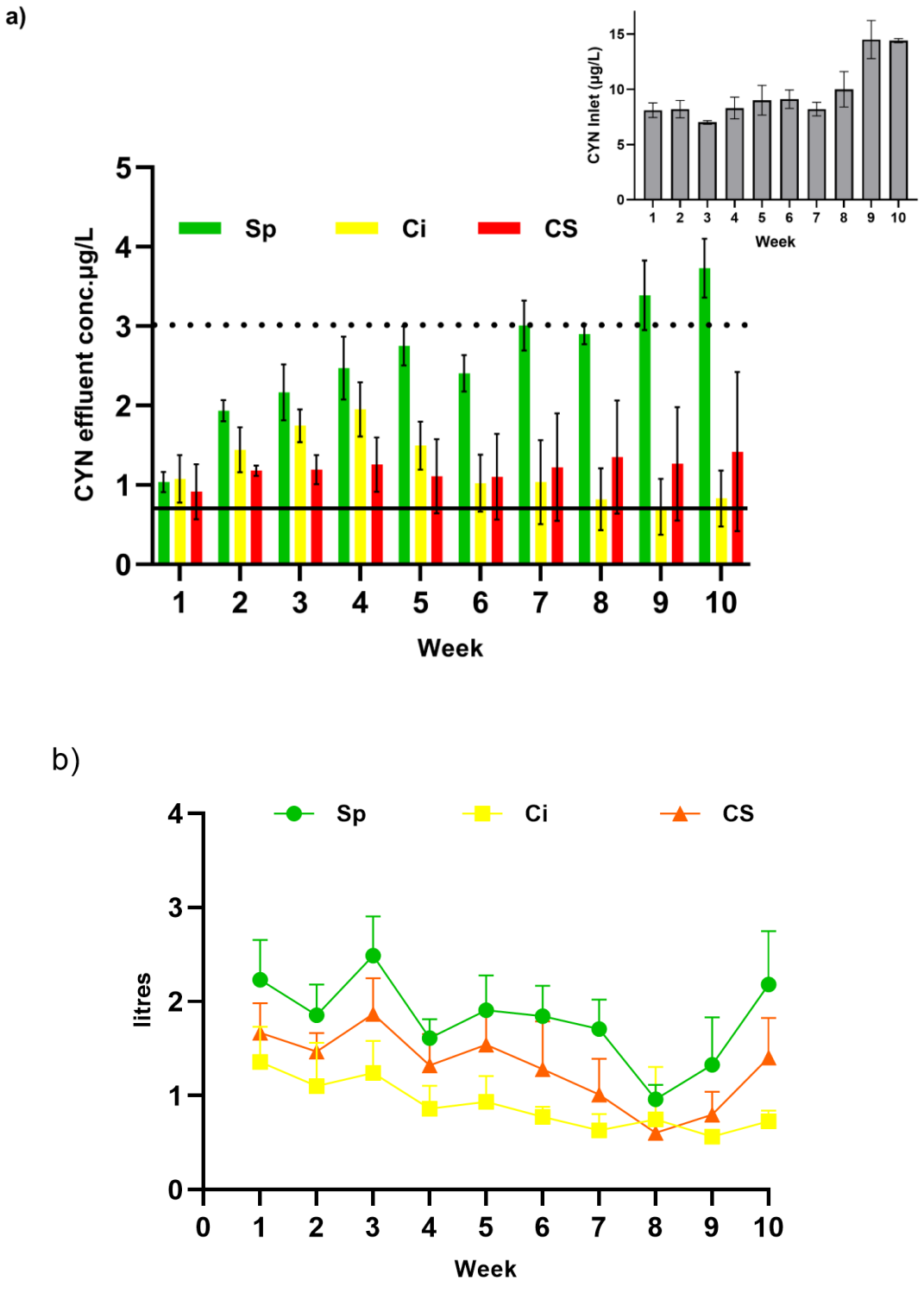


Figure 11. a) Average CYN effluent and inlet (onset) concentrations. The black solid line is a reference for the drinking water threshold value for chronic exposure (0.7 µg/L) and the dotted line the threshold values for short-term exposure (3 µg/L) , according to WHO guidelines; b) ET rates in the different plant compositions throughout the experiment. Error bars represent standard deviation (n=3). Sp indicates TW with S. erectum, Ci indicates TW with C. indica, and CS indicates TW with both S. erectum and C. indica

To investigate potential metabolites resulting from cyanotoxins degradation within the TW systems, the TPs were analyzed in the inlets and outlets of weeks 1 and week 10. The TP investigated were those reported in literature [8]. Some TPs could be found in the inlet waters, namely 7-deoxy-desulfo-CYN, TP4-CYN, TP2 MC-LR, and TP11 MC-LR [8]. This could be from using whole cell extracts of the cyanobacterial cultures for spiking the inlet waters. Of these TPs, only TP4-CYN were found in the outlets of week 1 and 10. However, it is uncertain if this TP is present as secondary metabolites of the whole extracts of the cyanobacterial strains used to spike the inlet water, or was formed in the systems as a result of toxin degradation.

3.3.2. MC-LR and CYN in the Substrate Matrices

The potential total cyanotoxin retention rates of the total cyanotoxin input were investigated by analyzing the toxins in the bulk substrate (sand) and rhizo-substrate (sand mixed with biochar) of the TWs after the 10 treatment cycles. Generally, there was less MC-LR retention (0.3 - 2.2 %) than CYN (0.1 - 7.4 %) in both substrates (Figure 12). MC-LR had significantly higher retention in sand than in rhizo-substrate, independently of the TW systems. Noticeably, in the rhizo-substrate, there was significantly higher CYN retention than MC-LR. This was not the case in the sands. MC-LR retention in the rhizo-substrate, TW-Sp had significantly higher MC-LR and CYN retention than TW-Ci and TW-CS, both in the sand and rhizo-substrate ($p < 0.05$). Toxins concentrations in substrates can be seen in appendix B, Table B6.

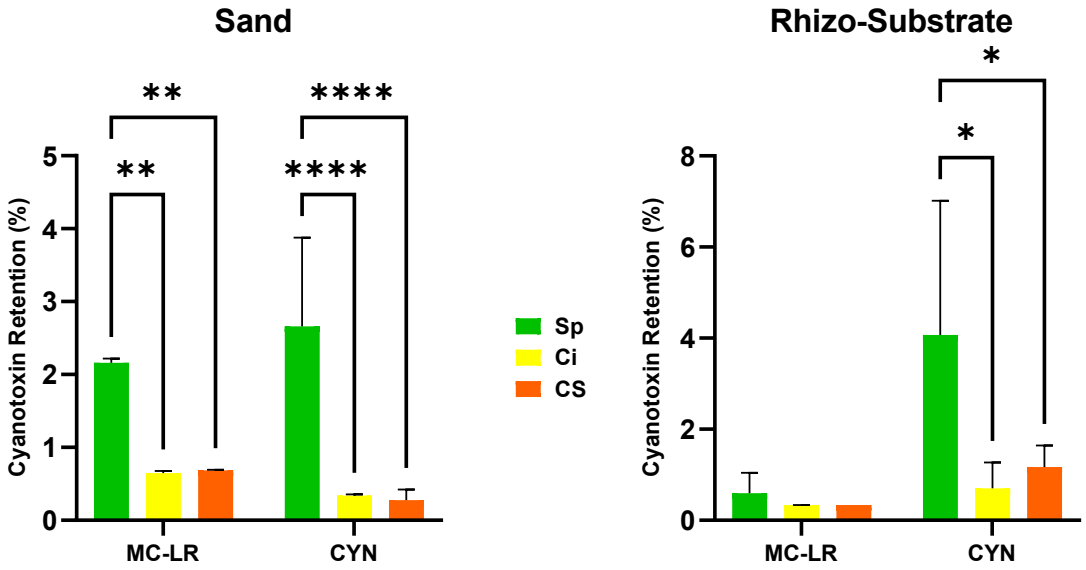


Figure 12. Average cyanotoxin retention rates in sand and rhizo-substrate samples in the different plant compositions at the end of the experiment. Error bars represent standard deviation (n=3). The asterisk (*) and lines show statistical significance between treat treatment groups (* p ≤ 0.05 to 0.01, ** p ≤ 0.01 to 0.001, **** p ≤ 0.001). Sp indicates TW with S. erectum, Ci indicates TW with C. indica, and CS indicates TW with both S. erectum and C. indica

3.3.3. MC-LR and CYN in Plant Tissues

Both MC-LR and CYN were not detected in any of the plant tissues (leaves and roots), being always below the detection limits (0.06 ng/g FM for MC-LR and 0.07 ng/ g FM for CYN). Even though cyanotoxins were not detected in the plant tissues, it cannot be dismissed that the plants were involved in toxins removal and their degradation within the system.

3.3.4. TW Microcosms' Performance

To estimate the overall TW performance over time, TSS, organic matter content, nutrients, pH and oxidative-reductive potential (ORP) in TW water inlet and outlets were measured throughout the experiment.

The TSS content in the inlet waters throughout the experiment were relatively low (appendix B- Table B3) (0.4- 2.9 mg/L), indicating clear water from the spring in general (Figure 13a). The TSS outlet levels ranged between 0- 1.9 mg/L in TW-Sp, 0.1- 1.7 mg/L in TW-Ci, and 0- 0.6 mg/L in TW-CS. The average TSS removal rates (appendix B- Table B3) varied between 30.5- 98.3 % in TW-Sp, 14.3- 91.2% in TW-Ci, and 60.7- 99.9% in TW-CS, during the experiment period, being significantly higher in TW-CS ($p < 0.05$), in comparison to TW-Sp and TW-Ci. Organic matter removals (measured through

COD) varied highly throughout the experimental period due to the varying COD inlets that were observed in the spring water. Total COD in the inlets (appendix B- Table B3) varied between 8- 188 mg/L during the 10-week treatment cycles, and COD in the outlets varied between 2- 94 mg/L in TW-Sp systems, 1- 81 mg/L in TW-Ci, and no detection-100 mg/L in TW-CS systems (Figure 13 b). The TW plant composition had no significant effect on COD removals (appendix B- Table B3).

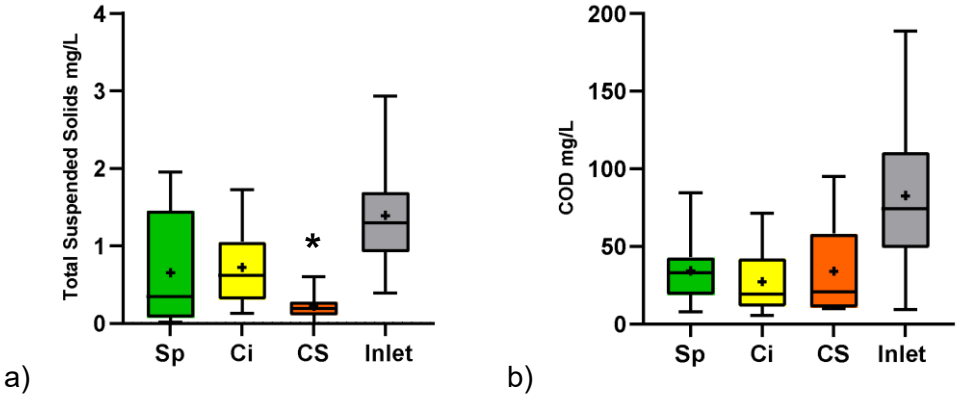


Figure 13. a) Box and whiskers plot of TSS and b) COD concentrations over the 10-week experimental cycle in the inlet and outlet water of TW systems. The median value is presented as the line inside the box, and the "+" represents the mean value (n=10). The whisker ends mark the minimum and maximum values, and the box ends mark the 25th and 75th percentile respectively. Sp indicates TW with *S. erectum*, Ci indicates TW with *C. indica*, and CS indicates TW with both *S. erectum* and *C. indica*

Nitrate ion concentration ranged between 21.5 and 56.3 mg/L in the inlet waters (appendix B- Table B3). In the outlet waters the concentration ranged from 0.5 and 4.3 mg/L in TW-Sp, 0.5 and 5.8 mg/L in TW-Ci, and 0.5 and 2.1 mg/L in TW-CS, throughout the experimental period (Figure 14a). Nitrate ion removals were on average >90 % throughout the experiment (appendix B- Table B3), with no significant difference among systems or time. Ammonia ion was not detected in the inlet waters nor in the outlets. Overall, total P levels in the inlet waters ranged between 0.2 and 1 mg/L, and between 0.1 and 0.6 mg/L for TW-Sp, 0.01 and 0.4 mg/L for TW-Ci, 0.02 and 0.5 mg/L for TW-CS in the outlet water throughout the experiment (appendix B- Table B3, Figure 14b). The average total P removal rates were 7.5 % - 63.5 % in TW-Sp systems, 55 % - 92.2 % in TW-Ci and 46.2%- 90.3% in TW-CS (appendix B- Table B3). Two-way RM- ANOVA revealed that there was a significant interaction effect ($p< 0.05$), indicating that both time and plant composition had an equal effect on total P removal. However, there were no significant differences among TW systems.

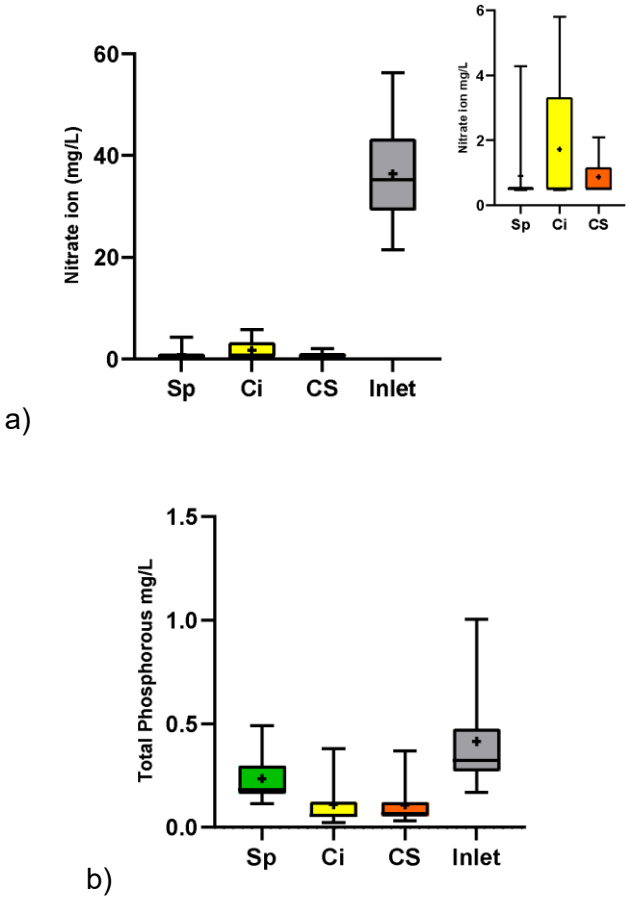


Figure 14. Box and whiskers plot of a) nitrate ion and b) total phosphorus concentrations over the 10-week experimental cycle in the inlet and outlet water of TW system. The median value is presented as the line inside the box, and the "+" represents the mean value (n=10). The whisker ends mark the minimum and maximum values, and the box ends mark the 25th and 75th percentile respectively. Sp indicates TW with S. erectum, Ci indicates TW with C. indica, and CS indicates TW with both S. erectum and C. indica

The average pH of the inlet waters during the 10 one-week cycles ranged from 5.2- 6.9, and the average pH of the outlet waters ranged from 6.7- 7.4 (appendix B- Table B3, Figure 15 a). Measured pH in the outlet water was higher than the inlet waters (TW-Sp: 7- 7.4, TW-Ci: 6.7- 7.1 and TW-CS: 6.7- 7.1), being more significant for TW-Sp (in weeks 3,4, 7, 9 and 10) and TW-Ci (in weeks 7, 9 and 10) ($p < 0.05$) than for TW-CS. The ORP levels ranged between 216 and 387 mV in TW-Sp, 250 and 324 mV in TW-Ci, 230 and 330 mV in TW-CS (Figure 15b), which indicates aerobic conditions. It significantly reduced over time for all the TWs ($p< 0.0001$), but with no significant difference among TW systems.

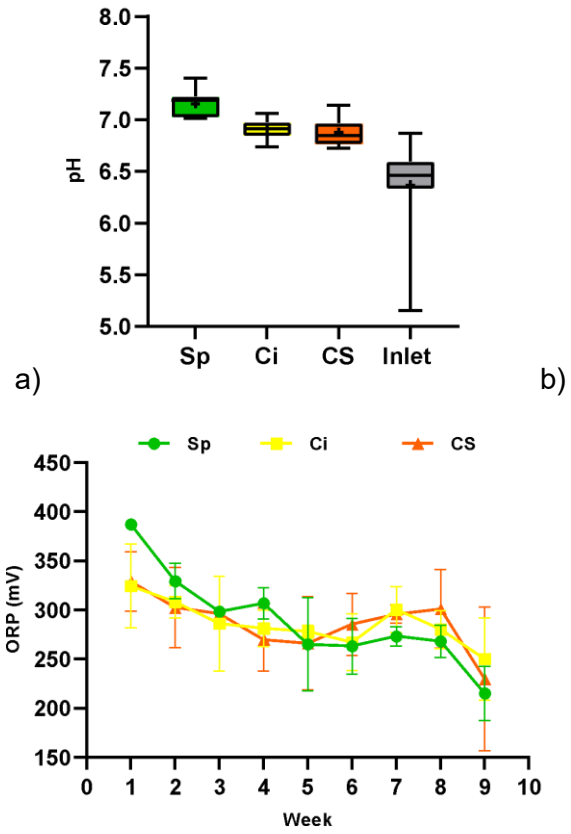


Figure 15a) Box and whiskers plot of total suspended solids (mg/L) over the 10-week experimental cycle. The median value is presented as the line inside the box, and the "+" represents the mean value (n=10). The whisker ends mark the minimum and maximum values, and the box ends mark the 25th and 75th percentile respectively; b) ORP levels (mV) throughout the 10-week experimental cycle. Sp indicates TW with S. erectum, Ci indicates TW with C. indica, and CS indicates TW with both S. erectum and C. indica

3.3.5. Plants Physiological Parameters

Visual changes of the plant physiological conditions were observed over time as a proxy of TW health conditions. Visual inspections showed signs of blight in *S. erectum* leaves from the fifth experiment cycle (week 5) onwards (appendix B, Figure B2a – 2i), indicating possible negative impacts. *C. indica* showed healthy signs throughout the experiment.

The maximum and effective quantum yield of PS II (Fv/Fm and ΦPSII, respectively) in plant leaves, and plant physiological parameters such as leaf pigments (chl a, chl b, anthocyanins and carotenoids), and sugars (total soluble and insoluble) were measured at T0, T-1/2, and Tf to track the plant status and stress indicators during the experiment.

Overall, the maximum and effective quantum yield of PSII were within the healthy levels (0.78 - 0.83) (appendix B- Figure B3). Chlorophyll a + b levels only showed significant decrease over time for *C. indica* of TW-Ci systems (Figure 16). The chlorophyll a and b

ratio on the other hand, had a significant increase at T-1/2 and a decrease at Tf for *C. indica* of TW-CS (Figure 16). Carotenoid levels had a significant decrease from T0 to T-1/2 in TW-Ci (Figure 17). Contrarily, carotenoid levels increased significantly from T0 to T-1/2 in *C. indica* of TW-CS, and then significantly decreased from T-1/2 to Tf. For anthocyanin there were no significant differences among TW treatments or time (Figure 17).

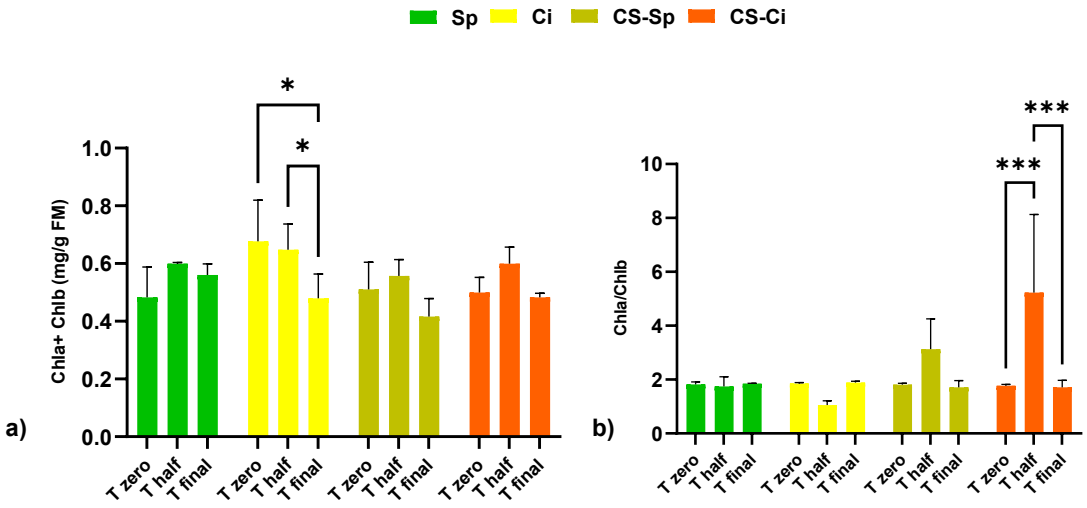


Figure 16. a) Sum of and b) ratio of chlorophyll a and chlorophyll b concentrations in plant leaves at T0 (before the experiment), T-1/2 (middle of the experiment) and Tf (end of the experiment). Error bars represent standard deviation (n=6). The asterisk (*) and lines show statistical significance between different sampling times ($p < 0.05$). FM stands for fresh mass. Sp indicates TW with S. erectum, Ci indicates TW with C. indica, CS-Sp indicates S. erectum from the polyculture TW, and CS-Ci indicates C. indica from the polyculture TW

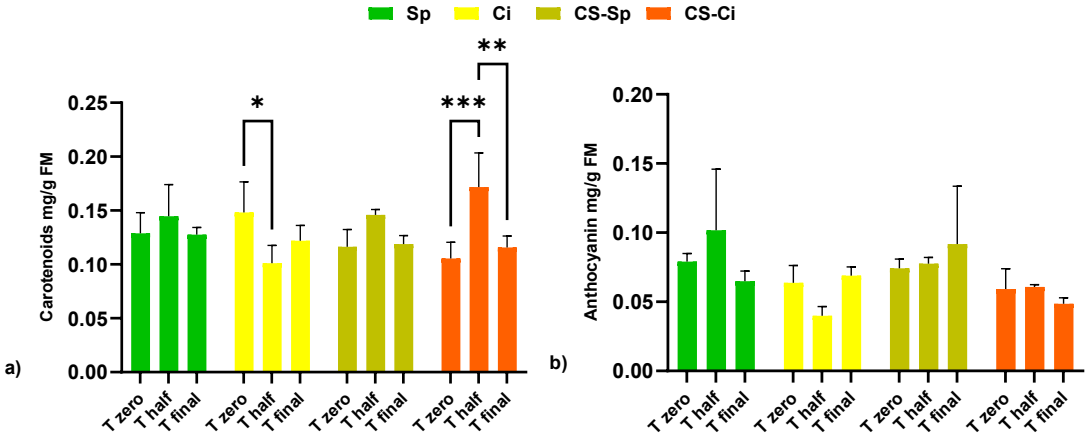


Figure 17a) Carotenoids and b) Anthocyanin concentrations in plant leaves at T0 (before the experiment), T-1/2 (middle of the experiment) and Tf (end of the experiment). Error bars represent standard deviation (n=6). The asterisk (*) and lines show statistical significance between different sampling times ($p < 0.05$). FM stands for fresh mass. Sp indicates TW with S. erectum, Ci indicates TW with C. indica, CS-Sp indicates S. erectum from the polyculture TW, and CS-Ci indicates C. indica from the polyculture TW

Two- way ANOVA showed that there was significant interaction effect of TW plant compositions and time on total soluble and insoluble sugar levels in plant leaves. Significant fluctuations of total soluble sugars between T0, T-1/2 and Tf in *C. indica* of TW-CS was observed (Figure 18). Insoluble sugars increased significantly from T0 to T-

1/2 and Tf in TW-Ci, and in C. indica of TW-CS increased from T0 to T-1/2 and decreased from T-1/2 to Tf (Figure 18).

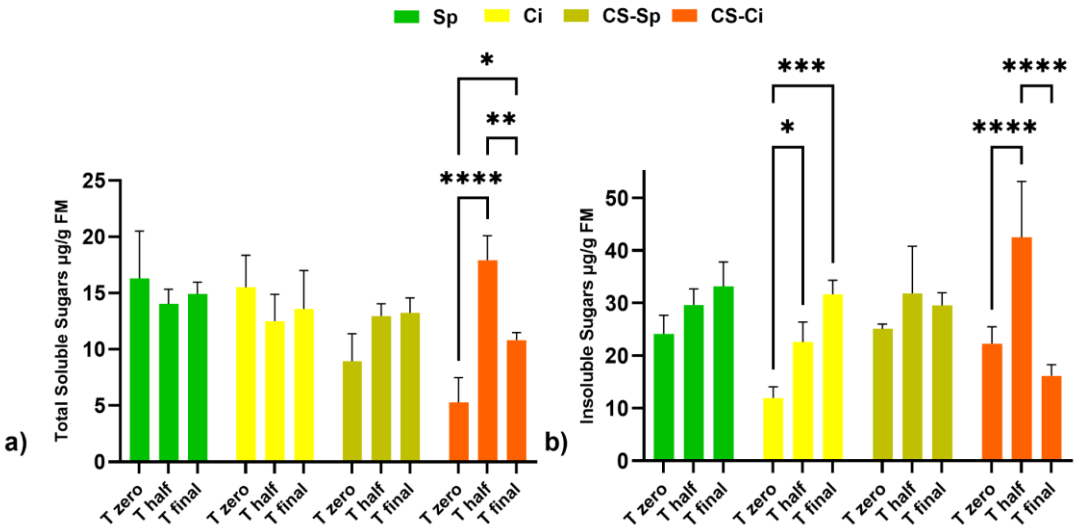


Figure 18a) Total soluble and b) insoluble sugars µg/g at T0, T-1/2 and Tf. Error bars represent standard deviation (n=6). The asterisk (*) and lines show statistical significance between different sampling times (* p ≤ 0.05 to 0.01, ** p ≤ 0.01 to 0.001, **** p≤ 0.001). FM stands for fresh mass. Sp indicates TW with S. erectum, Ci indicates TW with C. indica, CS-Sp indicates S. erectum from the polyculture TW, and CS-Ci indicates C. indica from the polyculture TW.

3.3.6. Presence of MC-LR- degrading Genes and Prokaryotic Community Composition in TWs Substrate

The results of the PCR screening for the mlrA gene revealed the absence of amplification of homologous gene sequences, which suggest the gene is absent in all the sand and rhizo-substrate samples.

For the prokaryotic community analysis in the substrate samples, there was a total of 16779 ASVs among 27 samples categorized by 3 sample variables- time (T0 and Tf), TW plant composition (Sp, Ci, CS), and substrate layer (sand and rhizo-sphere). Overall, 16394 taxa were classified across the 27 samples (41 unique phyla, 103 unique classes, 249 orders, 301 families, 624 genera). The most abundant phyla across all samples were *Proteobacteria, Actinobacteriota,* and *Cyanobacteria* (Figure 19). *Chloroflexi* and *Patescibacteria* were also found across all samples but at various abundances. *Planctomycetota* was found in all samples except one replicate of TW-Sp at T0. *Firmicutes* were found in all samples except one replicate of TW-Sp at T0 and another

replicate of TW-Sp's rhizo-substrate sample. *Acidobacteriota* was distributed among certain samples and not abundant in all samples and *Gemmatimonadota* was only abundant in one replicate of TW-Sp at Tf. The abundant phyla in the sand and rhizo-substrate at Tf, between the T0 and Tf sands, and only in the rhizosphere across the different TW plant composition, are in appendix B (Figures B4, B5 and B6).

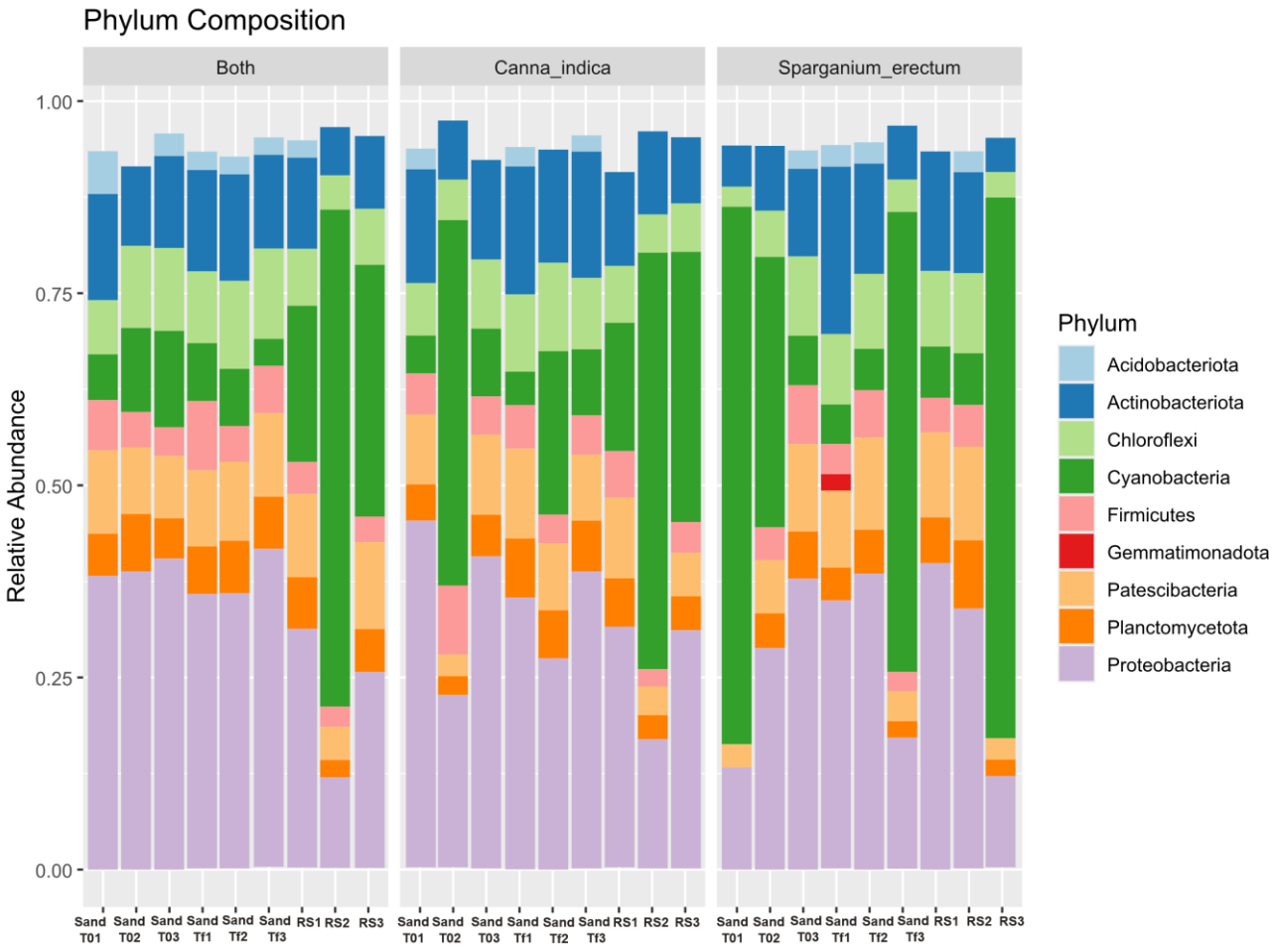


Figure 19. Prokaryotic phyla detected in TW substrate collected from replicates of TWs planted with C. indica, S. erectum and with both plants (> 2% relative abundance threshold). RS stands for rhizo-substrate, T0 for T-zero, and Tf for T- final)

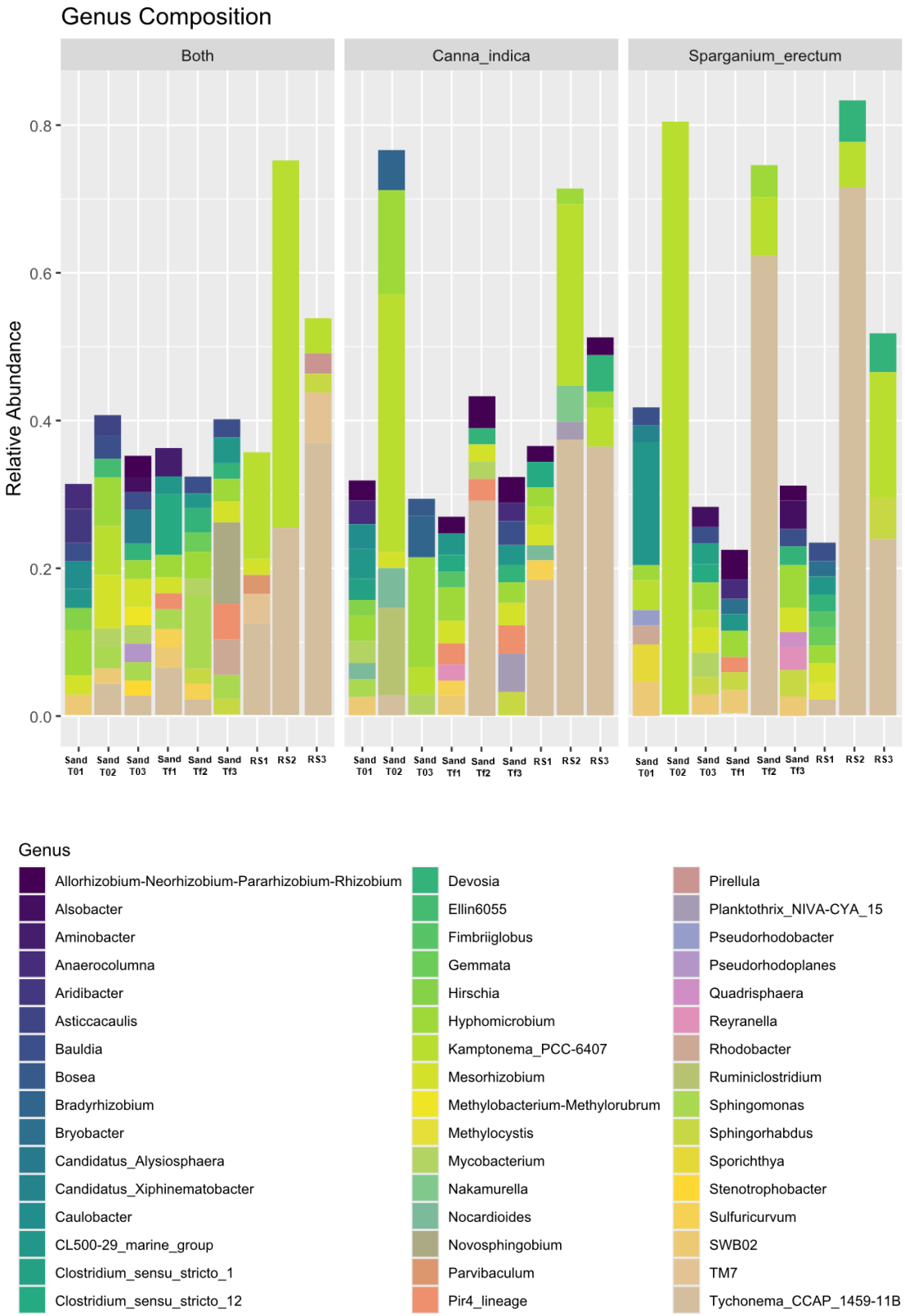


Figure 20. Prokaryotic genera (> 2% relative abundance threshold) in TW substrate collected from TW planted with C. indica, S. erectum and with both plants (Both). (RS stands for rhizo-substrate, T0 for T-zero, and Tf for T- final).

The most abundant genera found across all the samples are presented in Figure 20. *Sphingomonas* and *Novosphingobium,* two known MC degrading genera via the mlr degradation pathway were found across all sand samples. *Tychonema* and *Kamptonema,* were abundant across all rhizo-substrate samples. While they are both cyanotoxin producing genera, it is unlikely that the strains found here were cyanotoxin producing [56], [57]. There is clearly a higher number of different genera occurring in sand samples than rhizo-substrate (Figure 20). A shift in the abundant genera can also be seen between T0 and Tf (Figure 20). This is also in line with the beta-diversity (Figures 21 and 22) and alpha-diversity (Figures 23 and 24) analysis which is presented below.

Despite high diversity, prokaryotic community structure was strongly influenced by the different substrate matrices (sand and rhizo-substrate) and the time, but not by the TW plant composition (PERMANOVA p-values 0.001, 0.003 and 0.422, respectively) (Figure 21, appendix B- Table B7). Both the substrate matrix and time equally influenced the microbial community (R^2_{matrix} = 0.13, R^2_{time} = 0.11) (appendix B- Table B7). Indeed, at Tf, sand samples and rhizo-substrate samples were significantly different (PERMANOVA p = 0.002) (appendix B- Table B8). Although no differences were observed among TW plant compositions in the sands (PERMANOVA p= 0.169) (appendix B- Table B7), on the rhizo-substrate level, there was a strong influence of TW plant composition (Figure 22) (PERMANOVA p = 0.004) (appendix B- Table B8). On the other hand, among sand samples there was a strong influence of time (PERMANOVA p= 0.047).

The alpha-diversity metrics of all the samples grouped by plant composition and time can be seen in Figure 23 and grouped by plant composition and substrate matrices can be seen in Figure 24. When analyzing all samples together, only time significantly influenced the Chao1 and Observed alpha-diversity indices. However, a higher diversity is observed in sand than in rhizo-substrate samples (appendix B- Figure B8), especially in Chao1 and observed metrics. For sand samples alpha-diversity differences are only observed in *S. erectum* TW, which increased after cyanotoxins exposure (appendix B- Figure B9). On the rhizo-substrate level on the other hand, the alpha diversity indices were lower for *S. erectum* TWs (appendix B- Figure B10). Results are in line with ASV analysis.

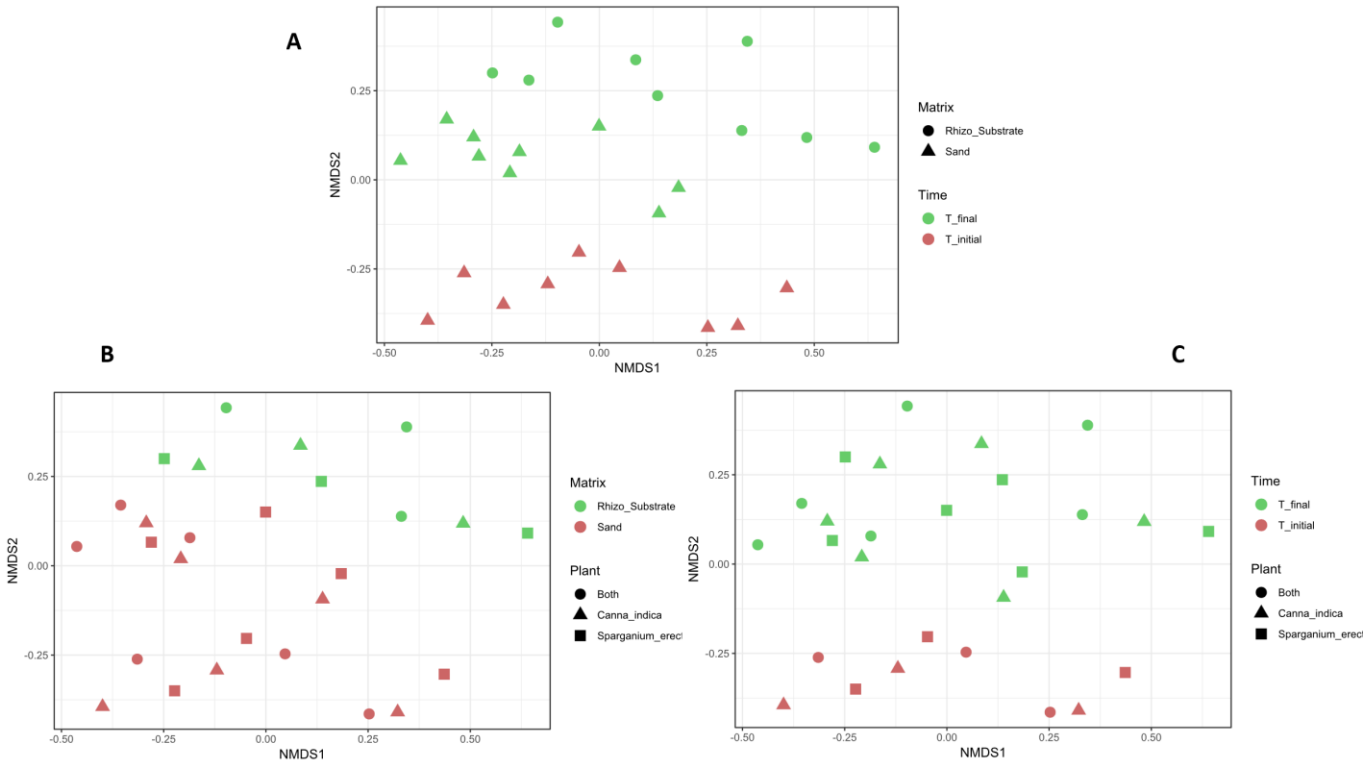


Figure 21. Non-metric Multidimensional Scaling (NMDS) plot showing Bray-Curtis distances for all samples, A) grouping time (T0 and Tf) and substrate matrices (sand and rhizo-substrate), B) grouping substrate matrices and plant compositions (TW planted with C. indica, TW planted with S. erectum and TW planted with both plants), and C) grouping time and plant compositions' influence on the microbial community

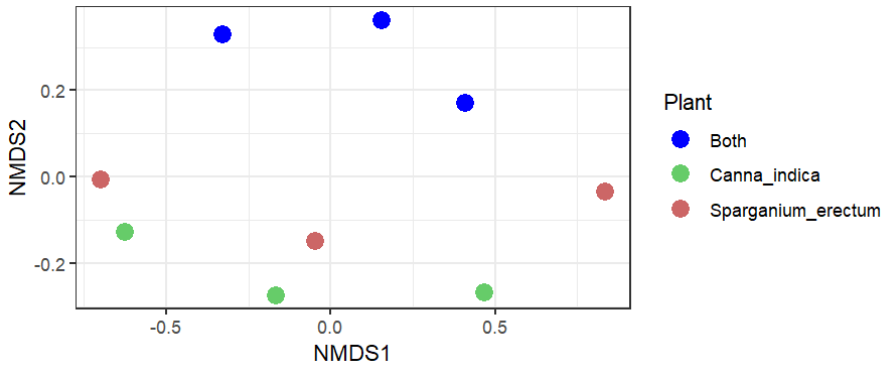


Figure 22. NMDS plot showing Bray-Curtis distances of rhizo-substrate samples at Tf, grouping plant compositions (TW planted with C. indica, TW planted with S. erectum, and TW planted with both plants).

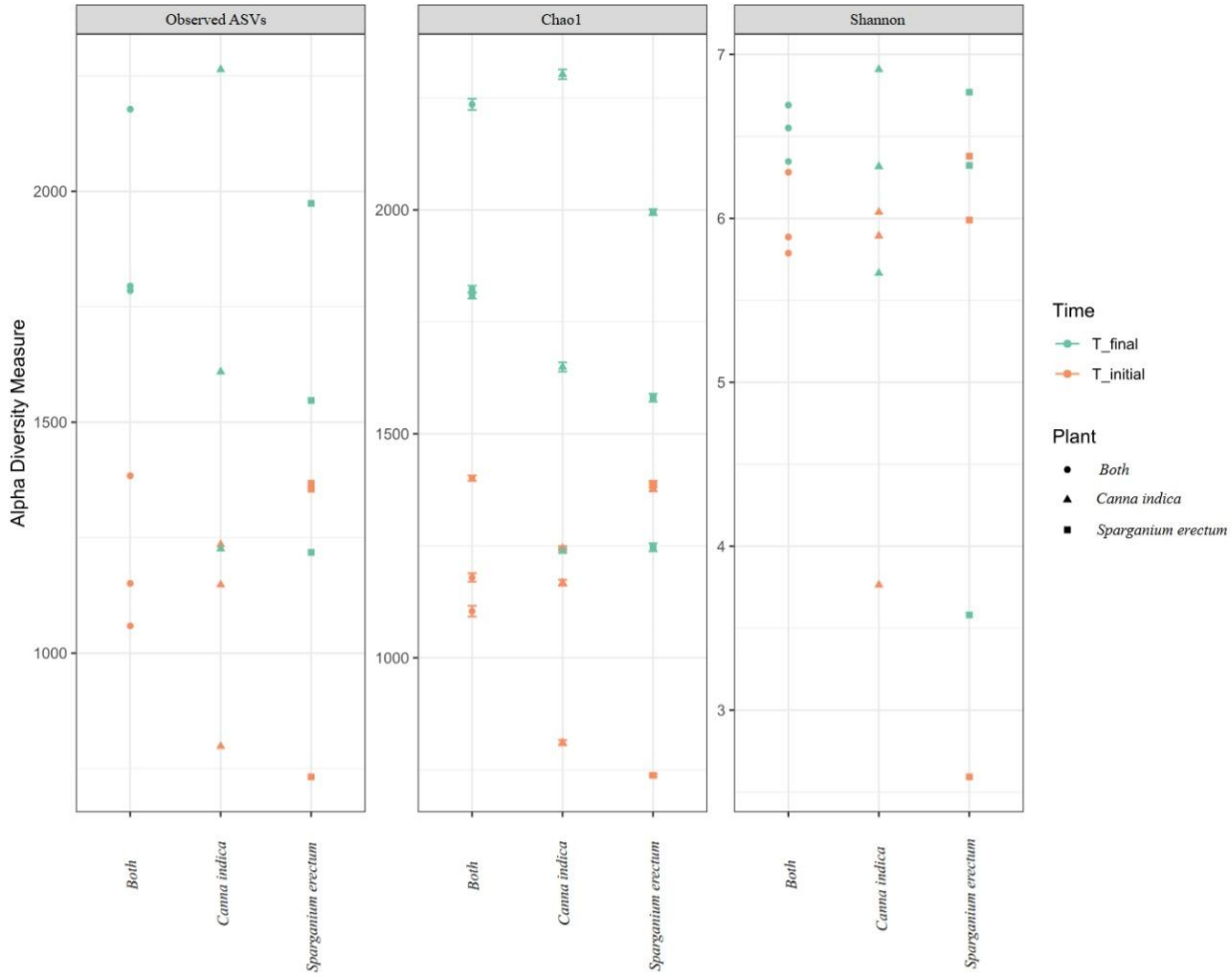


Figure 23. Alpha diversity indices (Chao1, Observed ASVs and Shannon) of samples grouped by Plant composition vs Time

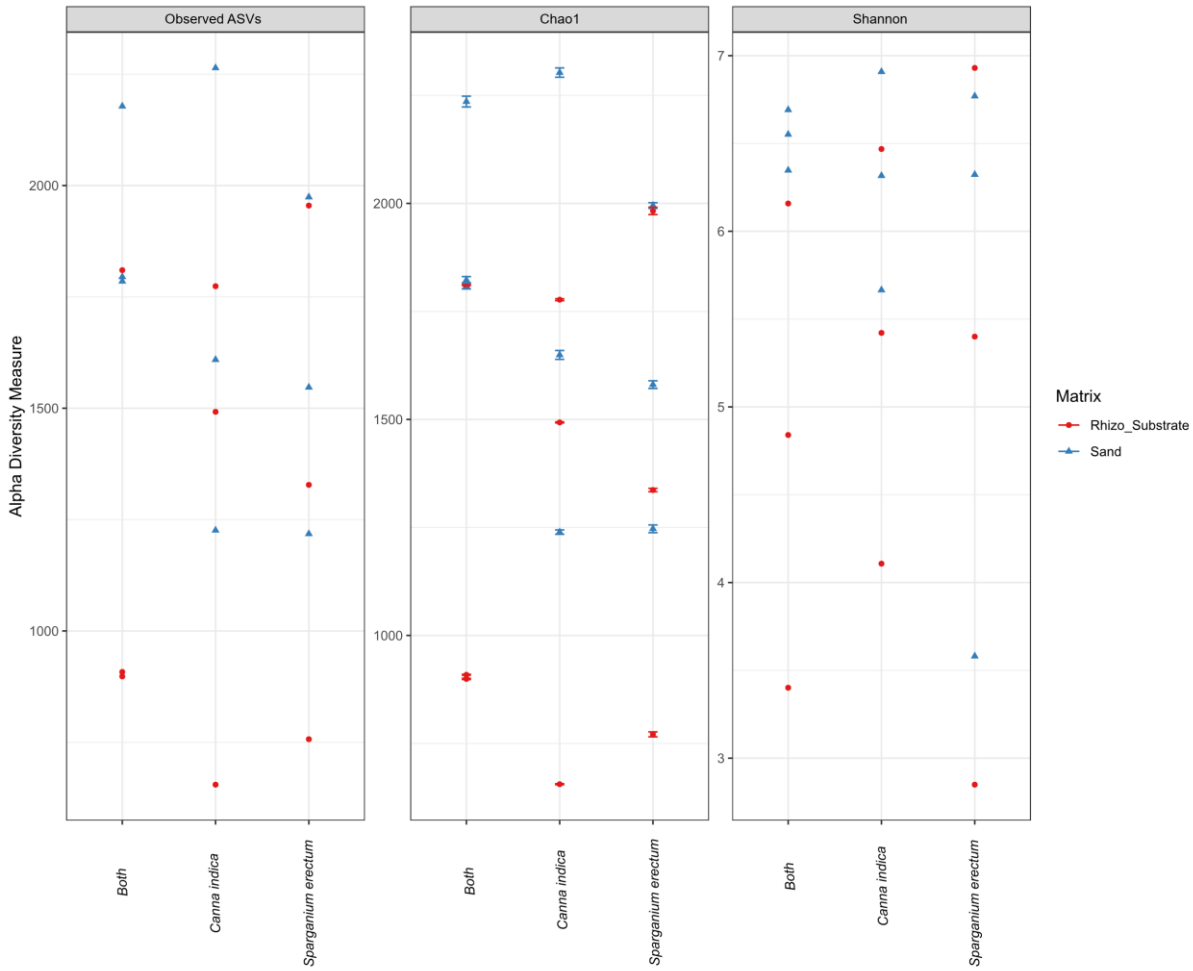


Figure 24. Alpha diversity indices (Chao1, Observed ASVs and Shannon) of samples grouped by Plant composition vs Substrate matrices

LEfSe analysis in the samples at Tf, showed that among the different TW plant conditions, *C. indica* TW substrates had the highest number of enriched genera (Figure 25). LEfSe analysis in the sand samples showed a higher number of enriched genera at Tf compared to T0, with 41 enriched groups in cyanotoxin contaminated sand (Figure 26).

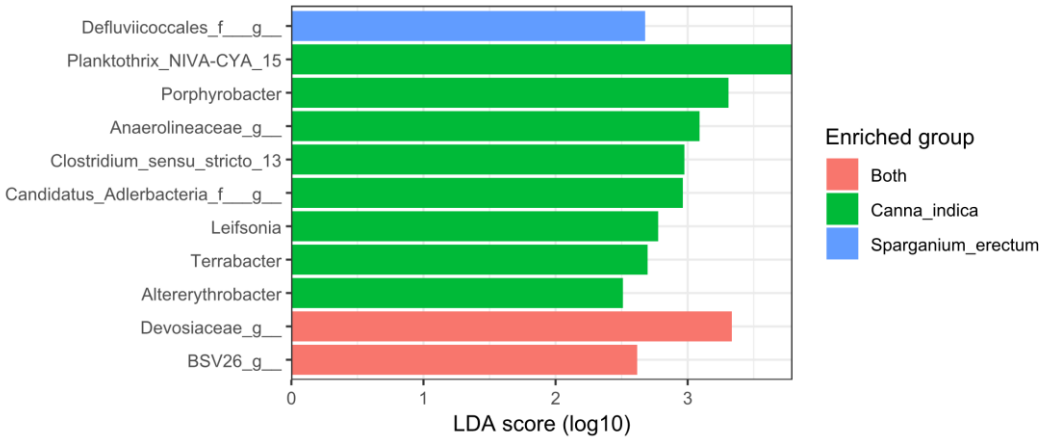


Figure 25. Enriched genera in substrate (sand and rhizo-substrate) of the different TW plant compositions TW planted with C. indica, TW planted with S. erectum, and TW planted with both plants) at Tf (LDA score 2.5)

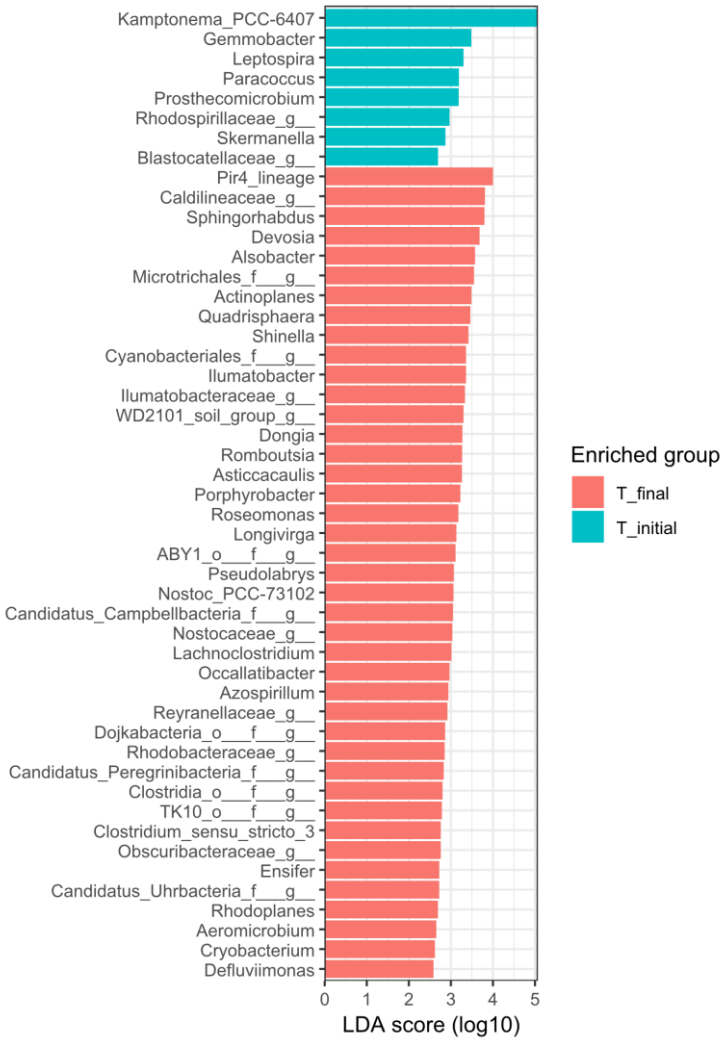


Figure 26. Enriched genera of TWs sand samples at T0 (T_initial) and at Tf (T_final) (LDA score 2.5)

LEfSe analysis in the sand and rhizo-substrate at Tf, showed that sand clearly had more enriched groups than the rhizo-substrate (Figure 27). This is in alignment with the alpha-diversity measures between the sand and rhizo-substrate (Figure 24 and appendix B-Figure B8).

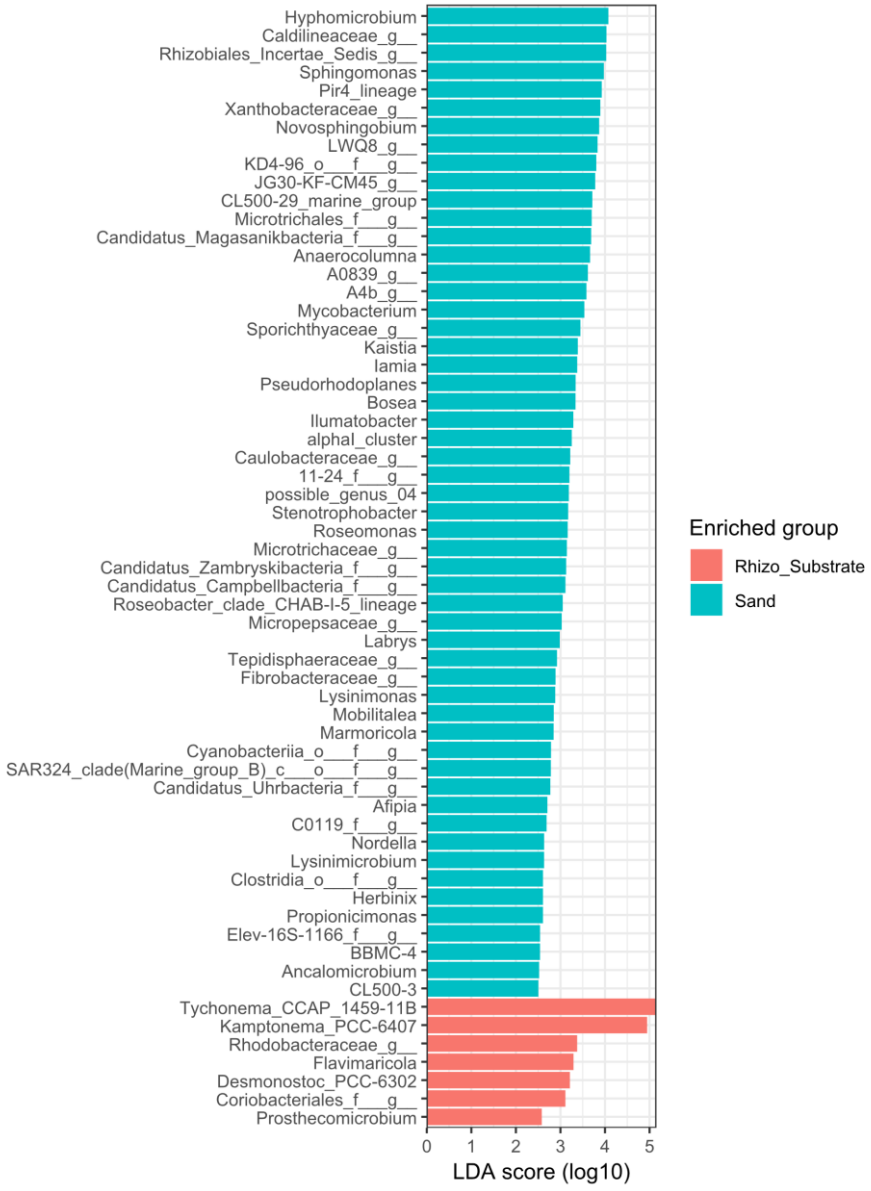


Figure 27. Enriched genera of sand and rhizo-substrate samples at Tf (LDA score 2.5)

3.4. Discussion

3.4.1. TW Performance

3.4.1.1. Cyanotoxins Removal

In this study, the performance of the TWs was influenced by the plant composition and time. The influence of time could be not only due to an adaption of the systems to the constant input of the cyanotoxins, but also due to the slight variations in cyanotoxin inlet concentrations and the spring water characteristics, as well as environmental factors such as temperature and light intensity (appendix B- Figure B1), over the experimental period. Variations in cyanotoxin doping concentrations were expected due to the inherent differences in concentrations of the freeze-thawed cyanobacterial cultures that were used to dope the spring water. However, harmful cyanobacterial blooms naturally vary due to several contributing factors including temporal, spatial, intra- or extra- cellular toxins, and environmental factors, resulting in varying toxin concentrations in the contaminated surface waters, [58], [59], [60]. As so, the experimental setup can represent real environmental conditions.

Generally, MC-LR removals were high in all the TW systems (> 96 % mass removal rates throughout the experiment), with outlet concentrations being below LOD (0.15 µg /L). This is consistent with previous studies evaluating MC-LR removal efficiencies in TWs [8], [9], [11], [12], [17], [31], despite the different TW design configurations, plant species and working modes tested. MC-LR concentrations in TW effluents in these studies are also comparable with ours, having also always been below the drinking water guideline value recommended by the WHO (1 µg/L).

CYN mass removals were higher in *C. indica* TW (TW-Ci: 73- 97 %, TW-CS: 81-98 %) than in those with *S. erectum* (TW-Sp: 70- 91 %). The CYN removal efficiency in TW-Sp decreased over time, whereas in TW-Ci and TW-CS systems removal increased over the treatment weeks. The reduced efficiency in *S. erectum* could be related with the exhibited plant senescence, probably also related to the decrease in temperature and light intensities over the course of the experimental period. In this regard, the TWs with *C. indica* were better adapted to the varying CYN input and environmental factors for CYN removal efficiency. However, there was no significant difference in removal efficiency between the TW-Ci and TW-CS systems, meaning the polyculture condition had no influence on cyanotoxin removal efficiency. Nevertheless, the removal rate was still higher than that reported for a VSSF-TW mesocosm planted with a wetland species (*Juncus effusus*), where a maximum removal rate of 52 % was achieved [17]. Another mesocosm study mimicking a horizontal subsurface flow TW, showed CYN removal

rates of 57 ± 12 % in spring and 77± 14 % in summer [10], which also correspond with the change in CYN removal efficiencies with varying climate conditions. In contrast to other studies that demonstrate that polyculture TW systems exhibit better pollutant removal efficiency [61], [62], there was no influence of using polyculture systems on cyanotoxin removal efficiency. However, it should be noted that the studies that have made comparisons between monocultures and polycultures were of pilot TWs that were run for at least a year. Since this study only lasted for 10 weeks, direct comparison needs to be made with caution and no deductions on the long-term functioning and stability of the TWs can be made.

Despite having high CYN mass removal rates, CYN concentration in the effluents were above the drinking water guideline values for chronic exposure (0.7 µg/L) [54]. In the case of TWs-Sp, towards the last weeks of the experiment, it was even above the drinking water guidelines for short term exposure (3 µg/L). ET rates in *S. erectum* are higher than in *C. indica*, which led to more concentrated effluents from TWs-Sp. This is similar to the pattern found in a study by A. Martinez i Quer *et al.*, 2024 [10], where despite having high mass removal rates, CYN concentrations in the outlets were above the guideline values for drinking water. However, in our study all effluent concentrations were below the guideline values for recreational waters (5.67 µg/L). It is to be noted that the dimensions of the TW systems in this study are small and installed above ground. TW systems at full scale would experience less heating, resulting in decreased ET rates and lower effluent concentrations. However, temperature differences can also potentially affect biodegradation rates. Since there are no standardized international guidelines for irrigation waters, we cannot discuss the possibilities of water re-use for crop irrigation. However, in the context of water reuse, using TWs planted with *C. indica* would exhibit better effluent quality.

3.4.1.2. Water Quality Parameters and TWs Working Conditions

Since natural spring water was used for the experiments, water that was collected every week to feed TWs, there was a high variability in the nutrients and organic matter content input in the TWs during the course of the experiment. Weather conditions mainly contributed to the variability in spring water composition, namely in nutrients and organic matter content, due to the occurrence of heavy precipitation in some periods. This resulted in high variations in the removal rates of nutrients and organic matter too.

The average TSS in the inlet waters were already low (0.4- 3.1 mg/L), indicating a good water quality. The removal rates varied among the TWs systems. However, the average

TSS in the outlets were below the inlets, showing no significant leaching from the TWs. The TSS in the polyculture systems (TW-CS) were also significantly lower than the other two TW conditions ($p < 0.05$), indicating a probability of better substrate stabilization due to the specific morphology or enmeshing of the roots in these systems. Regarding organic matter removal, measured through COD, the inlets varied between 8- 130 mg/L, which again resulted in variation in removal efficiencies, showing no removal in some cases, with COD levels being higher in the outlets than the inlets. This was however due to the already low COD levels in the inlet, rather than the low performance of the TWs. Moreover, the COD levels in the outlets were overall below the various global effluent discharge limits (≤ 100 mg/L). No significant differences were found among TW plant compositions in COD removal rates.

Nutrient removal rates were also evaluated (nitrate ion and total phosphorus) during the experiment to ensure proper functioning of the TWs. Nitrate ion removals were > 90 % in all TWs throughout the experiment, with no statistically significant differences among TWs. The total phosphorous removals varied with time but again no statistically significant differences among TWs were observed. pH levels in the outlets were also well within the safe levels, despite the inlet pH levels being slightly lower in some weeks.

The ORP is crucial for biochemical activity in TWs. Comprehending ORP levels in TWs is crucial for assessing the system's efficacy and treatment parameters. ORP gives information on the aerobic or anoxic conditions in TWs, influencing the efficacy of wetland treatment [63]. The ORP levels observed in the different TWs were still within aerated to moderately reduced conditions (100- 400 mV) [64], indicating good VSSF TW conditions. The significant decrease in ORP levels over time could be owed to the decreasing environmental temperatures, which can decrease plants activity [65], and the low COD levels in the spring water.

In summary, an overall good performance of the assembled TWs was observed, despite the varying inlet levels of nutrient and organic matter content, suggesting that the TW were simulating wetland conditions and could be extended for the treatment of water contaminated with cyanotoxins.

3.4.2. Cyanotoxin Retention in TW Substrate Matrices

While there was a significant removal of cyanotoxins from the spiked spring water, it is also important to determine if these toxins were retained inside the TW system, namely in the substrate or plant tissues, or if they were degraded.

There are several removal mechanisms of pollutants in TWs, which depend on factors including the pollutant/effluent being treated, hydraulic retention time, flow direction, substrate materials and plants used [66]. In this study, the role of substrate and plants in the removal of MC-LR and CYN from the waters was investigated, by analyzing the bulk substrate, the rhizo-substrate (in contact with plant roots), and plant tissues (leaves and roots) for possible retention of the cyanotoxins within the different TWs compartments. This contributes to the understanding of the cyanotoxin removal mechanisms in the TWs currently studied, which could help in estimating scale-up and design configurations of the TWs. It could also help investigate if novel components or factors of the TWs can increase removal efficiencies of the pollutants being targeted [66], [67].

The choice of substrates is known to have an important role in TWs, particularly in sub-surface flow wetlands [66]. They offer a supportive matrix for the establishment and development of plants and of the microbial community and provide the surface area for toxins sorptive interactions. Substrate is important for processes such as filtration, chemical transformations, permeability/ hydraulic control, and as a sink for contaminants, nutrients and organic matter [68]. Moreover, the use of a combinatorial substrate approach, combining different types of substrates, has shown to have synergistic effects, enhancing the overall efficacy of pollutant removal [69]. Two recent studies show that factors such as substrate arrangement, substrate-layer thickness ratio, substrate submergence depth, and hydraulic load also play a role in improving removal efficiencies of contaminants [70], [71], although these factors were not explored in the current study. In this study the substrate was composed of a top layer of sand (particle size of 0.4-0.8 mm) into which plants were transplanted, a second layer of biochar (particle size 1-4 mm), a third layer of light expanded clay aggregate (8- 13 mm), and a bottom layer of gravel (particle size 10-15 mm) as a support and drainage layer, following an ascending order of substrate particle size arrangement. This combination of substrate was selected as in a previous study both expanded clay and biochar have shown high cyanotoxins sorption [18].

The bulk-substrate and rhizo-substrate of each TW were analyzed for MC-LR and CYN levels at the end of the experiment. The bulk substrate contained only sand, whereas the rhizo-substrate (in contact with plant roots) contained a mix of sand and biochar as plant roots, initially in the sand layer, have grown into the below biochar layer. Results showed low retention rates (< 8%) of the cyanotoxins in the substrates. When comparing the two cyanotoxins, CYN had relatively higher retention than MC-LR, and when

comparing the bulk and rhizo-substrate, there was higher retention at the rhizo-substrate. The increased retention rates in the rhizosphere could be attributed to the plant action or the presence of biochar. Biochar has garnered interest as an effective substrate due to its large surface area, porosity, and functional groups that facilitate the adsorption and retention of contaminants [16], [72]. With regard to cyanotoxin adsorption by biochar, several studies demonstrate high sorption capacities of MC-LR [73], [74]. Biochar has also shown CYN sorption, with sorption capacity or removal efficiency being higher than that of MC-LR [18], as also observed in the current study, where there is significantly higher CYN retention in the rhizo-substrate, than MC-LR. However, most sorption investigations on MC-LR and CYN are batch studies rather than those involving adsorbents integrated into treatment systems and comparisons on sorption must be carefully made. Moreover, when incorporated in water treatment systems like TWs, other factors that influence pollutant retention or removal include the presence of vegetation and microbial degradation.

The presence of different plants had an influence on the cyanotoxin retention. In the sand, there was significantly higher retention of MC-LR and CYN ($p < 0.05$) in the TW-Sp systems, when compared to TW-Ci and TW-CS systems. In the rhizo-substrates, there was only significantly higher retention of CYN in the TW-Sp systems in comparison to TW-Ci and TW-CS. This difference in retention rates could be attributed to the distinct roots, rhizomes, and root exudates of *S .erectum* and *C. indica*, which can play an important role in the removal of contaminants. For instance, the root structure has shown to help improve hydraulic conductivity and prevent clogging in TWs [75], and larger root and rhizome distribution can improve surface area for contaminant adsorption [62], [66], [76], [77]. *S. erectum* has an extensive root system that is thick and deep, to support its tall growth habit in aquatic environments [78]. *C. indica* on the other hand, forms branched rhizomes that grow horizontally and are divided into bulbous segments rich in starch [79]. A study by T. Liffen et al., 2011 [80] demonstrates the resilience of the below-ground biomass of *S. erectum* to uprooting, indicating the species' potential to stabilise and fortify the typically soft, silty sediments it frequently inhabits, where its rhizomes and roots develop in low-energy river environments. Additionally, it demonstrates that the plant's rhizome strength stays consistent throughout the growing season, indicating its capacity to preserve and reinforce fine sediments throughout winter even when above-ground biomass is absent. This could explain the higher cyanotoxin retention in the substrate matrices of the TWs planted with *S. erectum*. Moreover, the chemical profiles of the root exudates of *S.erectum* and *C.indica* can differ significantly, and can contribute

to the differences observed in cyanotoxin retention and removal. While there is limited direct evidence available in scientific literature on the composition of root exudates for *S.erectum*, the root exudate composition of *C.indica* is well characterized. There is even evidence of specific root metabolites and rhizosphere bacteria that get recruited during metal stress in *C.indica* [81]. This difference in root exudates and rhizosphere composition could also contribute to the observed differences in cyanotoxin retention and removal, for instance, with higher cyanotoxin degradation in the substrates of TWs planted with *C. indica*, resulting in lower toxins levels in the rhizosphere of this plant. There was no significant difference in cyanotoxin retention in the substrate matrices of TW-Ci and TW-CS, indicating that plant diversity did not influence the cyanotoxin retention. Moreover, considering the high removal rates in water, and relatively low retention rates in the substrates, there is an indication that cyanotoxins could have been uptake by plants and/or degradation by TW microbial community.

3.4.3. Cyanotoxin in Plants Tissues and Impact on Plant Status

Plant uptake can play a role in pollutant removal, although its importance varies with the plant species and biology, the pollutant type and the TW system configuration [82]. Cyanotoxins may also be taken up and accumulated by plants, though their mechanisms and effects can vary. Strategic design and selection of plant species can enhance this process for specific pollutant removal.

The uptake and accumulation of cyanotoxins by plants is dependent upon parameters like toxin type/ concentration, plant's organ partition and capacity of metabolization of the cyanotoxin, and time of exposure, sometimes leading to various phytotoxic effects. Although limited, there are some studies on the uptake and accumulation of microcystins by aquatic plant species (emergent and submergent), which indicate bioaccumulation and unveil phytotoxic effects [83], [84], [85]. However, it is important to note that these studies were done in water systems and not water-soil systems, at non-ecologically relevant concentrations [86]. Thus, extrapolation to soil systems must be taken with caution, as soil may have additional effects towards reducing cyanotoxin bioavailable, which can decrease plant their uptake and toxicity.

In the current study, MC-LR and CYN were not detected in any of the plant tissues/organs, neither after 5 weeks nor after 10 weeks of exposure to cyanotoxin contaminated spring water. Although, the plants were exposed to relatively low levels of either cyanotoxin (2-10 µg/L MC-LR, 8-15 µg/L CYN), and the cyanotoxin detection limits in the plant tissues (0.06 ng/g FM for MC-LR and 0.07 ng/ g FM for CYN) might not allow detection of accumulation. Moreover, the TW system encompasses a complex

combination of mechanisms for pollutant removal and the contribution of the plants in this study to directly uptake and accumulate cyanotoxins might be limited. The absence of detectable MC-LR and CYN in either plant roots or plant leaves does not rule out uptake and metabolization within plant tissues could have occurred. Studies in the literature confirms plants' ability to uptake cyanotoxins, however, explicit metabolization pathways for their detoxification remain poorly characterized [8], [87]. Previous studies indicate the possible pathways of glutathione conjugation by specific glutathione-S-transferase enzymes [88], and cytochrome P450-mediated alterations similar to xenobiotic detoxification processes [89] in plants. The results obtained here open perspectives for future studies unveiling the metabolic pathways of cyanotoxin detoxification by *S. erectum* or *C. indica*.

The potential stress effects on the plants' biology and performance were monitored throughout the experiment, through leaves photosynthetic efficiency, pigment composition and carbohydrate metabolism, which are key endpoints of primary metabolism. By doing so, the adaptability of the different plant species and their polycultures in TW conditions could be investigated.

Chlorophyll analyses indicated that *C. indica* in monoculture might be more susceptible than *S. erectum*, but that these effects may be overcome in polyculture TWs. This higher susceptibility was for example demonstrated by the significant decrease in net chlorophyll levels (a + b) found in the *C. indica* species in the TW-Ci systems over time, but not in the polyculture system The chlorophyll a/b ratio increased in *C. indica* in TW-CS systems may indicate a response to mild stress conditions along the experimental weeks exposure condition. Considering that the plants used were already adult at the beginning of the experiment, and the leaves well expanded, the changes observed in the pigments suggest that no major impacts towards impairing photosynthesis are observed by the presence of cyanotoxins, and that the effects may be minorized when different plant species are grown together. This conclusion is in agreement with the Fv/Fm and ϕPSII results that indicate no impairment in PSII. The maximum and effective quantum yield of PSII were within healthy levels throughout the experiment. Nevertheless, further metabolic studies may be performed to validate this hypothesis, as other studies have been showing significant impacts of cyanotoxins on other plant species [90], [91], [92], [93], [94].

Carotenoids function as antenna pigments in plants and play a crucial role in stress indication. They are precursors in the biosynthesis of abscisic acid, which is a phytohormone that regulates stress responses [95], [96]. Here, carotenoid levels

significantly increased at T-1/2 in *C. indica* in TW-CS systems, indicating stress. But their levels decreased again at Tf, going back to initial levels. This initial increase, and subsequent decrease as plants acclimate to the experimental conditions, could signify the plant's adaptation to stress [97]. This was also observed in the *S. erectum*, although the fluctuations were not significant. Interestingly, this was not observed in the *C. indica* of the TW-Ci systems, demonstrating better adaptation of the *C. indica* of the polyculture systems. Anthocyanin, another important pigment in the indication of plant stress responses, was also evaluated, but the fluctuations were not statistically significant.

Soluble sugars in plants are essential for multiple structural and functional roles, including osmotic balance, energy supply and stress signalling, and are often used, together with other indicators, to make a global assessment of the photosynthetic status. The soluble sugar metabolism can be affected by environmental stresses such as variations in light, water, temperature and attack by pathogens [98][99]. In this study, total soluble sugars increased by four times in the leaves of TW-CS *C. indica* at T-1/2. This increase may have been a stress response aimed at contributing to for e.g., higher energetic demands when put on defense mechanisms, increased lignification of some cell walls towards controlling the stress pathways (e.g. apoplast/symplast), or to help mitigating damage caused by reactive oxygen species generated during stress conditions. However, in total soluble sugars a significant decrease again at Tf was observed, suggesting the plant's possible acclimation to the stress. A similar pattern was observed in the insoluble sugar levels in the *C. indica* of TW-CS systems. Insoluble sugars serve as long-term energy reserves and structural components of the plants. Insoluble, sugars are also known to accumulate in plant leaves due to various stress factors [98], [100].

So, *C. indica* of the polyculture TWs showed some signs of stress response with fluctuating levels of sugars and pigments which indicated its capability of plant stress adaptability. On the other hand, the absence of significant fluctuations in *S. erectum* and *C. indica* in monoculture indicates no signs of adaptability to stress effects. This demonstrates that using *C. indica* in polyculture would be a good prospective to use in TWs treating MC-LR and CYN. However, it should be noted that plants in the field are typically exposed to a mixture of many abiotic stressors such as nutrient imbalances, anoxia due to saturated TW conditions, pH fluctuations, varying temperatures and light intensities related to season, and other co-contaminants could be contributors of stress. The responses of plants to combined stressors are distinct, and the reactions to each stress cannot be considered in isolation [98]. Furthermore, not all plants have identical

responses to all combinations of stress, and there are considerable differences across species and genotypes [101]. Therefore, responses of plants to stresses are a very complex phenomenon and may also differ while scaling up of TWs. The absence of significant stress could also be attributed to the fact that no significant accumulation of cyanotoxins was observed, or that the cyanotoxin detoxification pathways in these plants were adequate, highlighting the potential of these two species to these types of TWs.

Plants' contribution to organic pollutant removal can be more indirect. For instance, they can support microbial communities in the roots (rhizosphere effect). These microbes degrade organic pollutants more efficiently than plant uptake alone [102]. In this study, as no accumulation by the plants was observed, one must not discard the relevance of the contribution of the associated microbial community in the cyanotoxin biodegradation, which deserves further studies.

3.4.4. Genes and Microbial Community Composition

Given the high removal rates of cyanotoxins from the water, low retention rates in the substrates, and no detected plant accumulation, microbial degradation of cyanotoxins seems the key removal mechanism. PCR screening for the mlrA gene confirmed the absence of amplification of homologous genes, rejecting the only known MC-LR degradation pathway, as seen in other studies [10]. However, the absence of the gene does not rule out microbial intervention, the promiscuous degradation by microbes through other unspecified pathways is a high possibility. Moreover, MC-LR could be co-metabolically degraded with carbon, nitrogen or phosphorus. Currently there are no known degradation pathways for CYN. Therefore, characterizing the microbial community within the TW substrate is important for understanding the impact of cyanotoxins and their potential degradation. It is well known that TWs rely heavily on their microbial community for pollutants removal/degradation. The microbial community composition of TWs can differ depending on various factors such as plant presence and species, season, substrate, and the specific pollutants being treated. Here, the microbial community composition, dominance, diversity and functional dynamics across various plant compositions, different TW matrices, and the impact of time was investigated.

3.4.4.1. Phylum and Genus Composition of Prokaryotic community

The most dominant phyla in all substrate samples were *Proteobacteria, Actinobacteriota*, and *Cyanobacteria*. *Chloroflexi, Patescibacteria, Planctomycetota, Firmicutes, Acidobacteriota,* and *Gemmatimonadota* were present in various abundances in specific samples. This agrees with other studies focused on microbial

ecology in TWs [103], [104], including one with the treatment of MC-LR and CYN [10]. *Proteobacteria, Actinobacteriota, Chloroflexi* are consistently identified as dominant phylum in vertical flow TW studies, having been known to play a vital role in nutrient cycling, complex organic matter degradation, biofilm formation, and overall water purification processes. There was no shift in the abundance at the phylum level when comparing TW plant compositions, time, or substrate type.

At the genus level, there were 48 different genera that were abundant across all the samples, with high diversity amongst the samples. It can be seen that certain genera dominated the rhizo-substrate, in comparison to the sand samples, where there was more diversity. This is likely due to the selection of certain bacteria in the rhizosphere from the bulk substrate. This trend of higher microbial diversity in bulk-soil than the rhizosphere is typical and has been observed in studies comparing microbial diversity in the rhizosphere and bulk soils [105], [106]. However, this might also be due to the presence of biochar. A study by Cakin et al., 2024 [107], noted increased TW performance but reduced microbial diversity with incorporating biochar in TW mesocosms. A shift in the genus composition can also be seen when comparing sand samples over time, and the different TW plant conditions at the rhizosphere level. This indicates that microbial composition varies according to the aforementioned parameters; nevertheless, further investigation through alpha and beta diversity allowed us to comprehend their significant shifts.

Beta-diversity analysis showed that there was indeed a strong shift in the microbial community over time. The microbial community was also significantly different between the two substrate types (at Tf). The TW plant compositions influenced the microbial community only at the rhizosphere level, with the TW-CS community being more dissimilar from the TW-Sp and TW-Ci communities, showing that different plants can influence the microbial community surrounding their roots. This could be related, for instance, to the exudation of different compounds by plant roots of different plant species [108].

Alpha-diversity analysis across all the samples conformed to this, with higher Observed ASVs and Chao1 indices at Tf than T0. Interestingly, when comparing T0 and Tf sand samples, only *S. erectum* TW showed an increase in alpha-diversity at Tf. Whereas at the rhizosphere level, *S. erectum* TW showed lower alpha-diversity indices compared to systems containing *C .indica* and both *C. indica* and *S. erectum.* This indicates that *C. indica* brings more diversity at the rhizosphere level than *S. erectum*. This correlates with

the plant health parameters where *C. indica* demonstrated better adaptation strategies through fluctuations in the pigments and sugar levels than *S. erectum*. When comparing sand and rhizo-substrate samples at Tf, sands had relatively higher alpha-diversity indices. Which reinforces that the bulk substrate has more microbial diversity than the rhizo-substrate matrix. The possible explanations for this could be the selective pressure in the rhizosphere due to plant root exudates, change in nutrient gradient and competition, and oxygen and redox gradients, thereby favouring specific microbial taxa leading to lower microbial diversity but higher functional specializations. On the other hand, the bulk substrate may have more varied micro-habitats, including biofilms, which can help form extensive multi-species communities leading to increased microbial diversity [109][106].

3.4.4.2. Enriched Taxa in Different Plant Compositions

Zooming in to taxa enriched under specific plant compositions, it can be seen that TW-Ci substrates had more enriched groups than the substrate of the other two TW types. The order *Defluviicoccales* was typical of the TW-Sp systems. Although this order has previously not been associated with *S. erectum*, they have been relatively abundant in enhanced biological phosphorus removal (EBPR) wastewater treatment plants [110]. They are recognized for accumulating glycogen and polyhydroxyalkanoates which potentially support their winter niche [111]. Their enriched presence here could be attributed to phosphorous removal and helping the plant survive during the dormant winter months [78].

Among the taxa enriched in TW-Ci systems, *Anaerolineaceae* was the only group previously linked to the presence of *C. indica*, having been a dominant family present in a TW beside Dianchi Lake in China planted with *C. indica* [112]. The enriched taxa typical to TW-Ci systems were mostly of plant-associated soil and rhizosphere habitats, characteristic to stress tolerance and plant-growth promoting activity such as nitrogen fixation, and pathogen resistance (*Porphyrobacter, Leifsonia, Terrabacter, Altererythrobacter*) [113], [114], [115]. Certain species of the *Porphyrobacter* genus also exhibit algicidal properties, having implications to control harmful algal blooms [116]. Some of the enriched taxa were also characteristic to anaerobic conditions (*Anaerolineaceae*, Clostridium_sensu_stricto_13, *Candidatus_Adlerbacteria*). The *Devosiaceae* and BSV26 family were enriched in TW-CS systems, both of which facilitate environmental adaptability and sensing. *Devosia*, the most common genus of *Devosiaceae* , thrive in various toxin-contaminated soils (e.g., hexachlorocyclohexane,

mycotoxins) and are best characterized for their bioremediation capabilities [117]. BSV26 families are found in diverse habitats, highlighting its ecological adaptability, however its metabolic functions are speculative. Its presence in activated sludge from petroleum facilities, deep layers of lakes, and caves suggest roles in elemental cycling, hydrocarbon degradation, and microbial ecosystem stability has been reported [118], [119], [120].

3.4.4.3. Enriched Taxa at T0 and Tf

3.4.4.3.a. At T0 –

It is to be noted that the taxa enriched at T0, before cyanotoxin exposure, could have a prominent influence from the spring water and the biofilms formed in the TWs during the acclimation stage of the TWs, or even the plant roots, as adult plants were transplanted into the TWs systems. It is difficult to clearly point out which was the driving influence for specific enriched taxon, without the microbial community data of the spring water or plant roots, which was not assessed in the current study. However, most of the enriched taxa typically found at T0 were characteristic to low nutrient freshwater environments, specializing in nutrient cycling and efficiently utilizing scarce resources. This could mean that the nutrients from the spring water were insufficient for the plants, resulting in the formation of a diverse microbial community with effective strategies for nutrient availability. Of the enriched taxa at T0, some were also previously identified in constructed or natural wetland ecosystems (*Leptospira, Paracoccus, Rhodospirillaceae, Blastocatellaceae*) [121], [122], [123]. *Leptospira,* a genus with several pathogenic species, were previously identified in sediments of wetlands affected by livestock raising intensification [121]. Given their ability to flourish in waterlogged soils and stagnant water, they were likely introduced into the spring water via urban runoff; and entered the TWs through the spring water. A potentially toxic strain of *Kamptonema (PCC 6407)*, known to inhabit diverse freshwater environments, including shallow pools, springs, and periodically flooded areas may have also been enriched through the spring water. The enriched genera *Gemmobacter* and *Paracoccus* have both been attributed to their versatile metabolic capabilities in diverse environments, resulting in their biodegradation potential of various organic pollutants in the environment [124], [125]. Other enriched taxa here such as *Prosthecomicrobium, Skermanella*, *Rhodospirillaceae,* and *Blastocatellaceae*, were all metabolically versatile, possessing adaptive traits such as enhanced surface area (prosthecae formation), biofilm formation, nitrogen fixation,

carbon decomposition, and host symbiosis to cope with low nitrogen and, or carbon environments [126], [127], [128], [129].

3.4.4.3.b. At Tf–

Some of the typical taxa enriched at Tf, after cyanotoxin exposure, *Sphingorhabdus, Shinella, Porphyrobacter* were associated with cyanotoxin degradation; while *Ilumatobacteraceae, Roseomonas,* and *Obscuribacteraceae* were known to be part of bacterial consortia co-existing with harmful algal blooms. *Sphingorhabdus* has already been identified as a potential MC degrader and has been found to be abundant in a biologically enhanced biochar environment exposed to MC-LR [130]. *Sphingorhabdus,* along with *Roseomonas, was* also found in abundance in a bacterial community associated with phytoplankton bloom in a eutrophic lake in South Norway, coincidentally when the cyanotoxin concentration was highest [131]. *Shinella* have also shown to be capable of degrading MCs [132], suggesting an adaptive role in mitigating cyanotoxin impacts. Interestingly, *Porphyrobacter*, a genus exhibiting algicidal properties, was also typical to the TW-Ci systems (refer section 3.1.1.), meaning the presence of MC-LR/ CYN were also drivers of their enrichment in the TW-Ci systems. Another study found *Ilumatobacteraceae* proliferated alongside the predominance of *Microcystis* spp. in coexisting *Synechococcus* and *Microcystis* blooms, where it was linked to high *Microcystis* and low *Synechococcus* samples [133]. *Obscuribacteraceae* was consistently found in the benthic sediment of a WWTP stabilization pond frequently disrupted by *Microcystis* blooms. While these bacterial groups are consistently found in occurrence with harmful algal blooms or cyanotoxin contaminated environments, the functional roles behind their associations are under explored. Their presence in TW ecosystems could suggest their potential as cyanotoxins degraders or that they have developed a microbial community resistant to cyanotoxin exposure.

Other enriched taxa at Tf included groups functional in nitrogen and phosphorous transformation. For instance, the bacterial groups *Microtrichales, Actinoplanes*, and *Quadrisphaera* are biomarkers for phosphorus accumulation and mobilization in soil ecosystems [134], [135], [136]. Known nitrogen-fixing and nitrogen transforming groups including *Roseomonas, Nostoc, Azospirillum, Ensifer, Rhodoplanes*, and *Defluviimonas* were also enriched [137], [138], [139], [140]. There were also enriched groups that have previously been identified specifically in constructed or natural wetland ecosystems- *Caldilineaceae, WD2101_soil_group, Dongia, Romboutsia, Reyranellaceae, Ilumatobacter* and *Rhodobacteraceae* [10], [109], [141], [142], [143], [144]. Plant-growth

promoting and environmental oxidative stress tolerant bacterial taxa such as *Devosia, Alsobacter, Occallatibac* [117] were also enriched at Tf. Numerous enriched taxa also belonged to an anaerobic niche, with some being identified specifically in anaerobic digester (AD) bacterial communities (*Pir4_lineage, Longivirga, Dojkabacteria, Candidatus_Campbellbacteria, Clostridium_sensu_stricto_3*) [145], [146], [147], [148], [149] indicating that certain sites in the TWs were functioning anaerobically. It is also to be noted that at Tf, *Cyanobacteriales* were enriched in the TWs substrates too.

3.4.4.4. Enriched Taxa in the Different Substrate Matrices at Tf

3.4.4.4.a. Enriched Groups in the Rhizo-substrate -

The presence of more enriched groups in the sand substrate than the rhizo-substrates further confirms higher microbial diversity in the bulk substrate than in the rhizo-substrate. In fact, numerous studies indicate that biodiversity is greater in bulk soil compared to rhizosphere soil, since the rhizosphere tends to be a more selective environment, leading to enrichment of certain microbial taxa that are particularly adapted to the root exudates and conditions near roots [99], [100], [102]. The enriched groups in the rhizo-substrate specifically belonged to the phyla *Cyanobacteria, Proteobacteria, and Actinobacteriota.* The cyanobacteria *Tychonema* and *Kamptonema* enriched at the rhizosphere belong to genera that are potential cyanotoxin producers, however it is unlikely that the strains found here are toxin producing [56], [57]. *Desmonostoc PCC-6302* on the other hand is a key component in microbial consortia designed for soil improvement, particularly in nitrogen fixation and carbon sequestration; and is not related to cyanotoxin production. This microbial consortium formulated for soil enhancement [150] includes other cyanobacteria like *Nostoc PCC-73102,* which was also enriched in our systems at Tf. Two genera (*Kamptonema PCC 6407* and *Prosthecomicrobium*) enriched in the rhizo-substrate, were also enriched in the bulk substrate at T0, indicating that they were probably from the spring water. It is likely that they were selected at the rhizosphere for specific plant growth or stress related functions. Other enriched groups like *Rhodobacteraceae, Flavimaricola,* and *Coriobacteriales* all contribute to the metabolic functions within the rhizosphere and endosphere of wetland plants, enhancing pollutant degradation processes [19], [151].

3.4.4.4.b. Enriched Groups in the Sand -

The genus *Sphingomonas* that is known to degrade MCs and nodularins; *Novosphingobium* and *CL500-29_marine_group*, identified in abundance during

M.aeruginosa blooms [152] were enriched in the sand, the bulk substrate. Strains of *Sphingomonas* and *Novosphingobium* are also known to have the *mlr* gene cluster. Mn-oxidizing bacteria (MOB) such as the *Hyphomicrobium* family were enriched in the sands. Previous studies have indicated that MOB has the potential to degrade CYN [153]. *Hyphomicrobium* and *Caldilineaceae* are enriched groups that have also been previously abundant in cyanotoxin treating TWs [10]. Enriched groups such as *Xanthobacteraceae*, *Bosea, Stenotrophobacter, A4b,* and *KD4-96* are identified in TW ecosystems known to degrade a wide range of contaminants [154], [155], [156], [157].

There was also enrichment of several oligotrophs of the biofilm niche, such as *Kaistia*, *Pseudorhodoplanes*, *Caulobacteraceae*, *Ancalomicrobium*, *CL500-3* [158], [159], [160], [161]. Enriched bacterial groups - *Pir4_lineage, Anaerocolumna, Microtrichaceae, Candidatus_Campbellbacteria, Tepidisphaeraceae, Mobilitalea, Herbinix, Propionicimonas, BBMC-4*, and a possible_genus_04 from *Fibrobacteraceae* family are all specific to complex organic matter degradation in anaerobic conditions [147], [149], [162], [163], [164], [165], [166], [167], [168], [169]. This could be correlated to the decaying plant biomass visually observed during the winter weeks of the experiment, especially in the *S. erectum* plants. Plant growth promoting bacteria normally found in contaminated sites were also enriched in the sands- *Rhizobiales_Incertae_Sedis*, *LWQ8* family*, Lamia, Labrys* and *Marmoricola* [19], [170], [171], [172], [173]. These bacteria are specific to plant biotic and abiotic stress responses. Pathogens such as *Mycobacterium, Clostridia, Bosea* and *Afipia* were also enriched in the sands [174], [175], [176], however their presence in the systems can probably be traced back to the spring water. JG30-KF-CM45, a family of bacteria increasingly found in biochar amended soils was also enriched in the sand samples [177]. The enriched presence of the members of the alphal cluster within the *Beijerinckiaceae* family (methylotrophs) in the sands again indicated the mitigation of methane emission by aerobic methane oxidation [178].

In summary, despite the differences in plant species, substrate type, and cyanotoxin presence, phylum-level relative abundance remained largely consistent among TW substrates. However, on the genus level there was significant variation in relative abundance, particularly between rhizo-substrate and sand samples, influenced by rhizosphere-specific selection and potentially biochar presence. Alpha and beta diversity analyses confirmed marked community shifts over time, between the bulk and rhizosphere substrates of the TWs, and the different plant compositions within the rhizosphere. TWs with *C. indica* exhibited higher microbial diversity and plant-growth

promoting taxa, correlating with improved physiological resilience. Taxa enriched at T0 were primarily associated with oligotrophic environments and efficient nutrient cycling, while at Tf communities included known cyanotoxin degraders and stress-tolerant plant-associated bacteria. Substrate specific enrichment at Tf revealed higher diversity in sands, which supported both cyanotoxin degrading taxa and anaerobic, complex organic matter degraders. Rhizo-substrates, in contrast, were dominated by functionally specialized taxa contributing to nutrient transformations and plant-microbe interactions.

3.5. Conclusion

The present work evaluated VF-TWs planted with monoculture and polyculture of *S. erectum* and *C. indica* plant species for the removal of MC-LR and CYN, and their mechanisms of removal in the TWs, including substrate retention and plant uptake. Microbialstatus communities in the substrate were also characterised, aiming to identify those responsible for cyanotoxin removal. MC-LR removals were high across all plant compositions (mass removal rates > 96% throughout the experimental period), with outlet concentrations from all TWs below LOD (0.15 µg /L). TWs with *C.indica* showed higher CYN mass removal rates (TW-Ci: 73- 97 %, TW-CS: 81-98 %) than TWs with just *S.erectum* (TW-Sp: 70- 91 %), and there was no influence of using polyculture systems on cyanotoxin removal efficiency. While substrate retention of MC-LR and CYN were generally low (< 8%), the plant composition had an impact on cyanotoxin retention at the bulk substrate and rhizo-substrate level. TW-Sp had significantly higher retention of both MC-LR and CYN in the sands, and just of CYN in the rhizo-substrate, than the other two plant compositions. The uptake and accumulation of MC-LR and CYN were not detected in the plant tissues, suggesting that microbial degradation could be the key removal mechanism of cyanotoxins. The microbial community showed a marked shift over the period of the experiment, between the bulk and rhizosphere of the TWs, and the rhizosphere of the different plants. Higher microbial diversity was observed at the bulk substrate than the rhizo-substrate. TW- Ci exhibited enriched plant-growth promoting taxa (*Leifsonia, Terrabacter, Altererythrobacter)*, which also correlated with the plant physiological tests, indicating that TW-Ci had higher stress tolerance to the overall TW conditions treating cyanotoxins. The bulk substrates were enriched with known MC degrading taxa *Sphingomonas, Shinella* and *Novosphingobium* at Tf. While they are known MC degrading genera via the mlr degradation pathway, the genes were not

detected in this study. MOBs that have known potential to degrade CYN, such as *Hyphomicrobium* were also enriched in the bulk substrate. Several taxa associated with mlr-independent cyanotoxin degradation such as *Sphingorhabdus* and *Porphyrobacter*, and HAB associated taxa such as *Ilumatobacteraceae, Roseomonas,* and *Obscuribacteraceae* were also enriched in the systems at Tf. This suggests that other unknown cyanotoxin degradation pathways were in play, a topic that deserves further investigation. Overall, VF- TWs planted with *C.indica* demonstrated better efficiency for CYN removal and adaptability in the treatment of surface waters contaminated with cyanotoxins. However, future studies with pilot scale TWs are suggested to monitor effluent quality for water-reuse, taking in consideration evapotranspiration rates.

Author Contributions:

G.B: Conceptualization, Methodology, Investigation, Validation, Formal analysis, Data curation, Writing - original draft, Visualisation; D.A.M.A: Methodology, Validation, Writing- review & editing; M.F.C: Methodology, Resources; R.J.M: Methodology, Validation, Writing- review & editing; C.S: Conceptualization, Methodology, Writing- review &editing, Resources; L.D-Q: Methodology, Validation; A.J: Methodology, Validation; A.M.C: Methodology, Resources; A.C: Conceptualization, Methodology, Validation, Resources, Writing - review & editing, Supervision, Project administration, Funding acquisition; P.N.C: Conceptualization, Methodology, Validation, Resources, Writing - review & editing, Supervision, Funding acquisition; C.M.R.A: Conceptualization, Methodology, Validation, Resources, Writing - review & editing, Supervision, Project administration, Funding acquisition.

References

[1] H. W. Paerl, R. S. Fulton, P. H. Moisander, and J. Dyble, “Harmful freshwater algal blooms, with an emphasis on cyanobacteria.,” 2001. doi: 10.1100/tsw.2001.16.

[2] F. Haque, S. Banayan, J. Yee, and Y. W. Chiang, “Extraction and applications of cyanotoxins and other cyanobacterial secondary metabolites,” 2017, *Elsevier Ltd*. doi: 10.1016/j.chemosphere.2017.05.106.

[3] Z. Svirčev *et al.*, “Global geographical and historical overview of cyanotoxin distribution and cyanobacterial poisonings,” Sep. 01, 2019, *Springer Verlag*. doi: 10.1007/s00204-019-02524-4.

[4] S. Merel, D. Walker, R. Chicana, S. Snyder, E. Baurès, and O. Thomas, “State of knowledge and concerns on cyanobacterial blooms and cyanotoxins,” 2013, *Elsevier Ltd*. doi: 10.1016/j.envint.2013.06.013.

[5] A. D. Chatziefthimiou, J. S. Metcalf, W. B. Glover, S. A. Banack, S. R. Dargham, and R. A. Richer, “Cyanobacteria and cyanotoxins are present in drinking water impoundments and groundwater wells in desert environments,” *Toxicon*, vol. 114, pp. 75–84, May 2016, doi: 10.1016/j.toxicon.2016.02.016.

[6] R. Melaram, A. R. Newton, and J. Chafin, “Microcystin Contamination and Toxicity: Implications for Agriculture and Public Health,” May 01, 2022, *MDPI*. doi: 10.3390/toxins14050350.

[7] P. K. Kulabhusan and K. Campbell, “Physico-chemical treatments for the removal of cyanotoxins from drinking water: Current challenges and future trends,” Mar. 20, 2024, *Elsevier B.V.* doi: 10.1016/j.scitotenv.2024.170078.

[8] A. Martinez i Quer, Y. Larsson, A. Johansen, C. A. Arias, and P. N. Carvalho, “Cyanobacterial blooms in surface waters – Nature-based solutions, cyanotoxins and their biotransformation products,” Mar. 01, 2024, *Elsevier Ltd*. doi: 10.1016/j.watres.2024.121122.

[9] G. Bavithra *et al.*, “Assessment of constructedwetlands’ potential for the removal of cyanobacteria and microcystins (MC-LR),” *Water (Switzerland)*, vol. 12, no. 1, 2020, doi: 10.3390/w12010010.

[10] A. Martinez i Quer *et al.*, “Saturated constructed wetlands for the remediation of cylindrospermopsin and microcystin-LR: Plants, microbes, and biodegradation

pathways," *Science of the Total Environment*, vol. 948, Oct. 2024, doi: 10.1016/j.scitotenv.2024.174745.

[11] R. Wang *et al.*, "Enhanced removal of Microcystis bloom and microcystin-LR using microcosm constructed wetlands with bioaugmentation of degrading bacteria," *Chemosphere*, vol. 210, pp. 29–37, Nov. 2018, doi: 10.1016/j.chemosphere.2018.06.140.

[12] R. Cheng, H. Zhu, B. Shutes, and B. Yan, "Treatment of microcystin (MC-LR) and nutrients in eutrophic water by constructed wetlands: Performance and microbial community," *Chemosphere*, vol. 263, Jan. 2021, doi: 10.1016/j.chemosphere.2020.128139.

[13] A. M. Gorito, A. R. Ribeiro, C. R. Gomes, C. M. R. Almeida, and A. M. T. Silva, "Constructed wetland microcosms for the removal of organic micropollutants from freshwater aquaculture effluents," *Science of the Total Environment*, vol. 644, pp. 1171–1180, Dec. 2018, doi: 10.1016/j.scitotenv.2018.06.371.

[14] C. M. R. Almeida, F. Santos, A. C. F. Ferreira, I. Lourinha, M. C. P. Basto, and A. P. Mucha, "Can veterinary antibiotics affect constructed wetlands performance during treatment of livestock wastewater?," *Ecol Eng*, vol. 102, pp. 583–588, May 2017, doi: 10.1016/j.ecoleng.2017.02.035.

[15] J. Vymazal, "Constructed wetlands for wastewater treatment: Five decades of experience," *Environ Sci Technol*, vol. 45, no. 1, pp. 61–69, Jan. 2011, doi: 10.1021/es101403q.

[16] J. Santos, S. Rodrigues, M. Magalhães, K. Rodrigues, L. Pereira, and G. Marinho, "A state-of-the-art review (2019–2023) on constructed wetlands for greywater treatment and reuse," Aug. 01, 2024, *Elsevier B.V.* doi: 10.1016/j.envc.2024.100973.

[17] L. A. Thyssen, A. Martinez i Quer, C. A. Arias, L. Ellegaard-Jensen, P. N. Carvalho, and A. Johansen, "Constructed wetland mesocosms improve the biodegradation of microcystin-LR and cylindrospermopsin by indigenous bacterial consortia," *Harmful Algae*, vol. 131, Jan. 2024, doi: 10.1016/j.hal.2023.102549.

[18] G. Bavithra, J. Azevedo, A. Campos, C. M. R. Almeida, and P. N. Carvalho, "Evaluating Agro-Based Waste Materials for Cyanotoxin Sorption for Future Incorporation in Nature-Based Solution Units (NBSUs)," *Water (Switzerland)*, vol. 17, no. 2, Jan. 2025, doi: 10.3390/w17020285.

[19] P. Porras-Socias *et al.*, “Removal of metals and emergent contaminants from liquid digestates in constructed wetlands for agricultural reuse,” *Front Microbiol*, vol. 15, 2024, doi: 10.3389/fmicb.2024.1388895.

[20] M. Senze, M. Kowalska-Góralska, and K. Czyż, “Emergent (branched bur-reed—Sparganium erectum L.) and submergent (river water-crowfoot—Ranunculus fluitans Wimm., 1841) aquatic plants as metal biosorbents under varying water pH conditions in laboratory conditions,” *Environmental Science and Pollution Research*, vol. 30, no. 40, pp. 92053–92067, Aug. 2023, doi: 10.1007/s11356-023-28752-x.

[21] F. Karam, R. Haddad, N. Amacha, W. Charanek, and J. Harmand, “Assessment of the Impacts of Phyto-Remediation on Water Quality of the Litani River by Means of Two Wetland Plants (Sparganium erectum and Phragmites australis),” *Water (Switzerland)*, vol. 15, no. 1, Jan. 2023, doi: 10.3390/w15010004.

[22] G. de Stefani, D. Tocchetto, M. Salvato, and M. Borin, “Performance of a floating treatment wetland for in-stream water amelioration in NE Italy,” *Hydrobiologia*, vol. 674, no. 1, pp. 157–167, Oct. 2011, doi: 10.1007/s10750-011-0730-4.

[23] A. Zorai, K. Benzahi, B. Labed, A. Ouakouak, M. Serraoui, and A. Bouhoreira, “Performance of Canna indica and Typha latifolia in mono and mixed culture for secondary wastewater treatment in constructed wetlands with vertical flows under arid conditions (Touggourt, Algeria),” *Water Pract Technol*, vol. 18, no. 1, pp. 53–67, Jan. 2023, doi: 10.2166/wpt.2022.163.

[24] R. Pinninti *et al.*, “Performance of Canna Indica based microscale vertical flow constructed wetland under tropical conditions for domestic wastewater treatment,” *Int J Phytoremediation*, vol. 24, no. 7, pp. 684–694, 2022, doi: 10.1080/15226514.2021.1962800.

[25] P. N. Karungamye, “Potential of Canna indica in Constructed Wetlands for Wastewater Treatment: A Review,” Sep. 01, 2022, *Multidisciplinary Digital Publishing Institute (MDPI)*. doi: 10.3390/conservation2030034.

[26] Y. Luo, Q. Chen, F. Liu, and C. Dai, “Both species richness and growth forms affect nutrient removal in constructed wetlands: A mesocosm experiment,” *Front Ecol Evol*, vol. 11, 2023, doi: 10.3389/fevo.2023.1139053.

[27] V. Carrillo, Y. Casas-Ledón, P. Neumann, and G. Vidal, “Environmental performance of constructed wetland planted with monocultures and polycultures for wastewater treatment,” *Ecol Eng*, vol. 193, Aug. 2023, doi: 10.1016/j.ecoleng.2023.107015.

[28] L. Y. Kochi, P. L. Freitas, L. T. Maranho, P. Juneau, and M. P. Gomes, “Aquatic macrophytes in constructed wetlands: A fight against water pollution,” Nov. 01, 2020, *MDPI*. doi: 10.3390/su12219202.

[29] A. M. Leiva, R. Núñez, G. Gómez, D. López, and G. Vidal, “Performance of ornamental plants in monoculture and polyculture horizontal subsurface flow constructed wetlands for treating wastewater,” *Ecol Eng*, vol. 120, pp. 116–125, Sep. 2018, doi: 10.1016/j.ecoleng.2018.05.023.

[30] Á. González Álvarez, A. Martinez i Quer, L. Ellegaard-Jensen, R. Sapkota, P. N. Carvalho, and A. Johansen, “Fungal removal of cyanotoxins in constructed wetlands: The forgotten degraders,” *Science of the Total Environment*, vol. 929, Jun. 2024, doi: 10.1016/j.scitotenv.2024.172590.

[31] R. Cheng, S. Hou, J. Wang, H. Zhu, B. Shutes, and B. Yan, “Biochar-amended constructed wetlands for eutrophication control and microcystin (MC-LR) removal,” *Chemosphere*, vol. 295, May 2022, doi: 10.1016/j.chemosphere.2022.133830.

[32] A. Llanos-Lizcano, E. Barraza, A. Narvaez, L. Varela, and A. Caselles-Osorio, “Efficiency of pilot-scale horizontal subsurface flow constructed wetlands and microbial community composition operating under tropical conditions,” *Int J Phytoremediation*, vol. 21, no. 1, pp. 34–42, Jan. 2019, doi: 10.1080/15226514.2018.1523874.

[33] F. Zhong *et al.*, “Nutrient Removal Process and Cathodic Microbial Community Composition in Integrated Vertical-Flow Constructed Wetland – Microbial Fuel Cells Filled With Different Substrates,” *Front Microbiol*, vol. 11, Aug. 2020, doi: 10.3389/fmicb.2020.01896.

[34] J. Ding *et al.*, “Microbial abundance and community in constructed wetlands planted with Phragmites australis and Typha orientalis in winter,” *Int J Phytoremediation*, vol. 23, no. 14, pp. 1476–1485, 2021, doi: 10.1080/15226514.2021.1907737.

[35] B. Wang *et al.*, “Characteristics of Greenhouse Gas Emissions from Constructed Wetlands Vegetated with Myriophyllum aquatic: The Effects of Influent C/N Ratio and Microbial Responses,” *Water (Switzerland)*, vol. 16, no. 2, Jan. 2024, doi: 10.3390/w16020308.

[36] S. Bai *et al.*, “Constructed Wetlands as Nature-Based Solutions for the Removal of Antibiotics: Performance, Microbial Response, and Emergence of Antimicrobial Resistance (AMR),” *Sustainability (Switzerland)*, vol. 14, no. 22, Nov. 2022, doi: 10.3390/su142214989.

[37] M. Hollstein, M. Comerford, M. Uhl, M. Abel, S. P. Egan, and L. B. Stadler, “Impact of a natural disturbance on the performance and microbial communities in a full-scale constructed wetland for industrial wastewater treatment,” *Front Environ Sci*, vol. 11, 2023, doi: 10.3389/fenvs.2023.1187143.

[38] F. Zhong *et al.*, “The use of vertical flow constructed wetlands for the treatment of hyper-eutrophic water bodies with dense cyanobacterial blooms,” *Water Science and Technology*, vol. 77, no. 5, pp. 1186–1195, Mar. 2018, doi: 10.2166/wst.2017.635.

[39] Q. Wang *et al.*, “Microbial abundance and community in subsurface flow constructed wetland microcosms: role of plant presence,” *Environmental Science and Pollution Research*, vol. 23, no. 5, pp. 4036–4045, Mar. 2016, doi: 10.1007/s11356-015-4286-0.

[40] D. R. Helsel, *Statistics for censored environmental data using Minitab and R (Vol. 77)*. John Wiley & Sons., 2011.

[41] S. Tisler, C. Liang, P. N. Carvalho, and K. Bester, “Identification of more than 100 new compounds in the wastewater: Fate of polyethylene/polypropylene oxide copolymers and their metabolites in the aquatic environment,” *Science of the Total Environment*, vol. 761, Mar. 2021, doi: 10.1016/j.scitotenv.2020.143228.

[42] L. A. Thyssen, A. Martinez i Quer, C. A. Arias, L. Ellegaard-Jensen, P. N. Carvalho, and A. Johansen, “Constructed wetland mesocosms improve the biodegradation of microcystin-LR and cylindrospermopsin by indigenous bacterial consortia,” *Harmful Algae*, vol. 131, Jan. 2024, doi: 10.1016/j.hal.2023.102549.

[43] L. Díez-Quijada *et al.*, “New method for simultaneous determination of microcystins and cylindrospermopsin in vegetable matrices by SPE-UPLC-MS/MS,” *Toxins (Basel)*, vol. 10, no. 10, Oct. 2018, doi: 10.3390/toxins10100406.

[44] R. J. Mendes *et al.*, “Fire Blight Management: Physiological Assessment of Cultural Control by Pruning in Pear Orchards,” *Agriculture (Pol’nohospodarstvo)*, vol. 66, no. 3, pp. 128–136, Nov. 2020, doi: 10.2478/agri-2020-0012.

[45] D. A. Sims and J. A. Gamon, “Relationships between leaf pigment content and spectral reflectance across a wide range of species, leaf structures and developmental stages.” [Online]. Available: www.elsevier.com/locate/rse

[46] M. C. Dias, S. Correia, J. Serôdio, A. M. S. Silva, H. Freitas, and C. Santos, “Chlorophyll fluorescence and oxidative stress endpoints to discriminate olive cultivars tolerance to drought and heat episodes,” *Sci Hortic*, vol. 231, pp. 31–35, Jan. 2018, doi: 10.1016/j.scienta.2017.12.007.

[47] J. Zhang, Q. Lu, Q. Ding, L. Yin, and Y. Pu, “A novel and native microcystin-degrading bacterium of Sphingopyxis sp. isolated from lake Taihu,” *Int J Environ Res Public Health*, vol. 14, no. 10, Oct. 2017, doi: 10.3390/ijerph14101187.

[48] T. Maruyama *et al.*, “Sphingosinicella microcystinivorans gen. nov., sp. nov., a microcystin-degrading bacterium,” *Int J Syst Evol Microbiol*, vol. 56, no. 1, pp. 85–89, Jan. 2006, doi: 10.1099/ijs.0.63789-0.

[49] H.-D. Park, Y. Sasaki, T. Maruyama, E. Yanagisawa, A. Hiraishi, and K. Kato, “Degradation of the Cyanobacterial Hepatotoxin Microcystin by a New Bacterium Isolated from a Hypertrophic Lake,” 2001.

[50] B. J. Callahan, P. J. McMurdie, M. J. Rosen, A. W. Han, A. J. A. Johnson, and S. P. Holmes, “DADA2: High-resolution sample inference from Illumina amplicon data,” *Nat Methods*, vol. 13, no. 7, pp. 581–583, Jun. 2016, doi: 10.1038/nmeth.3869.

[51] C. Quast *et al.*, “The SILVA ribosomal RNA gene database project: Improved data processing and web-based tools,” *Nucleic Acids Res*, vol. 41, no. D1, Jan. 2013, doi: 10.1093/nar/gks1219.

[52] P. J. McMurdie and S. Holmes, “Phyloseq: An R Package for Reproducible Interactive Analysis and Graphics of Microbiome Census Data,” *PLoS One*, vol. 8, no. 4, Apr. 2013, doi: 10.1371/journal.pone.0061217.

[53] Y. Cao, Q. Dong, D. Wang, P. Zhang, Y. Liu, and C. Niu, “microbiomeMarker: an R/Bioconductor package for microbiome marker identification and visualization,” *Bioinformatics*, vol. 38, no. 16, pp. 4027–4029, Aug. 2022, doi: 10.1093/bioinformatics/btac438.

[54] “Cyanobacterial toxins: cylindrospermopsins Background document for development of WHO,” 2020. [Online]. Available: http://apps.who.int/bookorders.

[55] “Cyanobacterial toxins: microcystins Background document for development of WHO,” 2020. [Online]. Available: http://apps.who.int/bookorders.

[56] S. Shams *et al.*, “Anatoxin-a producing Tychonema (cyanobacteria) in European waterbodies,” *Water Res*, vol. 69, pp. 68–79, Feb. 2015, doi: 10.1016/j.watres.2014.11.006.

[57] T. K. Shishido *et al.*, “A cylindrospermopsin-producing cyanobacterium isolated from a microbial mat in the Baltic Sea,” *Toxicon*, vol. 232, Aug. 2023, doi: 10.1016/j.toxicon.2023.107205.

[58] R. U. Tanvir, Z. Hu, Y. Zhang, and J. Lu, “Cyanobacterial community succession and associated cyanotoxin production in hypereutrophic and eutrophic freshwaters,” Dec. 01, 2021, *Elsevier Ltd*. doi: 10.1016/j.envpol.2021.118056.

[59] T. Wu, B. Qin, G. Zhu, L. Luo, Y. Ding, and G. Bian, “Dynamics of cyanobacterial bloom formation during short-term hydrodynamic fluctuation in a large shallow, eutrophic, and wind-exposed Lake Taihu, China,” *Environmental Science and Pollution Research*, vol. 20, no. 12, pp. 8546–8556, Dec. 2013, doi: 10.1007/s11356-013-1812-9.

[60] M. Guan *et al.*, “Quantifying the effects of wind wave on cyanobacterial blooms in large shallow lake from 10 years high frequency satellite observation,” *Hydrobiologia*, Feb. 2024, doi: 10.1007/s10750-024-05735-8.

[61] M. Licata, M. C. Gennaro, T. Tuttolomondo, C. Leto, and S. la Bella, “Research focusing on plant performance in constructed wetlands and agronomic application of treated wastewater - A set of experimental studies in Sicily (Italy),” *PLoS One*, vol. 14, no. 7, Jul. 2019, doi: 10.1371/journal.pone.0219445.

[62] I. Arliyani, B. V. Tangahu, and S. Mangkoedihardjo, “Plant Diversity in a Constructed Wetland for Pollutant Parameter Processing on Leachate: A Review,” *Journal of Ecological Engineering*, vol. 22, no. 4, pp. 240–255, 2021, doi: 10.12911/22998993/134041.

[63] M. E. Rahman *et al.*, “Design, operation and optimization of constructed wetland for removal of pollutant,” Nov. 02, 2020, *MDPI AG*. doi: 10.3390/ijerph17228339.

[64] O. Husson, “Redox potential (Eh) and pH as drivers of soil/plant/microorganism systems: A transdisciplinary overview pointing to integrative opportunities for agronomy,” Jan. 01, 2013, *Kluwer Academic Publishers*. doi: 10.1007/s11104-012-1429-7.

[65] Z. Fan, S. Yang, B. Yang, and X. Yang, “Understanding the interaction between soil ORP and enzyme activity: How does wetland type affect constructed wetland performance for the advanced treatment of swine wastewater?,” *Chemical Engineering Journal*, vol. 499, Nov. 2024, doi: 10.1016/j.cej.2024.156353.

[66] S. K. Malyan, S. Yadav, V. Sonkar, V. C. Goyal, O. Singh, and R. Singh, “Mechanistic understanding of the pollutant removal and transformation processes in the constructed wetland system,” Oct. 01, 2021, *John Wiley and Sons Inc*. doi: 10.1002/wer.1599.

[67] S. G. Abdelhakeem, S. A. Aboulroos, and M. M. Kamel, “Performance of a vertical subsurface flow constructed wetland under different operational conditions,” *J Adv Res*, vol. 7, no. 5, pp. 803–814, Sep. 2016, doi: 10.1016/j.jare.2015.12.002.

[68] L. Davis, “A HANDBOOK OF CONSTRUCTED WETLANDS a guide to creating wetlands for,” 1995.

[69] J. Santos, S. Rodrigues, M. Magalhães, K. Rodrigues, L. Pereira, and G. Marinho, “A state-of-the-art review (2019–2023) on constructed wetlands for greywater treatment and reuse,” Aug. 01, 2024, *Elsevier B.V.* doi: 10.1016/j.envc.2024.100973.

[70] C. Shiwei, J. Zhaoqian, Y. Peng, W. Yue, and W. Yin, “Performance of constructed wetlands with different substrates for the treated effluent from municipal sewage plants,” *Journal of Water Reuse and Desalination*, vol. 9, no. 4, pp. 452–462, Dec. 2019, doi: 10.2166/wrd.2019.032.

[71] P. Zhao *et al.*, “Study on the optimization of hydrodynamic characteristics and pollutant removal efficiency in integrated vertical flow constructed wetlands,” *Sci Rep*, vol. 15, no. 1, p. 6017, Dec. 2025, doi: 10.1038/s41598-025-88785-9.

[72] L. L. Zhuang *et al.*, “The performance and mechanism of biochar-enhanced constructed wetland for wastewater treatment,” Feb. 01, 2022, *Elsevier Ltd*. doi: 10.1016/j.jwpe.2021.102522.

[73] C. Chambers, S. Grimes, S. Fire, and M. T. Reza, “Influence of biochar on the removal of Microcystin-LR and Saxitoxin from aqueous solutions,” *Sci Rep*, vol. 14, no. 1, Dec. 2024, doi: 10.1038/s41598-024-61802-z.

[74] J. Li, L. Cao, Y. Yuan, R. Wang, Y. Wen, and J. Man, “Comparative study for microcystin-LR sorption onto biochars produced from various plant- and animal-wastes at different pyrolysis temperatures: Influencing mechanisms of biochar properties,” *Bioresour Technol*, vol. 247, pp. 794–803, 2018, doi: 10.1016/j.biortech.2017.09.120.

[75] D. Ventura *et al.*, “On the performance of a pilot hybrid constructed wetland for stormwater recovery in Mediterranean climate,” *Water Science and Technology*, vol. 79, no. 6, pp. 1051–1059, Mar. 2019, doi: 10.2166/wst.2019.103.

[76] H. Brix, “FUNCTIONS OF MACROPHYTES IN CONSTRUCTED WETLANDS,” 1994. [Online]. Available: https://iwaponline.com/wst/article-pdf/29/4/71/15729/71.pdf

[77] Y. Yang, Y. Zhao, R. Liu, and D. Morgan, “Global development of various emerged substrates utilized in constructed wetlands,” Aug. 01, 2018, *Elsevier Ltd*. doi: 10.1016/j.biortech.2018.03.085.

[78] E. A. Belyakov and A. G. Lapirov, “Modular and structural–functional organization of G. sparganium L. species in different ecological conditions,” *Contemp Probl Ecol*, vol. 8, no. 5, pp. 647–659, Sep. 2015, doi: 10.1134/S1995425515050030.

[79] A. Hassan and M. Oliur Rahman, “MORPHOLOGICAL AND ANATOMICAL INVESTIGATION AMONG SIX VARIANTS OF CANNA INDICA L. NAHID SULTANA, SABIHA SULTANA AKHI,” 2019.

[80] T. Liffen, A. M. Gurnell, M. T. O’Hare, N. Pollen-Bankhead, and A. Simon, “Biomechanical properties of the emergent aquatic macrophyte Sparganium erectum: Implications for fine sediment retention in low energy rivers,” *Ecol Eng*, vol. 37, no. 11, pp. 1925–1931, Nov. 2011, doi: 10.1016/j.ecoleng.2011.06.015.

[81] H. Mao, W. Zhao, X. Yang, L. Sheng, and S. Zhu, “Recruitment and metabolomics between Canna indica and rhizosphere bacteria under Cr stress,” *Front Microbiol*, vol. 14, 2023, doi: 10.3389/fmicb.2023.1187982.

[82] G. Langergraber, “The role of plant uptake on the removal of organic matter and nutrients in subsurface flow constructed wetlands: a simulation study,” 2005. [Online]. Available: https://iwaponline.com/wst/article-pdf/51/9/213/434817/213.pdf

[83] S. M. Mitrovic, O. Allis, A. Furey, and K. J. James, “Bioaccumulation and harmful effects of microcystin-LR in the aquatic plants Lemna minor and Wolffia arrhiza and the filamentous alga Chladophora fracta,” *Ecotoxicol Environ Saf*, vol. 61, no. 3, pp. 345–352, Jul. 2005, doi: 10.1016/j.ecoenv.2004.11.003.

[84] J. Nimptsch, C. Wiegand, and S. Pflugmacher, “Cyanobacterial toxin elimination via bioaccumulation of MC-LR in aquatic macrophytes: An application of the ‘green liver concept,’” *Environ Sci Technol*, vol. 42, no. 22, pp. 8552–8557, Nov. 2008, doi: 10.1021/es8010404.

[85] G. K. Pindihama and M. W. Gitari, “Uptake and Growth Effects of Cyanotoxins on Aquatic Plants Ludwigia Adscendens and Amaranthus Hybridus in Raw Surface Waters,” *International Journal of Environmental Science and Development*, vol. 8, no. 2, pp. 93–101, 2017, doi: 10.18178/ijesd.2017.8.2.928.

[86] J. Machado, A. Campos, V. Vasconcelos, and M. Freitas, “Effects of microcystin-LR and cylindrospermopsin on plant-soil systems: A review of their relevance for agricultural plant quality and public health,” 2017, *Academic Press Inc.* doi: 10.1016/j.envres.2016.09.015.

[87] C. Weralupitiya, R. P. Wanigatunge, D. Gunawardana, M. Vithanage, and D. Magana-Arachchi, “Cyanotoxins uptake and accumulation in crops: Phytotoxicity and implications on human health,” May 01, 2022, *Elsevier Ltd*. doi: 10.1016/j.toxicon.2022.03.003.

[88] S. Pflugmacher, C. Wiegand, K. A. Beattie, E. Krause, C. E. W. Steinberg, and G. A. Codd, “UPTAKE, EFFECTS, AND METABOLISM OF CYANOBACTERIAL TOXINS IN THE EMERGENT REED PLANT PHRAGMITES AUSTRALIS (CAV.) TRIN. EX STEUD,” 2001. [Online]. Available: https://academic.oup.com/etc/article/20/4/846/7830940

[89] C. Máthé, M. M-Hamvas, G. Vasas, T. Garda, and C. Freytag, “Subcellular alterations induced by cyanotoxins in vascular plants—a review,” May 01, 2021, *MDPI AG*. doi: 10.3390/plants10050984.

[90] C. Wiegand, A. Peuthert, S. Pflugmacher, and S. Carmeli, “Effects of microcin SF608 and microcystin-LR two cyanotobacterial compounds produced by Microcystis sp., on aquatic organisms,” *Environ Toxicol*, vol. 17, no. 4, pp. 400–406, 2002, doi: 10.1002/tox.10065.

[91] Sana Saqrane, “Cyanobacterial toxins: A short review on phytotoxic effect in an aquatic environment,” *Afr J Environ Sci Tech*, vol. 5, no. 13, Dec. 2012, doi: 10.5897/ajestx11.008.

[92] S. Saqrane *et al.*, “Phytotoxic effects of cyanobacteria extract on the aquatic plant Lemna gibba: Microcystin accumulation, detoxication and oxidative stress induction,” *Aquatic Toxicology*, vol. 83, no. 4, pp. 284–294, Aug. 2007, doi: 10.1016/j.aquatox.2007.05.004.

[93] S. Pflugmacher, “Possible allelopathic effects of cyanotoxins, with reference to microcystin-LR, in aquatic ecosystems,” *Environ Toxicol*, vol. 17, no. 4, pp. 407–413, 2002, doi: 10.1002/tox.10071.

[94] M. C. Perron, B. Qiu, N. Boucher, F. Bellemare, and P. Juneau, “Use of chlorophyll a fluorescence to detect the effect of microcystins on photosynthesis and photosystem II energy fluxes of green algae,” *Toxicon*, vol. 59, no. 5, pp. 567–577, Apr. 2012, doi: 10.1016/j.toxicon.2011.12.005.

[95] L. Pizarro and C. Stange, “Light-dependent regulation of carotenoid biosynthesis in plants,” 2009. [Online]. Available: www.rcia.puc.cl

[96] J. Mi *et al.*, “A manipulation of carotenoid metabolism influence biomass partitioning and fitness in tomato,” *Metab Eng*, vol. 70, pp. 166–180, Mar. 2022, doi: 10.1016/j.ymben.2022.01.004.

[97] F. Ramel, S. Birtic, C. Ginies, L. Soubigou-Taconnat, C. Triantaphylidès, and M. Havaux, “Carotenoid oxidation products are stress signals that mediate gene responses to singlet oxygen in plants,” *Proc Natl Acad Sci U S A*, vol. 109, no. 14, pp. 5535–5540, Apr. 2012, doi: 10.1073/pnas.1115982109.

[98] M. Rosa *et al.*, “Soluble sugars-metabolism, sensing and abiotic stress a complex network in the life of plants,” *Plant Signal Behav*, vol. 4, no. 5, pp. 388–393, May 2009, doi: 10.4161/psb.4.5.8294.

[99] A. Campos *et al.*, “Impacts of microcystins on morphological and physiological parameters of agricultural plants: A review,” Apr. 01, 2021, *MDPI AG*. doi: 10.3390/plants10040639.

[100] J. Zhu *et al.*, “Characterization of sugar contents and sucrose metabolizing enzymes in developing leaves of Hevea brasiliensis,” *Front Plant Sci*, vol. 9, Feb. 2018, doi: 10.3389/fpls.2018.00058.

[101] M. R. Morsy, L. Jouve, J. F. Hausman, L. Hoffmann, and J. M. D. Stewart, “Alteration of oxidative and carbohydrate metabolism under abiotic stress in two rice (Oryza sativa L.) genotypes contrasting in chilling tolerance,” *J Plant Physiol*, vol. 164, no. 2, pp. 157–167, Feb. 2007, doi: 10.1016/j.jplph.2005.12.004.

[102] R. Haberl *et al.*, “Constructed Wetlands for the Treatment of Organic Pollutants,” 2003.

[103] Y. Wu *et al.*, “Correlating microbial community with physicochemical indices and structures of a full-scale integrated constructed wetland system,” *Appl Microbiol Biotechnol*, vol. 100, no. 15, pp. 6917–6926, Aug. 2016, doi: 10.1007/s00253-016-7526-4.

[104] K. Kumari *et al.*, “Evaluation of Three Prokaryote Primers for Identification of Prokaryote Community Structure and Their Abode Preference in Three Distinct Wetland Ecosystems,” *Front Microbiol*, vol. 12, Jul. 2021, doi: 10.3389/fmicb.2021.643945.

[105] X. Fu *et al.*, “Critical transition of soil microbial diversity and composition triggered by plant rhizosphere effects,” *Front Plant Sci*, vol. 14, 2023, doi: 10.3389/fpls.2023.1252821.

[106] N. Ling, T. Wang, and Y. Kuzyakov, “Rhizosphere bacteriome structure and functions,” *Nat Commun*, vol. 13, no. 1, Dec. 2022, doi: 10.1038/s41467-022-28448-9.

[107] I. Cakin, B. Morrissey, L. Marcello, P. P. J. Gaffney, S. Pap, and M. A. Taggart, “A comparison between constructed wetland substrates: Impacts on microbial community and wastewater treatment,” *Chemosphere*, vol. 364, Sep. 2024, doi: 10.1016/j.chemosphere.2024.143179.

[108] J. Sasse, E. Martinoia, and T. Northen, “Feed Your Friends: Do Plant Exudates Shape the Root Microbiome?,” Jan. 01, 2018, *Elsevier Ltd*. doi: 10.1016/j.tplants.2017.09.003.

[109] N. Seth *et al.*, “Microbial community diversity of an integrated constructed wetland used for treatment of sewage,” *Front Microbiol*, vol. 15, 2024, doi: 10.3389/fmicb.2024.1355718.

[110] L. C. Burow, Y. Kong, J. L. Nielsen, L. L. Blackall, and P. H. Nielsen, “Abundance and ecophysiology of Defluviicoccus spp., glycogen-accumulating organisms in full-scale wastewater treatment processes,” *Microbiology (N Y)*, vol. 153, no. 1, pp. 178–185, Jan. 2007, doi: 10.1099/mic.0.2006/001032-0.

[111] M. Wietz *et al.*, “The polar night shift: seasonal dynamics and drivers of Arctic Ocean microbiomes revealed by autonomous sampling,” *ISME Communications*, vol. 1, no. 1, Dec. 2021, doi: 10.1038/s43705-021-00074-4.

[112] G. YU, “Macrophyte succession in Dianchi Lake and relations with the environment,” *J. Lake Sci.*, vol. 12, pp. 73–80, 2000, Accessed: Jul. 31, 2025. [Online]. Available: https://cir.nii.ac.jp/crid/1574231876078055040.bib?lang=en

[113] B. Guo, H. Zhang, Y. Liu, J. Chen, and J. Li, “Drought-resistant trait of different crop genotypes determines assembly patterns of soil and phyllosphere microbial communities,” *Microbiol Spectr*, vol. 11, no. 5, Oct. 2023, doi: 10.1128/spectrum.00068-23.

[114] S. M. Kang *et al.*, “Gibberellins and indole-3-acetic acid producing rhizospheric bacterium Leifsonia xyli SE134 mitigates the adverse effects of copper-mediated stress on tomato,” *J Plant Interact*, vol. 12, no. 1, pp. 373–380, 2017, doi: 10.1080/17429145.2017.1370142.

[115] S. Akter *et al.*, “Ecofriendly synthesis of silver nanoparticles by Terrabacter humi sp. nov. and their antibacterial application against antibiotic-resistant pathogens,” *Int J Mol Sci*, vol. 21, no. 24, pp. 1–19, Dec. 2020, doi: 10.3390/ijms21249746.

[116] S. Kristyanto, S. D. Lee, and J. Kim, “Porphyrobacter algicida sp. nov., an algalytic bacterium isolated from seawater,” *Int J Syst Evol Microbiol*, vol. 67, no. 11, pp. 4526–4533, Nov. 2017, doi: 10.1099/ijsem.0.002324.

[117] C. Talwar, S. Nagar, R. Kumar, J. Scaria, R. Lal, and R. K. Negi, “Defining the Environmental Adaptations of Genus Devosia: Insights into its Expansive Short Peptide Transport System and Positively Selected Genes,” *Sci Rep*, vol. 10, no. 1, Dec. 2020, doi: 10.1038/s41598-020-58163-8.

[118] S. Zhou *et al.*, “A comprehensive analysis of microbial community differences in four morphologies of mainstream anaerobic ammonia oxidation systems using big-data mining and machine learning,” *Front Mar Sci*, vol. 11, Sep. 2024, doi: 10.3389/fmars.2024.1458853.

[119] M. M. Maroneze, L. Q. Zepka, J. G. Vieira, M. I. Queiroz, and E. Jacob-Lopes, “A tecnologia de remoção de fósforo: Gerenciamento do elemento em resíduos industriais,” *Revista Ambiente e Agua*, vol. 9, no. 3, pp. 445–458, 2014, doi: 10.4136/1980-993X.

[120] J. L. Gonzalez-Pimentel *et al.*, “Prokaryotic communities from a lava tube cave in La Palma Island (Spain) are involved in the biogeochemical cycle of major elements,” *PeerJ*, vol. 9, May 2021, doi: 10.7717/peerj.11386.

[121] Y. T. Chiani, P. Jacob, G. Mayora, D. S. Aquino, R. D. Quintana, and L. Mesa, “Presence of Leptospira spp. in a Mosaic of Wetlands Used for Livestock Raising under Differing Hydroclimatic Conditions,” *Appl Environ Microbiol*, vol. 89, no. 6, Jun. 2023, doi: 10.1128/aem.01971-22.

[122] M. V. Garibay *et al.*, “Characterization of the spatial variation of microbial communities in a decentralized subtropical wastewater treatment plant using passive methods,” *Water (Switzerland)*, vol. 13, no. 9, May 2021, doi: 10.3390/w13091157.

[123] L. Zhong *et al.*, “New insights into substrates shaped nutrients removal, species interactions and community assembly mechanisms in tidal flow constructed wetlands treating low carbon-to-nitrogen rural wastewater,” *Water Res*, vol. 256, Jun. 2024, doi: 10.1016/j.watres.2024.121600.

[124] L. Jin, C. Z. Jin, H. G. Lee, and C. S. Lee, “Genomic insights into denitrifying methane-oxidizing bacteria gemmobacter fulva sp. Nov., isolated from an anabaena culture,” *Microorganisms*, vol. 9, no. 12, Dec. 2021, doi: 10.3390/microorganisms9122423.

[125] L. Dziewit *et al.*, “Genome-guided insight into the methylotrophy of Paracoccus aminophilus JCM 7686,” *Front Microbiol*, vol. 6, no. AUG, 2015, doi: 10.3389/fmicb.2015.00852.

[126] A. A. Ivanova, A. D. Zhelezova, T. I. Chernov, and S. N. Dedysh, “Linking ecology and systematics of acidobacteria: Distinct habitat preferences of the Acidobacteriia and Blastocatellia in tundra soils,” *PLoS One*, vol. 15, no. 3, 2020, doi: 10.1371/journal.pone.0230157.

[127] Y. Zhang *et al.*, “Metagenomic insights into microbial variation and carbon cycling function in crop rotation systems,” *Science of the Total Environment*, vol. 947, Oct. 2024, doi: 10.1016/j.scitotenv.2024.174529.

[128] M. Williams *et al.*, “Short-stalked Prosthecomicrobium hirschii cells have a Caulobacter-like cell cycle,” *J Bacteriol*, vol. 198, no. 7, pp. 1149–1159, 2016, doi: 10.1128/JB.00896-15.

[129] M. Madigan, S. S. Cox, and R. A. Stegeman, “Nitrogen Fixation and Nitrogenase Activities in Members of the Family Rhodospirillaceae,” 1984. [Online]. Available: https://journals.asm.org/journal/jb

[130] J. Moore, A. Jayakumar, S. Soldatou, O. Mašek, L. A. Lawton, and C. Edwards, “Nature-Based Solution to Eliminate Cyanotoxins in Water Using Biologically Enhanced Biochar,” *Environ Sci Technol*, vol. 57, no. 43, pp. 16372–16385, Oct. 2023, doi: 10.1021/acs.est.3c05298.

[131] N. Nitin Parulekar *et al.*, “Characterization of bacterial community associated with phytoplankton bloom in a eutrophic lake in South Norway using 16S rRNA gene amplicon sequence analysis,” *PLoS One*, vol. 12, no. 3, Mar. 2017, doi: 10.1371/journal.pone.0173408.

[132] M. Haida *et al.*, “Effects of Irrigation with Microcystin-Containing Water on Growth, Physiology, and Antioxidant Defense in Strawberry Fragaria vulgaris under Hydroponic Culture,” *Toxins (Basel)*, vol. 14, no. 3, Mar. 2022, doi: 10.3390/toxins14030198.

[133] S. H. Te *et al.*, “Coexistence of Synechococcus and Microcystis Blooms in a Tropical Urban Reservoir and Their Links with Microbiomes,” *Environ Sci Technol*, vol. 57, no. 4, pp. 1613–1624, Jan. 2023, doi: 10.1021/acs.est.2c04943.

[134] T. Hao *et al.*, “Effects of low temperature on rhizosphere phosphate-mineralizing microbial populations in constructed wetlands,” *J Environ Manage*, vol. 376, Mar. 2025, doi: 10.1016/j.jenvman.2025.124243.

[135] Z. Xiao, S. Zhang, P. Yan, J. Huo, and M. Aurangzeib, “Microbial Community and Their Potential Functions after Natural Vegetation Restoration in Gullies of Farmland in Mollisols of Northeast China,” *Land (Basel)*, vol. 11, no. 12, Dec. 2022, doi: 10.3390/land11122231.

[136] A. M. Maszenan, J. H. Tay, P. Schumann, H. L. Jiang, and S. T. L. Tay, “Quadrisphaera granulorum gen. nov., sp. nov., a Gram-positive polyphosphate-accumulating coccus in tetrads or aggregates isolated from aerobic granules,” *Int J Syst Evol Microbiol*, vol. 55, no. 5, pp. 1771–1777, Sep. 2005, doi: 10.1099/ijs.0.63583-0.

[137] C. Huo, J. Zhang, X. Yang, X. Li, Y. Su, and Z. Chen, “Dry season irrigation promotes nutrient cycling by reorganizing Eucalyptus rhizosphere microbiome,” *Science of the Total Environment*, vol. 954, Dec. 2024, doi: 10.1016/j.scitotenv.2024.176307.

[138] F. Cassán and M. Diaz-Zorita, “Azospirillum sp. in current agriculture: From the laboratory to the field,” Dec. 01, 2016, *Elsevier Ltd*. doi: 10.1016/j.soilbio.2016.08.020.

[139] C. Fagorzi *et al.*, “Symbiotic and nonsymbiotic members of the genus ensifer (syn. Sinorhizobium) are separated into two clades based on comparative genomics and high-throughput phenotyping,” *Genome Biol Evol*, vol. 12, no. 12, pp. 2521–2534, 2020, doi: 10.1093/GBE/EVAA221.

[140] L. Ran, F. Huang, M. Ekman, J. Klint, and B. Bergman, “Proteomic analyses of the photoauto- and diazotrophically grown cyanobacterium Nostoc sp. PCC 73102,” *Microbiology (N Y)*, vol. 153, no. 2, pp. 608–618, Feb. 2007, doi: 10.1099/mic.0.29198-0.

[141] Y. Huang *et al.*, “Functionality of bacterial communities in constructed wetlands used for water purification: influence of root components and seasonality,” *Front Plant Sci*, vol. 16, 2025, doi: 10.3389/fpls.2025.1480099.

[142] S. N. Dedysh *et al.*, “Wide distribution of Phycisphaera-like planctomycetes from WD2101 soil group in peatlands and genome analysis of the first cultivated representative,” *Environ Microbiol*, vol. 23, no. 3, pp. 1510–1526, Mar. 2021, doi: 10.1111/1462-2920.15360.

[143] K. S. Baik, Y. M. Hwang, J. S. Choi, J. Kwon, and C. N. Seong, “Dongia rigui sp. nov., isolated from freshwater of a large wetland in Korea,” *Antonie van Leeuwenhoek, International Journal of General and Molecular Microbiology*, vol. 104, no. 6, pp. 1143–1150, Dec. 2013, doi: 10.1007/s10482-013-0036-9.

[144] Z. M. Gao, X. Xu, and L. W. Ruan, “Enrichment and characterization of an anaerobic cellulolytic microbial consortium SQD-1.1 from mangrove soil,” *Appl Microbiol Biotechnol*, vol. 98, no. 1, pp. 465–474, Jan. 2014, doi: 10.1007/s00253-013-4857-2.

[145] W. Ren, Y. Zhang, X. Liu, S. Li, H. Li, and Y. Zhai, “Peracetic acid pretreatment improves biogas production from anaerobic digestion of sewage sludge by promoting organic matter release, conversion and affecting microbial community,” *J Environ Manage*, vol. 349, Jan. 2024, doi: 10.1016/j.jenvman.2023.119427.

[146] A. Detman *et al.*, “Dynamics of dark fermentation microbial communities in the light of lactate and butyrate production,” *Microbiome*, vol. 9, no. 1, Dec. 2021, doi: 10.1186/s40168-021-01105-x.

[147] S. N. Dedysh *et al.*, “Lacipirellula parvula gen. nov., sp. nov., representing a lineage of planctomycetes widespread in low-oxygen habitats, description of the family Lacipirellulaceae fam. nov. and proposal of the orders Pirellulales ord. nov., Gemmatales ord. nov. and Isosphaerales ord. nov.,” *Syst Appl Microbiol*, vol. 43, no. 1, Jan. 2020, doi: 10.1016/j.syapm.2019.126050.

[148] S. Campanaro *et al.*, “New insights from the biogas microbiome by comprehensive genome-resolved metagenomics of nearly 1600 species originating from multiple anaerobic digesters,” *Biotechnol Biofuels*, vol. 13, no. 1, Feb. 2020, doi: 10.1186/s13068-020-01679-y.

[149] M. Langone, R. Ferrentino, W. Mertz, D. Mattioli, L. Petta, and G. Andreottola, “Microbial community composition from full-scale reactors treating mature landfill leachate through

innovative biological processes and its importance in mathematical modeling," *Biochem Eng J*, vol. 204, Apr. 2024, doi: 10.1016/j.bej.2024.109226.

[150] B. Wolf *et al.*, "WO2023183783A2," Mar. 2023.

[151] X. Lv, Z. Kankan, H. Li, and B. Ma, "Niche partition of phenanthrene-degrading bacteria along a Phragmites australis rhizosphere gradient," *Biol Fertil Soils*, vol. 54, no. 5, pp. 607–616, Jul. 2018, doi: 10.1007/s00374-018-1287-4.

[152] M. Kang *et al.*, "Novosphingobium cyanobacteriorum sp. nov., isolated from a eutrophic reservoir during the Microcystis bloom period," *Int J Syst Evol Microbiol*, vol. 73, no. 9, Sep. 2023, doi: 10.1099/ijsem.0.006042.

[153] A. Larasati, O. Bernadet, G.-J. W. Euverink, H. P. J. van Veelen, and M. C. Gagliano, "Production of birnessite-type manganese oxides by biofilms from oxygen-supplemented biological activated carbon (BAC) filters," *Environ Sci (Camb)*, 2024, doi: 10.1039/d4ew00208c.

[154] C. Zhao *et al.*, "Innovative utilization of plant-derived dissolved organic matter to promote polycyclic aromatic hydrocarbons removal in constructed wetlands: Unraveling the synergy among substrate adsorption, plant uptake, and microbial degradation," *Process Safety and Environmental Protection*, vol. 190, pp. 1590–1600, Oct. 2024, doi: 10.1016/j.psep.2024.08.012.

[155] X. Zhang *et al.*, "The environmental risk assessment of constructed wetlands filled with iron and manganese ores in typical antibiotic treatment," *Environ Res*, vol. 240, Jan. 2024, doi: 10.1016/j.envres.2023.117567.

[156] N. Dai *et al.*, "Enhanced adaptability of pyrite-based constructed wetlands for low carbon to nitrogen ratio wastewater treatments: Modulation of nitrogen removal mechanisms and reduction of carbon emissions," *Bioresour Technol*, vol. 395, Mar. 2024, doi: 10.1016/j.biortech.2024.130348.

[157] Z. Feng *et al.*, "Microbial survival strategies in biological soil crusts of polymetallic tailing wetlands," *Geoderma*, vol. 443, Mar. 2024, doi: 10.1016/j.geoderma.2024.116852.

[158] Y. Usui, T. Shimizu, A. Nakamura, and M. Ito, "Metabolites produced by kaistia sp. 32k promote biofilm formation in coculture with methylobacterium sp. me121," *Biology (Basel)*, vol. 9, no. 9, pp. 1–14, Sep. 2020, doi: 10.3390/biology9090287.

[159] R. C. Wilhelm, “Following the terrestrial tracks of Caulobacter - redefining the ecology of a reputed aquatic oligotroph,” *ISME Journal*, vol. 12, no. 12, pp. 3025–3037, Dec. 2018, doi: 10.1038/s41396-018-0257-z.

[160] D. van der Kooij, H. R. Veenendaal, R. Italiaander, E. J. van der Mark, and M. Dignum, “Primary colonizing Betaproteobacteriales play a key role in the growth of Legionella pneumophila in biofilms on surfaces exposed to drinking water treated by slow sand filtration,” *Appl Environ Microbiol*, vol. 84, no. 24, Dec. 2018, doi: 10.1128/AEM.01732-18.

[161] J. T. Staley’, “Prosthecomicrobium and Ancalomicrobiutm: New Prosthecate Freshwater Bacteria,” 1968. [Online]. Available: https://journals.asm.org/journal/jb

[162] R. Guo *et al.*, “Microbial community structures and important taxa across oxygen gradients in the Andaman Sea and eastern Bay of Bengal epipelagic waters,” *Front Microbiol*, vol. 13, Nov. 2022, doi: 10.3389/fmicb.2022.1041521.

[163] A. Ueki, A. Tonouchi, N. Kaku, and K. Ueki, “Anaerocolumna chitinilytica sp. nov., a chitin-decomposing anaerobic bacterium isolated from anoxic soil subjected to biological soil disinfestation,” *Int J Syst Evol Microbiol*, vol. 71, no. 9, 2021, doi: 10.1099/ijsem.0.004999.

[164] W. B. Lenferink, T. A. van Alen, M. S. M. Jetten, H. J. M. Op den Camp, M. A. H. J. van Kessel, and S. Lücker, “Genomic analysis of the class Phycisphaerae reveals a versatile group of complex carbon-degrading bacteria,” *Antonie van Leeuwenhoek, International Journal of General and Molecular Microbiology*, vol. 117, no. 1, Dec. 2024, doi: 10.1007/s10482-024-02002-7.

[165] O. A. Podosokorskaya, E. A. Bonch-Osmolovskaya, A. V. Beskorovaynyy, S. V. Toshchakov, T. V. Kolganova, and I. V. Kublanov, “Mobilitalea sibirica gen. nov., sp. nov., a halotolerant polysaccharide-degrading bacterium,” *Int J Syst Evol Microbiol*, vol. 64, no. PART 8, pp. 2657–2661, 2014, doi: 10.1099/ijs.0.057109-0.

[166] E. E. Ziganshina, D. E. Belostotskiy, S. S. Bulynina, and A. M. Ziganshin, “Effect of magnetite on anaerobic digestion of distillers grains and beet pulp: Operation of reactors and microbial community dynamics,” *J Biosci Bioeng*, vol. 131, no. 3, pp. 290–298, Mar. 2021, doi: 10.1016/j.jbiosc.2020.10.003.

[167] G. W. Zhou *et al.*, “Propionicimonas ferrireducens sp. nov., isolated from dissimilatory iron(III)-reducing microbial enrichment obtained from paddy soil,” *Int J Syst Evol Microbiol*, vol. 68, no. 6, pp. 1914–1918, Jun. 2018, doi: 10.1099/ijsem.0.002766.

[168] K. Kujala, A. Mikkonen, K. Saravesi, A. K. Ronkanen, and M. Tiirola, “Microbial diversity along a gradient in peatlands treating mining-affected waters,” *FEMS Microbiol Ecol*, vol. 94, no. 10, Oct. 2018, doi: 10.1093/femsec/fiy145.

[169] E. Ransom-Jones, “The ecology of Fibrobacter spp. in cellulose-degrading microbial communities,” 2014. [Online]. Available: http://www.llgc.org.uk/index.php?id=4676

[170] M. Ornik, R. Salinas, G. Antonacci, M. Schädler, and H. Azarbad, “The stress history of soil bacteria under organic farming enhances the growth of wheat seedlings,” *Front Microbiol*, vol. 15, 2024, doi: 10.3389/fmicb.2024.1355158.

[171] E. Y. Tapia-García *et al.*, “Plant growth-promoting bacteria isolated from wild legume nodules and nodules of Phaseolus vulgaris L. trap plants in central and southern Mexico,” *Microbiol Res*, vol. 239, Oct. 2020, doi: 10.1016/j.micres.2020.126522.

[172] M. Iniesta-Pallarés *et al.*, “Changes in rice rhizosphere and bulk soil bacterial communities in the Doñana wetlands at different growth stages,” *Applied Soil Ecology*, vol. 190, Oct. 2023, doi: 10.1016/j.apsoil.2023.105013.

[173] L. N. Zhang *et al.*, “Long-Term Field Application of a Plant Growth-Promoting Rhizobacterial Consortium Suppressed Root-Knot Disease by Shaping the Rhizosphere Microbiota,” *Plant Dis*, vol. 108, no. 1, pp. 94–103, Jan. 2024, doi: 10.1094/PDIS-09-22-2196-RE.

[174] R. J. Moore and J. A. Lacey, “Genomics of the Pathogenic Clostridia,” *Microbiol Spectr*, vol. 7, no. 3, May 2019, doi: 10.1128/microbiolspec.gpp3-0033-2018.

[175] T. P. Primm, C. A. Lucero, and J. O. Falkinham, “Health Impacts of Environmental Mycobacteria,” Jan. 2004. doi: 10.1128/CMR.17.1.98-106.2004.

[176] V. Thomas, N. Casson, and G. Greub, “New Afipia and Bosea strains isolated from various water sources by amoebal co-culture,” *Syst Appl Microbiol*, vol. 30, no. 7, pp. 572–579, Nov. 2007, doi: 10.1016/j.syapm.2007.06.004.

[177] M. Li *et al.*, “Effects of biochar amendment and organic fertilizer on microbial communities in the rhizosphere soil of wheat in Yellow River Delta saline-alkaline soil,” *Front Microbiol*, vol. 14, 2023, doi: 10.3389/fmicb.2023.1250453.

[178] J. Pratscher, J. Vollmers, S. Wiegand, M. G. Dumont, and A. K. Kaster, “Unravelling the Identity, Metabolic Potential and Global Biogeography of the Atmospheric Methane-Oxidizing Upland Soil Cluster α,” *Environ Microbiol*, vol. 20, no. 3, pp. 1016–1029, Mar. 2018, doi: 10.1111/1462-2920.14036.

Chapter 4. General Discussion and Future Perspectives

This thesis explored strategies to enhance NBS, such as TWs, to address the complex dynamics of CyanoHABs and ensure their effective treatment. In the first experimental study, it was aimed to test agro-based waste materials that were locally relevant, for their sorption potential of the most prevalent cyanotoxins (MC-LR and CYN), and the sorption kinetics and mechanisms of the two best candidates were studied for their subsequent incorporation into NBS such as TWs and biofilters. We then aimed to test and compare monocultures and polycultures of two plant species (*S.erectum* and *C.indica*) in TWs for the removal of MC-LR and CYN in a second experimental study. The two best sorbent materials from the first experiment were incorporated into the TW systems, thereby linking the two aims of the thesis and studying a new TW configuration for cyanotoxin removal. Finally, the removal mechanisms of MC-LR and CYN in this specific TW design was studied, contributing to the knowledge on TW performance that could be valuable for scale-up of these systems.

The research described in this thesis provides knowledge and steps for the enhancement of NBS through valorisation of local resources, testing of different plant species, their effectiveness in the removal of cyanotoxins, and the processes involved in cyanotoxin removal.

4.1. Valorisation of Agro-based Waste Materials as Cyanotoxin Sorbents in NBS

Low-cost sorbents, namely raw un-modified agro based waste materials were explored for the sorption of cyanotoxins MC-LR and CYN, along with some benchmark substrates used in NBS (biochar, LECA, and sand). The work is positioned at the intersection of resource-efficient material use and NBS, with the objective of guiding the sustainable incorporation of sorptive materials into NBS for enhanced cyanotoxin mitigation.

4.1.1. Sorption Performance and Material Viability

The findings demonstrate that biochar and LECA are promising materials for inclusion in NBS due to their notable sorption capacities for both MC-LR and CYN. The kinetic and isotherm modelling indicated that chemisorption was the dominant mechanism

governing the uptake of cyanotoxins, with the Freundlich and D-R models best describing the experimental data, underscoring the heterogeneous and microporous nature of the materials. In contrast, unmodified agro-waste materials such as cork granules, OP, and rice husk exhibited negligible sorption potential under the tested conditions. These results underscore a critical trade-off: while agro-wastes are attractive from a sustainability and circular economy perspective, their unmodified state appears insufficient for practical application in cyanotoxin removal.

4.1.2. Interpretation of Sorption Mechanisms

The higher cyanotoxin sorption observed with biochar and LECA is attributable not only to their surface area and pore characteristics but also to their surface chemistry and morphological heterogeneity, as revealed by SEM/EDS analysis. While attempts to correlate the elemental composition of the material directly to their sorption performance were inconclusive, the high content of positively charged elements on the sorbent surfaces may have enhanced the interaction with negatively charged MC-LR at neutral pH. However, CYN, being zwitterionic at pH 7, warrants deeper mechanistic investigation, particularly regarding its interaction with variable surface functional groups.

4.1.3. Implications for Integrating into NBS

The incorporation of non-functionalized biochar and LECA into NBS aligns with the principles of low-input, scalable, and adaptive water treatment strategies, especially for rural and resource-constrained settings. Their sorption characteristics, coupled with physical durability and commercial availability, support their incorporation into modular NBS designs aimed at tackling seasonal or episodic cyanotoxin peaks. However, the sorption performance under dynamic field conditions, where multiple pollutants coexist and biogeochemical processes interact, remains to be validated. For instance, the influence of TWs operational parameters such as hydraulic retention time, redox gradients, microbial activity, and plant-substrate-water interactions must be systematically studied to extrapolate laboratory findings to field-scale applications.

4.1.4. Future Perspectives

The promising sorbents identified should be incorporated into pilot or mesocosm scale NBS under environmentally relevant conditions. This would allow for monitoring of long-term sorption stability of the cyanotoxins, and performance under varying hydraulic and pollutant loading regimes. Future investigations should address competitive sorption of cyanotoxins, either when multiple cyanotoxins are present or in the presence of other organic micropollutants, nutrients (e.g., ammonium, nitrates, phosphate), and natural organic matter, which may inhibit or alter cyanotoxin sorption. Moreover, understanding

whether sorbed toxins remain intact, undergo transformation, or become bioavailable for microbial degradation is essential for risk mitigation and the long-term safety of NBS effluents. Integration of sorption studies with microbial community analyses would help elucidate how sorptive substrates contribute to or hinder microbial degradation pathways. The synergy between abiotic (sorption) and biotic (biodegradation) processes may be critical for complete cyanotoxin removal.

Without resorting to chemical activation, alternative low-energy modifications such as steam treatment, physical milling, or enzymatic pretreatment of agro-waste materials could be explored to enhance their sorption performance while maintaining ecological compatibility.

4.2. Comparing a Natural Wetland and an Ornamental Species in TWs as Monocultures and Polycultures

VSSF TWs planted with monoculture and polyculture of *S. erectum,* and *C. indica* plant species were studied for MC-LR and CYN removal, as well as the processes of removal in the TWs, such as substrate retention and plant uptake. Microbial communities in the substrate were also investigated in order to identify those responsible for cyanotoxin removal. This was an initial lab-scale study with the goal of enabling further pilot-scale investigations to assess evapotranspiration dynamics and effluent quality for safe water reuse.

4.2.1. Performance of TWs and Cyanotoxin Removal Mechanisms

All TWs consistently achieved > 96% removal of MC-LR, with effluent concentrations falling below the LOD and well under WHO drinking water guidelines [1]. This aligns with previous studies and reinforces the robustness of vertical-flow TW configurations for MC-LR elimination. CYN removal was plant-species dependent, with TWs planted with *C. indica* (monoculture or polyculture) showing improved mass removal (73–98%), outperforming systems with *S. erectum* over time. However, CYN concentrations in TW effluents still exceeded WHO drinking water guidelines for chronic exposure limits in all treatments [2]. *S. erectum* had higher evapotranspiration and reduced effluent dilution capacity. It should be mentioned that TW systems at full scale would be less heated, resulting in lower ET rates and effluent concentrations, when compared to microcosm studies such as this.

Despite variations in inlet water quality and environmental conditions, TWs maintained > 90% nitrate ion removal and consistently met the various global effluent discharge limits for COD (≤ 100 mg/L) and TSS (< 2 mg/L). Substrate stabilization was notably effective in polyculture systems. Low cyanotoxin retention in substrates (< 8%) and non-detectable levels of MC-LR and CYN in plant tissues indicate that microbial degradation within substrates is likely the primary removal mechanism. Absence of mlrA gene amplification, a gene commonly associated with cyanotoxins degradation, suggests alternative microbial degradation pathways at work.

4.2.2. Plant Role and Stress Response

C. indica in polyculture TWs exhibited stress adaptation, as indicated by temporary variations in pigment and sugar levels, indicating robust physiological responses. In contrast, *S. erectum* showed minimal stress response and visual signs of declining over the experimental period.

The absence of detectable toxin in plant tissues may reflect rapid metabolism (e.g., glutathione conjugation or P450 activity) , limited uptake, or bioavailability. These detoxification pathways remain speculative and require deeper investigation.

4.2.3. Microbial Community Dynamics

TW substrate microbial communities were dominated by *Proteobacteria, Actinobacteriota,* and *Cyanobacteria*. Significant plant species– and substrate–driven shifts occurred at the genus level, particularly over time. *C. indica* systems (both in monoculture and polyculture) exhibited enrichment of plant-growth–promoting taxa (e.g., *Leifsonia, Terrabacter, Altererythrobacter*). Bulk substrates hosted a broader range of degradative, decomposing, and anaerobic microbial consortia, suggesting complementary functional zones within TWs substrate.

4.2.4. Future Perspectives

Pilot scale up testing needs to be done across seasonal cycles and open-ground installations to capture reduced evapotranspiration, stratification, and their effects on CYN removal and microbial activity. Moreover, long-term stability extended over monitoring beyond 10 weeks (ideally >1 year) to assess TW resilience, pigment stability in plants, root longevity, and pollutant removal consistency, as seen in long-term pilot studies, should be considered.

The P-k–C* model was fitted using the experimental data to provide area-based rate constants (k_a) and facilitate area demand calculation [3] (Appendix 4- S1, Table S1). For this, the average effluent concentrations of MC-LR and CYN over the 10-week experimental cycle was used and the area was calculated considering the treatment of

1m^3/day of water with an initial MC-LR and CYN concentration of 10 µg/L, under two different WHO GV scenarios (recreational and drinking water). For reaching the recreational GV (MC-LR- 5.67 µg/L and CYN- 3 µg/L), an estimated area of 0.7 m^2 for MC-LR and 1.4-1.5 m^2 for CYN was calculated, and for reaching the drinking water GV (MC-LR- 1µg/L and CYN- 0.7 µg/L) an estimated area of 4.4 m^2 for MC-LR and 4.7- 5.2 m^2 for CYN was calculated. While TWs are not usually used for treatment of drinking water, this GV helps create a stricter standard for the effluent water quality, since there are no GVs for cyanotoxins in irrigation waters.

The area demand calculated in this study is lower compared to another study by Martinez i Quer et al., 2024 [4] where mesocosms HSSF TWs with different plants and substrates were studied (7 to 30 m^2 for MC-LR, and 36 to 415 m^2 for CYN for reaching the WHO drinking water GV), showing that VSSF TWs require lesser area. However, it should also be noted that this study has a hybrid substrate VSSF TW design configuration. There is negligible difference between the area requirements among the different plant compositions in this study, and only a slight difference in the area requirements between MC-LR and CYN. On the other hand, there is a bigger difference between the two regulation scenarios (0.7- 1.5 m^2 for recreational water quality and 4.4-5.2 m^2 for drinking water quality). For estimating a scale up to a pilot scale TW, an area in between these two values (3 m^2 c.a.) would be ideal.

In terms of plant selection strategies, including or preferring stress adapted species such as *C.indica* can help balancing physiological resilience with microbial diversity. Moreover, studies testing combined substrate composition effects, including stability, spatial distribution, layering effects, and microbial colonization, needs to be done.

Further probing techniques, including shotgun metagenomic or meta-transcriptomic analysis, to characterize unknown cyanotoxin degrading enzymes and pathways, especially for CYN, are needed. Moreover, targeted isolation by cultivating and testing cyanotoxin-degrading isolated strains (e.g. *Shinella, Sphingorabdus*), could help transferability to engineered consortia or bioaugmentation strategies that could boost TWs performance, enhancing cyanotoxins removal.

Finally, a more thorough ecotoxicological evaluation (e.g. pathogen risk, microbiome re-establishment kinetics, nutrient load effects) is needed before re-use of TW effluents for irrigation (landscape or agricultural). Moreover, more empirical evidence is needed, especially on chronic cyanotoxin exposure limits, to provide support to cyanotoxin guideline value standards for irrigation waters.

4.3. Final Remarks

Overall, this thesis advances the use of TWs as NBS for decentralized treatment of cyanotoxins, addressing a critical gap in water management. By systematically linking sorption studies with plant and microbe interactions, the study demonstrates that no single mechanism can ensure safe cyanotoxin removal; rather, effective treatment requires a combination of abiotic sorption, microbial degradation, and plant-mediated resilience. Initially, the viability of local agro-waste materials and commercially available substrates were clarified, establishing biochar and LECA with efficient sorption capacity for cyanotoxins. It then shows how substrate selection interacts with plant composition and the respective microbial community in TWs, influencing treatment outcomes. The research establishes that VSSF TWs incorporated with sorptive substrates such as biochar and LECA, and resilient plant species like *C. indica*, provide a promising foundation for scalable systems capable of complete MC-LR removal and improved, though still incomplete, CYN mitigation. In doing so, it moves the field beyond proof-of-concept demonstrations to concrete plans for pilot scale installation.

Specifically, the results highlight that while MC-LR can be consistently reduced below guideline thresholds in this TW design configuration, CYN remains more recalcitrant and could require additional optimization. This cyanotoxin-specific performance underscores the need to move beyond generalized “one-size-fits-all” TWs towards targeted, adaptive designs.

The thesis also recognizes limitations that laboratory scale TW studies bring, particularly batch-loaded microcosms. This includes the inability to reflect the ecological and operational complexity of field circumstances, such as seasonal fluctuation, pollutant mixes, and variable redox gradients. Moreover, while biochar and LECA show promise, it raises questions of sourcing, cost, and regeneration under dynamic conditions. Microbial degradation emerged as a predominant removal pathway, yet the specific enzymes and metabolic routes remain unresolved, particularly for CYN. Despite these limitations, the thesis serves as a crucial stepping-stone towards the practical use of TWs for cyanotoxin mitigation. It highlights that implementation will require:

i) pilot-scale validation across climatic and seasonal contexts
ii) elucidation of microbial degradation mechanisms through functional genomics and strain isolation
iii) optimization of substrate configurations to balance sorption and microbial colonization

iv) screening of effluents for pathogens in the context of water re-use, including development of regulatory GVs for cyanotoxins in irrigation and reuse waters

v) socio-economic evaluation to demonstrate feasibility, co-benefits, and stakeholder acceptance

This study also attempts to create a paradigm shift, wherein TWs are viewed as customizable, multifunctional ecological infrastructures capable of tackling specific contaminants of emerging concern. By bridging sustainable material valorisation, ecological resilience, and microbial functionality, it offers both scientific insights and applied design criteria that move the field closer to using TWs as a viable, adaptive solution for cyanotoxin management in surface waters.

References

[1] “Cyanobacterial toxins: microcystins Background document for development of WHO,” 2020. [Online]. Available: http://apps.who.int/bookorders.

[2] “Cyanobacterial toxins: cylindrospermopsins Background document for development of WHO,” 2020. [Online]. Available: http://apps.who.int/bookorders.

[3] R. H. Kadlec and S. Wallace, “TREATMENT WETLANDS 2nd Ed,” 2008.

[4] A. Martinez i Quer *et al.*, “Saturated constructed wetlands for the remediation of cylindrospermopsin and microcystin-LR: Plants, microbes, and biodegradation pathways,” *Science of the Total Environment*, vol. 948, Oct. 2024, doi: 10.1016/j.scitotenv.2024.174745.

Appendix A— Evaluating Agro-based Waste Materials for Cyanotoxin Sorption for Future Incorporation in Nature-based Solution (NBS)

Table A1. Molecules addressed in the LC-MS/MS method and their MS operating parameters

Molecule	Retention Time	Mass-to-charge ratio (*m/z*) transition	Cone (V)	Collision energy (V)
MC-LR	**15,88**	**995.5>135** 995.5>599	**20**	**20**
CYN	**9,14**	415.9>194 **415.9>336.1**	**20**	**80**

Table A2. Average cyanotoxins removal in pre-screening of sorbents (*- matrix not suitable for CYN detection) (mean and standard deviation n=3)

SORBENT	*CYN REMOVAL (%)*		*MC-LR REMOVAL (%)*	
	after 24 h	after 48 h	after 24 h	after 48 h
Rice husk	9.1	7.6	2.9	-
Cork	5.3	8.2	5.4	0.6
LECA	68.1	79.7	62.1	72.9
Sand	3.4	3.7	25.7	26.5
Biochar	> 98	> 98	> 86	> 86
OP	*	*	1.36	15.12

Table A3. Statistical details of pre-screening of sorbents

PHASE 1 (24 h) MC-LR- Control, Rice husk, Cork, LECA

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	2404.118	3	801.373	64.667	5.96E-06	4.066
Within Groups	99.138	8	12.392			
Total	2503.256	11				

Tukey post-hoc

Group pairs	**Absolute difference**	**Standard error**	**q Tukey**	**q Critical**	**Significance**
Control (with gravel) vs Rice Husk	0.818	2.032	0.402	4.041	No
Control (with gravel) vs Cork	0.522	4.892	0.107	4.041	No
Control (with gravel) vs LECA	32.571	4.892	6.658	4.041	Yes
Rice Husk vs Cork	32.049	4.892	6.552	4.041	Yes
Rice Husk vs LECA	33.389	4.892	6.826	4.041	Yes
Cork vs LECA	32.049	4.892	6.552	4.041	Yes

PHASE 1 (48 h) MC-LR- Control, Rice husk, Cork, LECA

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	12723.25	3	4241.082	106.607	8.64E-07	4.066
Within Groups	318.261	8	39.783			
Total	13041.51	11				

Tukey post-hoc

Group pairs	**Absolute difference**	**Standard error**	**q Tukey**	**q Critical**	**Significance**
Control (with gravel) vs Rice Husk	14.380	3.642	3.949	4.041	No
Control (with gravel) vs Cork	5.144	5.181	0.993	4.041	No

Control (with gravel) vs LECA	67.743	5.181	13.075	4.041	Yes
Rice Husk vs Cork	27.708	5.181	5.348	4.041	Yes
Rice Husk vs LECA	82.123	5.181	15.850	4.041	Yes
Cork vs LECA	72.887	5.181	14.068	4.041	Yes

PHASE 2 (24 h) MC-LR- Control, Sand, OP, Biochar

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	12419.37	3	4139.789	206.790	6.43E-08	4.066
Within Groups	160.154	8	20.01925			
Total	12579.52	11				

Tukey post-hoc

Group pairs	**Absolute difference**	**Standard error**	**q Tukey**	**q Critical**	**Significance**
Control (with gravel) vs Sand	18.355	2.583	7.106	4.041	Yes
Control (with gravel) vs Biochar	75.770	2.583	29.332	4.041	Yes
Control (with gravel) vs OP	5.411	2.583	2.095	4.041	No
Sand vs Biochar	57.415	2.583	22.226	4.041	Yes
Sand vs OP	23.767	2.583	9.200	4.041	Yes
Biochar vs OP	81.182	2.583	31.426	4.041	Yes

PHASE 2 (48 h) MC-LR- Control, Sand, OP, Biochar

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	12059.55	3	4019.849	493.136	2.05E-09	4.066
Within Groups	65.213	8	8.152			
Total	12124.76	11				

Tukey post-hoc

Group pairs	**Absolute difference**	**Standard error**	**q Tukey**	**q Critical**	**Significance**

Control (with gravel) vs Sand	23.555	1.648	14.290	4.041	Yes
Control (with gravel) vs Biochar	83.822	1.648	50.851	4.041	Yes
Control (with gravel) vs OP	16.396	1.648	9.947	4.041	Yes
Sand vs Biochar	60.267	1.648	36.561	4.041	Yes
Sand vs OP	7.159	1.648	4.343	4.041	Yes
Biochar vs OP	67.426	1.648	40.904	4.041	Yes

PHASE 1 (24 h) CYN- Control, Rice husk, Cork, LECA

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	9271.04	3	3090.347	285.957	1.79E-08	4.066
Within Groups	86.456	8	10.80704			
Total	9357.496	11				

Tukey post-hoc

Group pairs	**Absolute difference**	**Standard error**	**q Tukey**	**q Critical**	**Significance**
Control (with gravel) vs Rice Husk	10.267	1.898	5.409	4.041	Yes
Control (with gravel) vs Cork	19.427	1.898	10.236	4.041	Yes
Control (with gravel) vs LECA	207.628	1.898	109.394	4.041	Yes
Rice Husk vs Cork	11.374	1.898	5.993	4.041	Yes
Rice Husk vs LECA	176.827	1.898	93.166	4.041	Yes
Cork vs LECA	188.201	1.898	99.159	4.041	Yes

PHASE 1 (48 h) CYN- Control, Rice husk, Cork, LECA

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	12855.01	3	4285.005	657.2591	6.55E-10	4.066
Within Groups	52.156	8	6.520			
Total	12907.17	11				

Tukey post-hoc

Group pairs	**Absolute difference**	**Standard error**	**q Tukey**	**q Critical**	**Significance**
Control (with gravel) vs Rice Husk	9.276	1.474	6.292	4.041	Yes
Control (with gravel) vs Cork	9.967	1.474	6.761	4.041	Yes
Control (with gravel) vs LECA	81.453	1.474	55.253	4.041	Yes
Rice Husk vs Cork	0.691	1.474	0.469	4.041	No
Rice Husk vs LECA	72.177	1.474	48.961	4.041	Yes
Cork vs LECA	71.486	1.474	48.492	4.041	Yes

PHASE 2 (24 h) CYN- Control, Sand, Biochar

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	17899.56	2	8949.78	3628.327	5.64E-10	5.143
Within Groups	14.799	6	2.467			
Total	17914.36	8				

Tukey post-hoc

Group pairs	**Absolute difference**	**Standard error**	**q Tukey**	**q Critical**	**Significance**
Control (with gravel) vs Sand	0.845	0.907	0.932	4.339	NO
Control (with gravel) vs Biochar	95.023	0.907	104.794	4.339	Yes
Sand vs Biochar	94.178	0.907	103.862	4.339	Yes

PHASE 2 (48 h) CYN- Control, Sand, Biochar

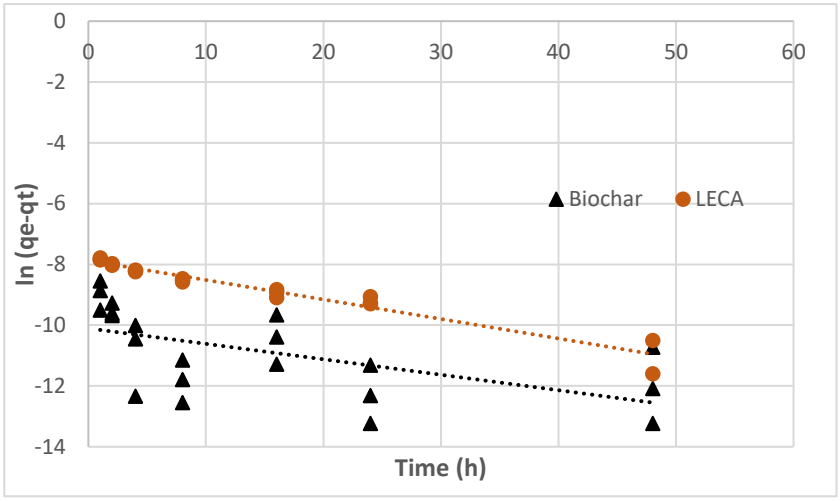
ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	17960.8	2	8980.401	943.455	3.18E-08	5.143
Within Groups	57.112	6	9.519			
Total	18017.91	8				

Tukey post-hoc

Group pairs	**Absolute difference**	**Standard error**	**q Tukey**	**q Critical**	**Significance**
Control (with gravel) vs Sand	0.851	1.781	0.478	4.339	No
Control (with gravel) vs Biochar	94.337	1.781	52.961	4.339	Yes
Sand vs Biochar	95.188	1.781	53.438	4.339	Yes

a)



b)

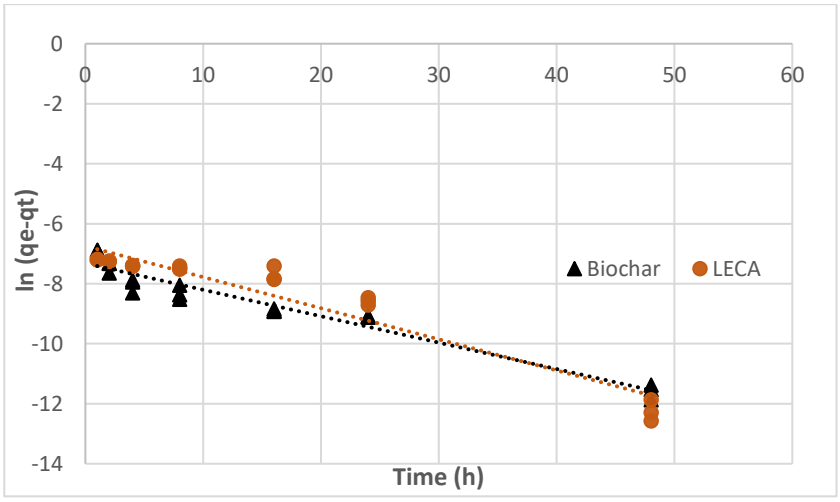
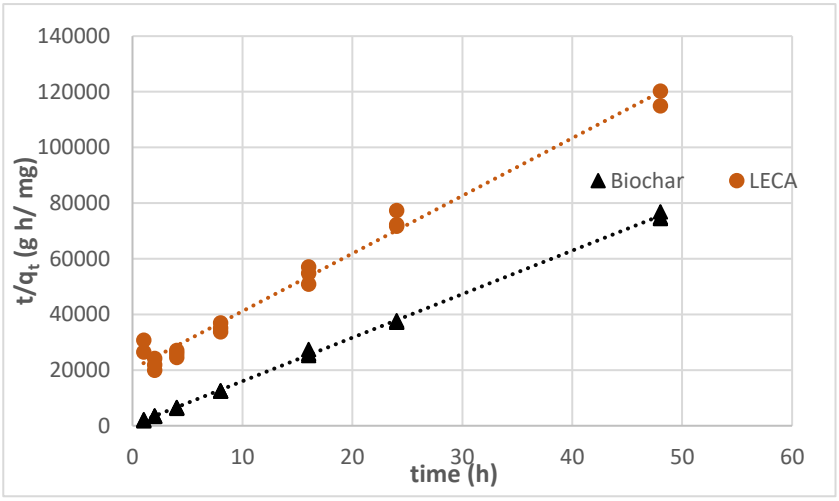


Figure A1. Pseudo-first-order sorption kinetic models for a) MC-LR and b) CYN onto sorbents biochar and LECA

a)



b)

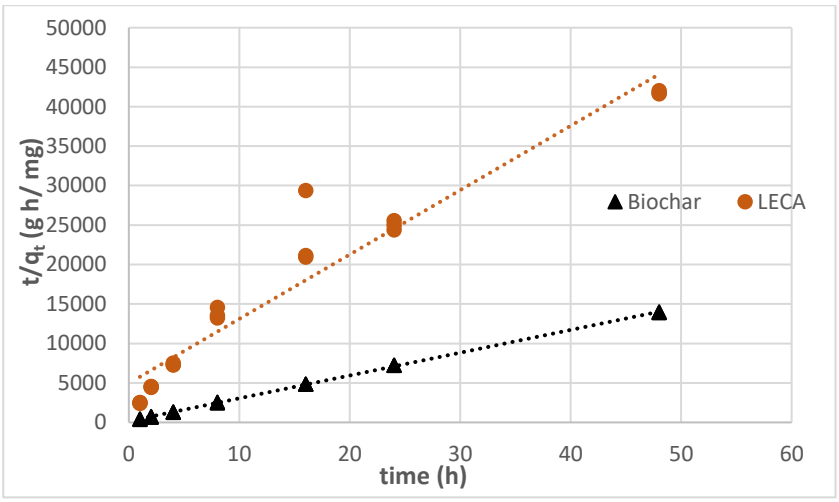
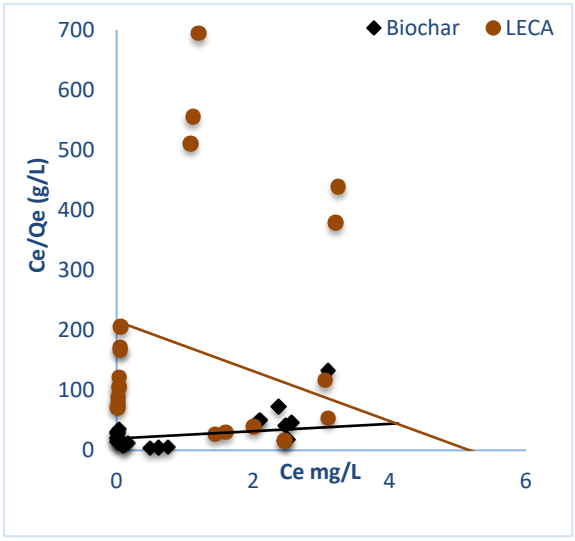


Figure A2. Pseudo-second-order sorption kinetic models for a) MC-LR and b) CYN onto sorbents biochar and LECA

a)



b)

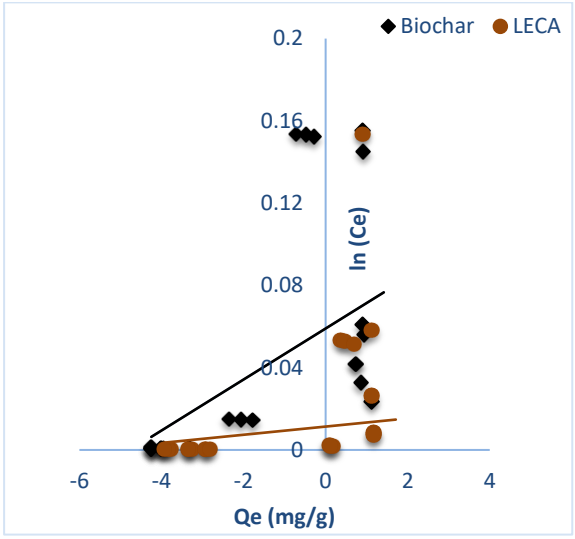
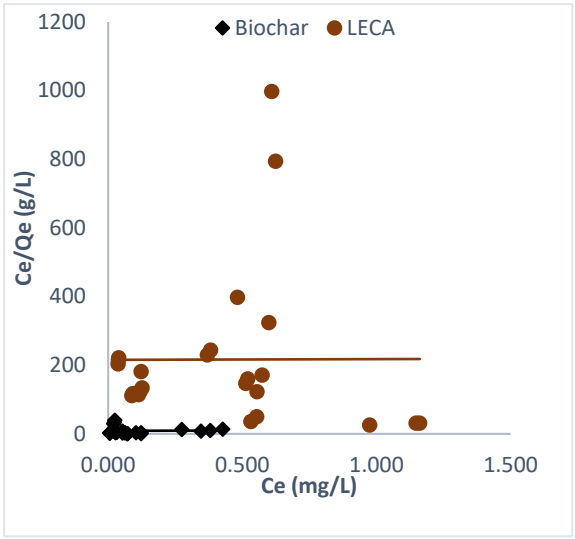
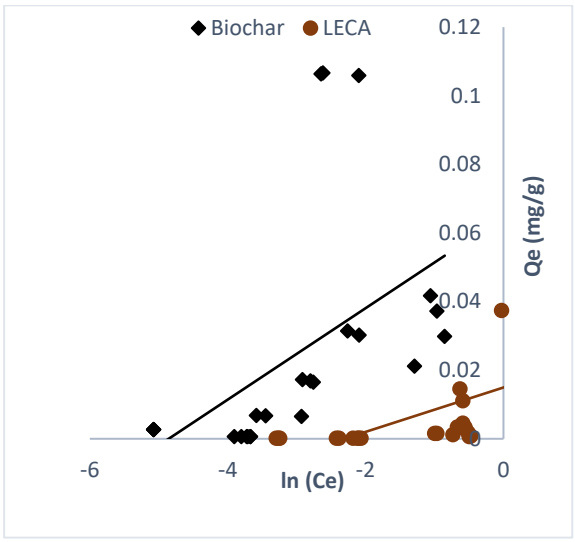


Figure A3. Sorption isotherm plots of MC-LR onto biochar and LECA: a) Langmuir and b) Temkin



a)



b)

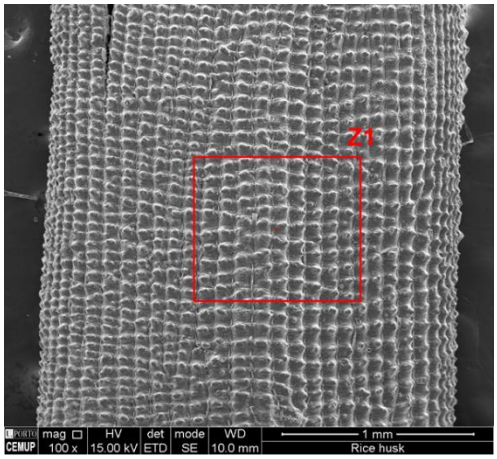
Figure A4. Sorption isotherm plots of CYN onto biochar and LECA: a) Langmuir and b) Temkin

A1. SEM/EDS of Sorbents

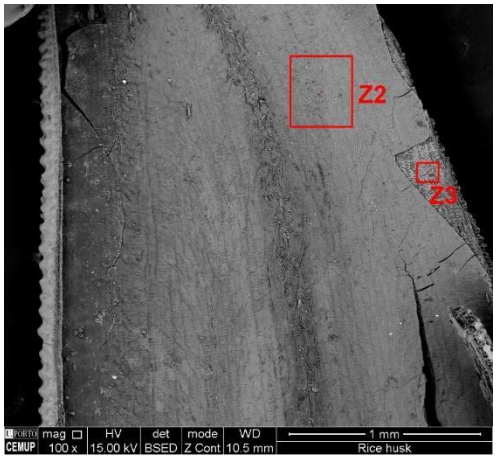
A1.1. Rice husk:

The surface morphology of raw, untreated RH differed on the outer and inner sides. From the SEM images in Figure A5a and A5b, it could be seen that the outer surface had a homogenous layer without porosity. The inner surface was covered by a homogeneous cellulosic membrane with a porous layer underneath that was only minutely visible in some places. The element composition represented by EDS graphs in Figure A5c-e shows that it is similar throughout all the surfaces.

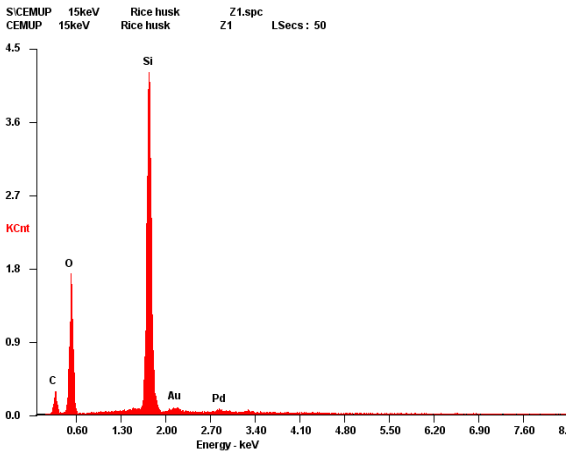
a)


Z1
CEMUP mag 100 x | HV 15.00 kV | det ETD | mode SE | WD 10.0 mm | 1 mm | Rice husk

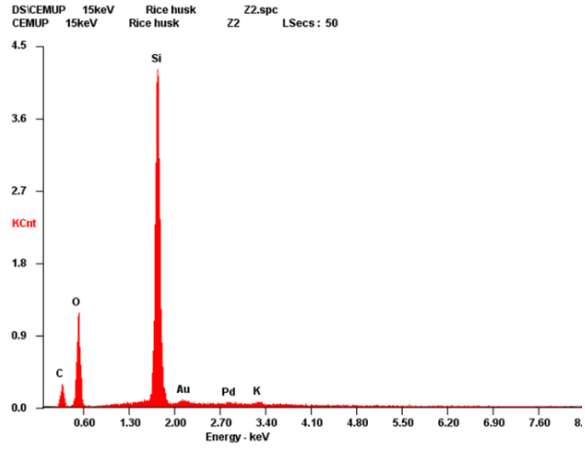
b)


Z2
Z3
CEMUP mag 100 x | HV 15.00 kV | det BSED | mode Z Cont | WD 10.5 mm | 1 mm | Rice husk

c)


S\CEMUP 15keV Rice husk Z1.spc
CEMUP 15keV Rice husk Z1 LSecs : 50
KCnt
Si
O
C
Au
Pd
Energy - keV

d)


DS\CEMUP 15keV Rice husk Z2.spc
CEMUP 15keV Rice husk Z2 LSecs : 50
KCnt
Si
O
C
Au
Pd
K
Energy - keV

e)

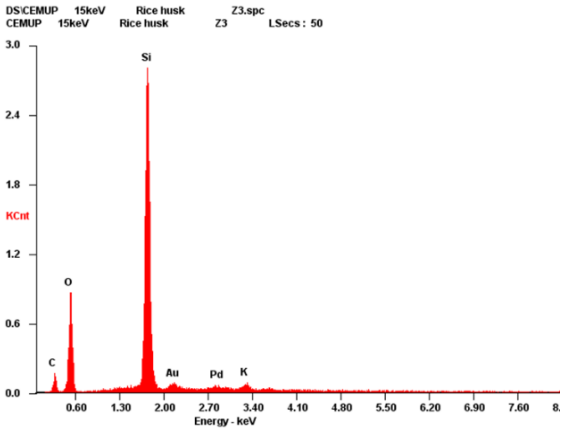
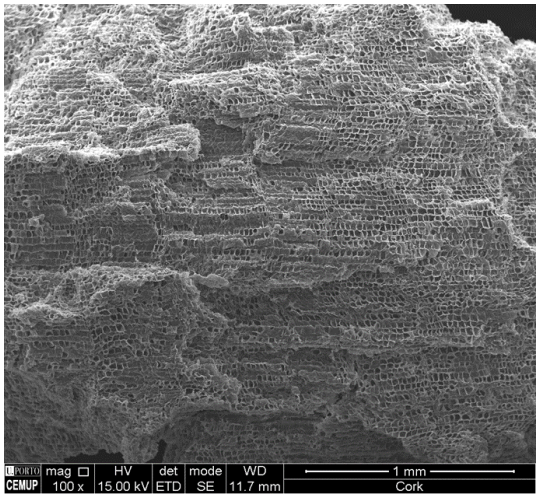


Figure A5. a) SEM image of outer side of RH; b) SEM image of inner side of RH; c) EDS graph of Z1; d) EDS graph of Z3; e) EDS graph of Z3

A1.2. Cork

From Figure A6a, it can be seen that the surface of the cork had geometrical cavities that had a regularity in pattern. It also had uniform element composition throughout its surface, similar to that shown in Figure A6b.

a)



b)

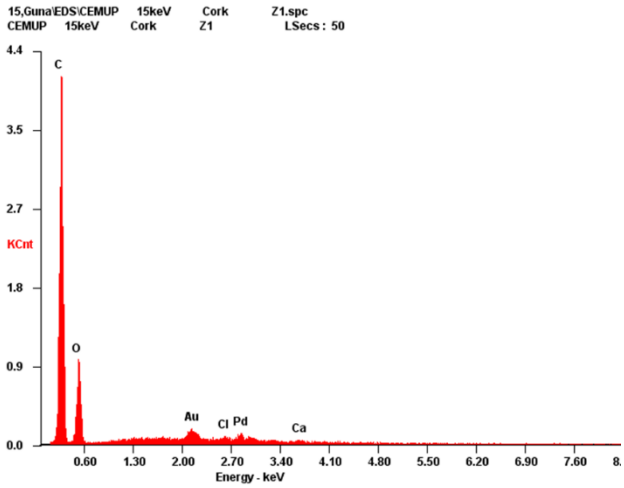
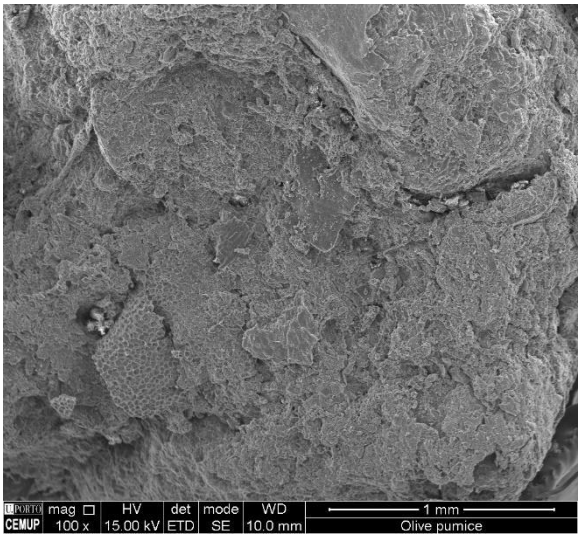


Figure A6. a) SEM image of cork b) EDS graph of cork

A1.3. Dried Olive Pomace Pellets

Figure A7a shows that although there were many irregularities in the surface morphological structure of olive pomace pellets, its elemental composition was homogenous throughout its surface.

a)



b)

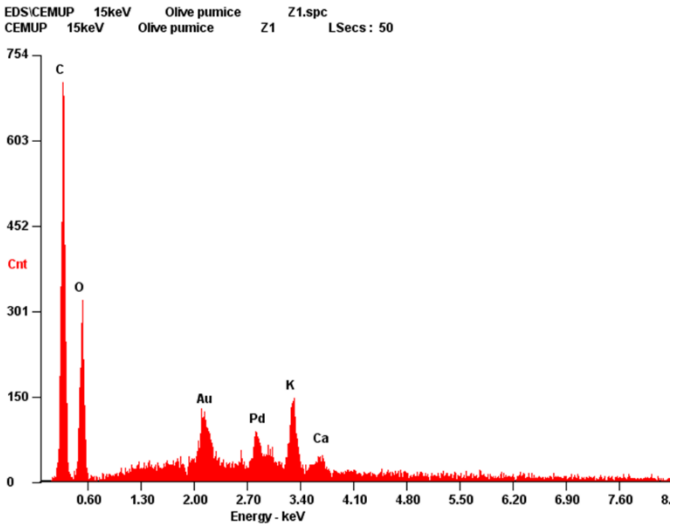
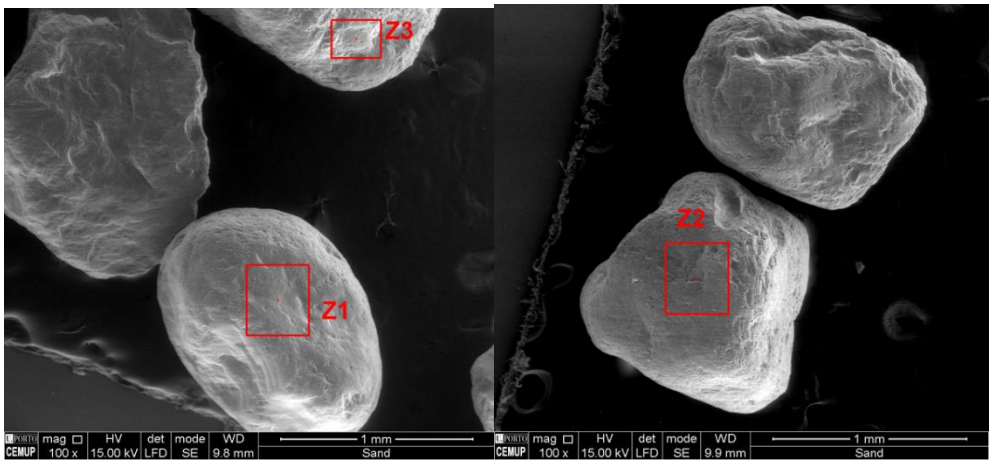


Figure A7. a) SEM image of OP b) EDS graph of OP

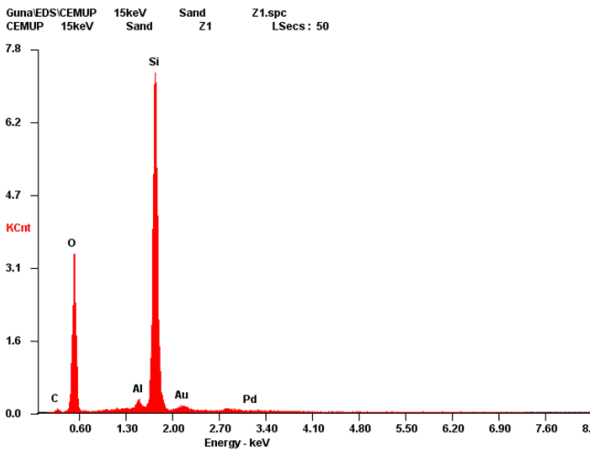
A1.4. Sand

Figure A8a shows that sand has an irregular surface with no porosity. Z1, Z2 and Z3 are filter sand particles that visually differed in colours. The elemental composition also differed accordingly (Figure A8b-A8d).

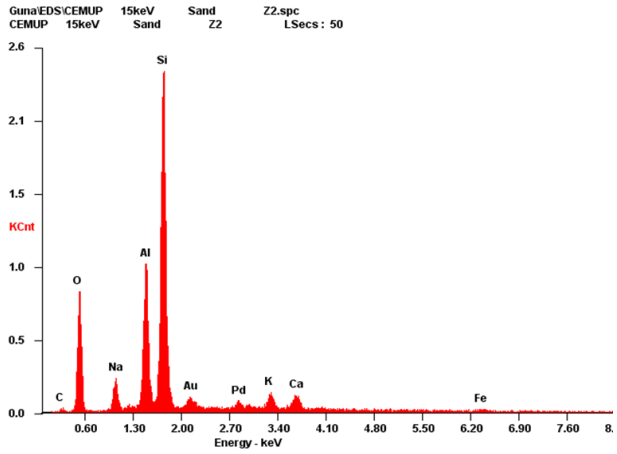
a)



b)



c)



d)

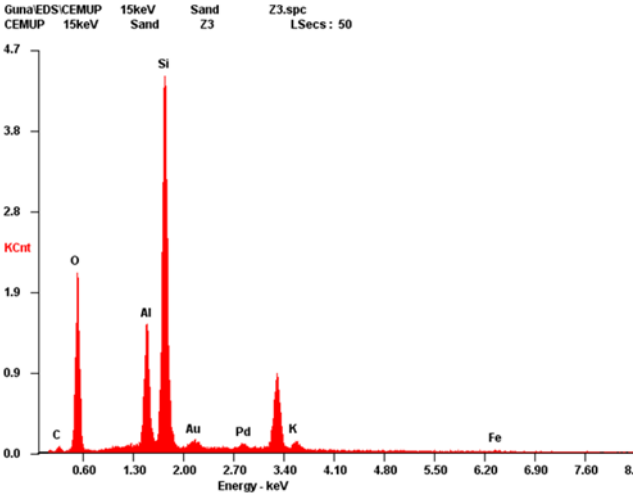
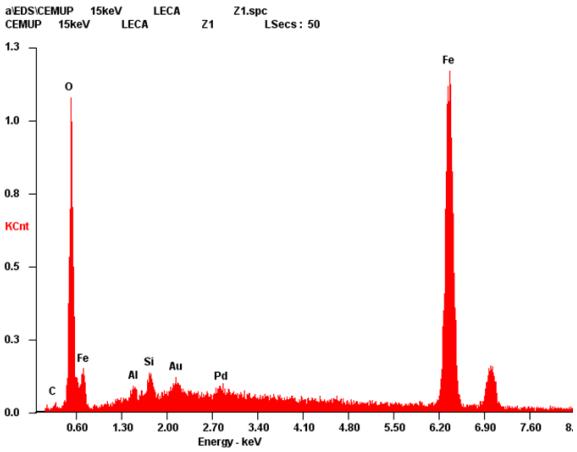


Figure A8.a) SEM images of sand; b) EDS graph of Z1; c) EDS graph of Z2; d) EDS graph of Z3

a)



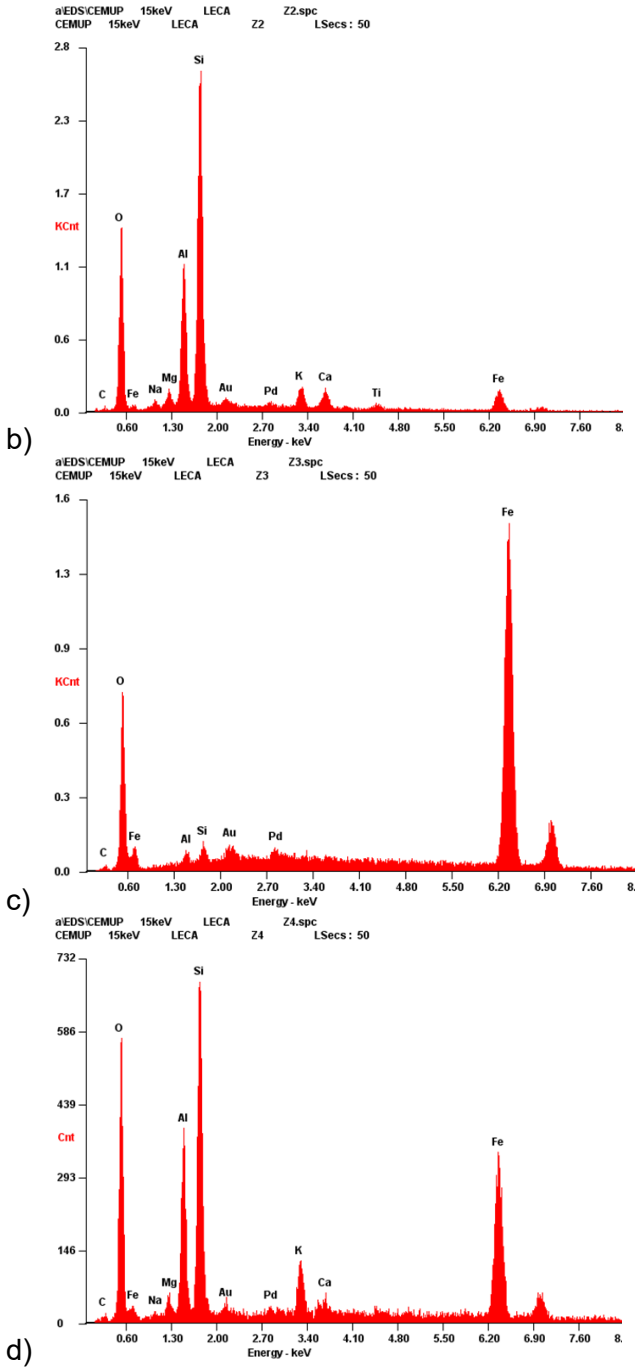


Figure A9. EDS graph of a) Z1; b) Z2; c) Z3; d) Z4 of LECA

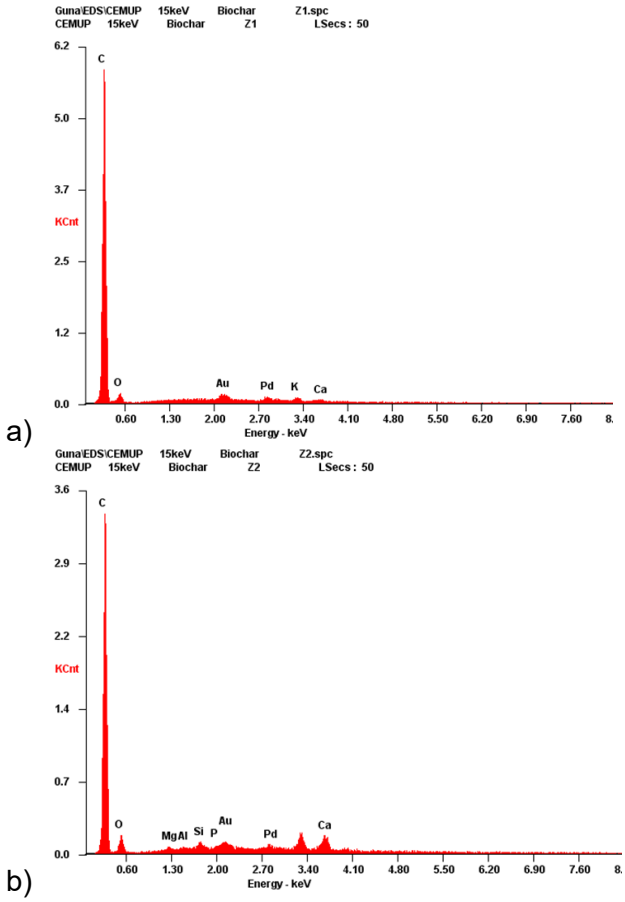


Figure A10. EDS graph of a) Z1; b) Z2 of biochar

Appendix B— Hybrid Vertical Subsurface Flow Treatment Wetlands for Cyanotoxin Remediation: Plant Composition, Microbial Dynamics, and Removal Efficiency

Table B1. Chemical oxygen demand (COD), nitrate ion and total phosphorus levels (mean and standard deviation, n=3) in the Spring Water used in the TW systems. Acc stands for acclimation period.

	COD (mg/L)	Nitrate ion (mg/L)	Total phosphorous (mg/L)
Acc. Week 1	3 (2)	14.0 (0.4)	0.1 (0.1)
Acc. Week 2	29 (9)	18 (2)	0.5 (0.1)
Week 1	37 (8)	16 (6)	0.2 (0.1)
Week 2	22 (4)	15 (3)	0.4 (0.1)
Week 3	18 (4)	16 (3)	0.1 (0.2)
Week 4	54 (6)	16 (1)	0.1 (0.2)
Week 5	71 (6)	9 (0.5)	0.1 (0.1)
Week 6	34 (6)	13 (2)	0.1 (0.0)
Week 7	17 (5)	15 (2)	0.1 (0.0)
Week 8	5 (4)	16 (1)	0.1 (0.0)
Week 9	5 (1)	15 (2)	0.0 (0.2)
Week 10	12 (5)	15.0 (0.9)	0.1 (0.0)

Table B2. Cyanotoxin concentrations in the inlet and outlet waters (mean and standard deviation, n=3) throughout the experimental weeks. LOD = 0.15 µg/ L for MC-LR and 0.03 µg/ L for CYN. TW-Sp indicates TW planted with *S.erectum*, TW-Ci indicates TW planted with *C.indica* and TW-CS indicates TW planted with *S.erectum* and *C.indica.*

	MC-LR				**CYN**			
	Inlet (µg/L)	Outlet (µg/L)			Inlet (µg/L)	Outlet (µg/L)		
		TW-Sp	TW-Ci	TW-CS		TW-Sp	TW-Ci	TW-CS
Week 1	2.0 (0.3)	<LOD	<LOD	<LOD	8.1 (1)	0.8 (0.1)	1.0 (0.2)	0.8 (0.3)
Week 2	3.7 (0.3)	<LOD	<LOD	<LOD	8.2 (0.8)	1.5 (0.1)	1.3 (0.3)	1.0 (0.1)
Week 3	2.6 (0.1)	<LOD	<LOD	<LOD	7 (0.2)	1.2 (0.1)	1.3 (0.3)	0.8 (0.1)
Week 4	1.7 (0.3)	<LOD	<LOD	<LOD	8.3 (1)	2.1 (0.2)	1.9 (0.4)	1.1 (0.3)
Week 5	9.6 (2)	<LOD	<LOD	<LOD	9 (1)	2.0 (0.3)	1.4 (0.2)	1.1 (0.4)
Week 6	8.8 (2)	<LOD	<LOD	<LOD	9.1 (1)	1.8 (0.1)	0.9 (0.3)	1.0 (0.5)
Week 7	8.3 (1)	<LOD	<LOD	<LOD	8.2 (1)	2.4 (0.2)	1.0 (1)	1.1 (1)
Week 8	7.6 (0.4)	<LOD	<LOD	<LOD	10 (4)	2.6 (0.1)	0.8 (0.4)	1.2 (1)
Week 9	6.7 (0.8)	<LOD	<LOD	<LOD	14.5 (1.7)	2.5 (0.7)	0.6 (0.3)	0.9 (0.4)
Week 10	9.5 (0.2)	<LOD	<LOD	<LOD	14.4 (0.2)	2.9 (0.4)	0.7 (0.3)	1.1 (0.7)

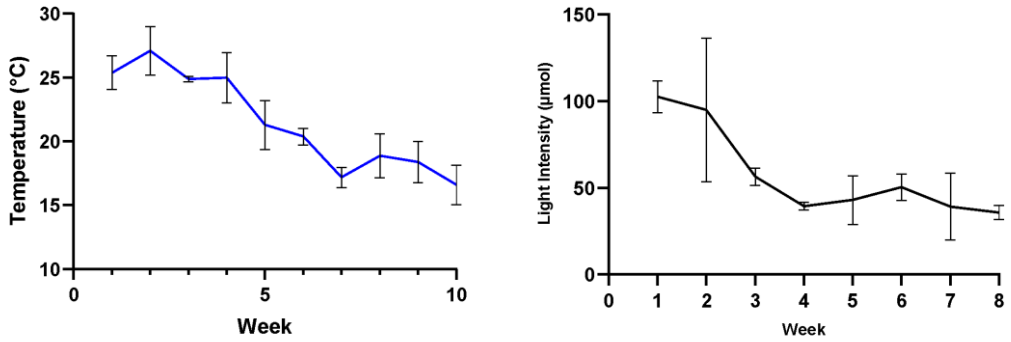


Figure B1. Temperature log and light intensities throughout the experiment. Error bars represent standard deviation; n=5 (for temperature) and n=10 for light intensities.

Table B3.Average inlets and average removal rates (standard deviation, n=3) of TW outlet water quality parameters. COD indicates chemical oxygen demand, TSS indicates total suspended solids, TW-Sp indicates TW planted with *S. erectum*, TW-Ci indicates TW planted with *C. indica* and TW-CS indicates TW planted with *S. erectum* and *C. indica.*

	Average Inlets	**Average removal rates %**		
Week	**COD (mg/L)**	**TW-Sp**	**TW-Ci**	**TW-CS**
1	103 (19)	68 (4)	66 (18)	60 (18)
2	90 (10)	57 (28)	38 (15)	32 (4)
3	59 (11)	33 (6)	53 (13)	3 (4)
4	133 (22)	44 (8)	35 (15)	29 (12)
5	189 (37)	94 (6)	96 (5)	95 (10)
6	103 (19)	83 (21)	71 (16)	87 (9)
7	57 (10)	88 (8)	74 (1)	82 (10)
8	26 (2)	12 (15)	41 (31)	56 (22)
9	10 (2)	-37 (20)	33 (20)	-75 (5)
10	58 (8)	42 (24)	71 (26)	59 (28)
Week	**Nitrate ion (mg/L)**	**Sp**	**Ci**	**CS**
1	47 (8)	98 (0.1)	97 (0.2)	98 (0.1)
2	42 (7)	98 (0.0)	99 (0.2)	98 (0.1)
3	56 (10)	99 (0.1)	88 (7)	96 (4)
4	40 (6)	91 (8)	92 (6)	96 (2)
5	22 (4)	96 (2)	95 (1)	94 (5)
6	30 (7)	97 (0.2)	96 (0.1)	97 (0.3)
7	33 (8)	98 (0.2)	96 (0.2)	95 (4)
8	27 (6)	97 (0.1)	96 (0.6)	96 (0.2)
9	30 (7)	97 (0.3)	96 (0.1)	96 (0.2)
10	38 (11)	98 (0.2)	96 (0.1)	97 (0.2)
Week	**Total Phosphorous (mg/L)**	**Sp**	**Ci**	**CS**
1	0.8 (0.1)	48 (22)	60 (5)	70 (9)

2	1.0 (0.2)	56 (17)	55 (6)	63 (14)
3	0.4 (0.1)	63 (20)	83 (10)	82 (15)
4	0.3 (0.0)	24 (40)	76 (12)	78 (17)
5	0.3 (0.0)	47 (23)	92 (4)	90 (15)
6	0.3 (0.0)	51 (31)	76 (13)	76 (8)
7	0.3 (0.1)	64 (29)	77 (7)	81 (9)
8	0.3 (0.0)	32 (35)	81 (8)	75 (21)
9	0.2 (0.0)	8 (8)	60 (23)	68 (27)
10	0.3 (0.1)	54 (19)	78 (7)	83 (9)
Week	**TSS (mg/L)**	**Sp**	**Ci**	**CS**
1	0.9 (0.2)	98 (3)	86 (25)	79 (37)
2	0.9 (0.2)	96 (6)	47 (5)	80 (21)
3	1.0 (0.3)	80 (34)	64 (6)	82 (18)
4	0.4 (0.1)	79 (30)	22 (28)	100 (0.2)
5	2.1 (0.1)	53 (47)	77 (5)	99 (2)
6	1.6 (0.1)	94 (8)	50 (5)	89 (14)
7	1.3 (0.0)	94 (4)	65 (3)	90 (17)
8	1.3 (0.1)	52 (21)	62 (12)	63 (8)
9	2.9 (0.4)	64 (33)	91 (2.0)	92 (7)
10	1.5 (0.1)	31 (38)	36 (35)	61 (18)
Week	**pH**	**Sp**	**Ci**	**CS**
1	6.9 (0.1)	7.0 (0.1)	7.0 (0.2)	6.8 (0.1)
2	6.1 (0.7)	7.0 (0.2)	6.7 (0.2)	6.7 (0.1)
3	6.5 (0.1)	7.1 (0.1)	6.8 (0.3)	6.8 (0.1)
4	6.5 (0.1)	7.0 (0.1)	6.9 (0.2)	6.8 (0.1)
5	6.5 (0.5)	7.2 (0.0)	6.9 (0.1)	6.8 (0.1)
6	6.6 (0.3)	7.2 (0.1)	6.9 (0.1)	6.9 (0.1)
7	6.5 (0.1)	7.3 (0.1)	7.1 (0.1)	7.0 (0.3)
8	6.1 (1.2)	7.2 (0.1)	6.9 (0.1)	7.0 (0.3)
9	6.7 (0.1)	7.4 (0.2)	7.1 (0.1)	7.1 (0.2)
10	6.4 (0.1)	7.2 (0.0)	6.9 (0.2)	6.9 (0.3)

Table B4. Cyanotoxin mass removal rates' two -way RM ANOVA table

MC-LR Mass Removal rates						
Parameter	**SS**	**DF**	**MS**	**F (DFn, DFd)**	**P value**	**Geisser-Greenhouse's epsilon**
Time x Plant Treatment	2.844	18	0.158	F (18, 54) = 4.000	P<0.0001	
Time	114	9	12.67	F (1.000, 6.000) = 359.4	P<0.0001	0.1111
Plant Treatment	3.173	2	1.587	F (2, 6) = 4.000	P=0.0787	

Subject	0.301	6	0.05017	F (6, 54) = 1.000	P=0.4350	
Residual	2.957	54	0.05476			
CYN Mass Removal rates						
Parameter	**SS**	**DF**	**MS**	**F (DFn, DFd)**	**P value**	**Geisser-Greenhouse's epsilon**
Time x Plant Treatment	890.1	18	49.45	F (18, 54) = 5.340	P<0.0001	
Time	1001	9	111.2	F (2.004, 12.02) = 11.28	P=0.0017	0.2227
Plant Treatment	635	2	317.5	F (2, 6) = 14.78	P=0.0048	
Subject	278.4	6	46.41	F (6, 54) = 5.113	P=0.0003	
Residual	554.4	54	10.27			

Table B5. TWs outlet water quality parameters' two-way RM ANOVA table

COD Removal rates						
Parameter	**SS**	**DF**	**MS**	**F (DFn, DFd)**	**P value**	**Geisser-Greenhouse's epsilon**
Time x Plant treatment	21355	18	1186	F (18, 54) = 1.368	P=0.1860	
Time	161525	9	17947	F (1.796, 10.78) = 20.70	P=0.0003	0.1995
Plant treatment	1280	2	639.9	F (2, 6) = 0.8176	P=0.4853	
Subject	4695	6	782.6	F (6, 54) = 0.9025	P=0.5000	
Residual	46823	54	867.1			
Nitrate ion Removal rates						
Parameter	**SS**	**DF**	**MS**	**F (DFn, DFd)**	**P value**	**Geisser-Greenhouse's epsilon**
Time x Plant treatment	704	18	39.11	F (18,54) = 1.184	P=0.3064	
Time	438.5	9	48.72	F (1.418, 8.506) = 1.475	P=0.2717	0.1575
Plant treatment	177.1	2	88.53	F (2, 6) = 1.313	P=0.3366	
Subject	404.7	6	67.45	F (6, 54) = 2.042	P=0.0757	

Residual	1784	54	33.03			
Total Phosphorous Removal rates						
Parameter	**SS**	**DF**	**MS**	**F (DFn, DFd)**	**P value**	**Geisser-Greenhouse's epsilon**
Time x Plant treatment	0.03913	18	0.002174	F (18, 54) = 1.038	P=0.4356	
Time	1.09	9	0.1211	F (1.625, 9.750) = 57.85	P<0.0001	0.1806
Plant treatment	0.3158	2	0.1579	F (2, 6) = 5.324	P=0.0468	
Subject	0.178	6	0.02966	F (6, 54) = 14.17	P<0.0001	
Residual	0.113	54	0.002094			
TSS Removal rates						
Parameter	**SS**	**DF**	**MS**	**F (DFn, DFd)**	**P value**	**Geisser-Greenhouse's epsilon**
Time x Plant treatment	24930	18	1385	F (18, 54) = 1.701	P=0.0681	
Time	17490	9	1943	F (3.443, 20.66) = 2.387	P=0.0916	0.3825
Plant treatment	13509	2	6754	F (2, 6) = 5.788	P=0.0398	
Subject	7002	6	1167	F (6, 54) = 1.433	P=0.2191	
Residual	43966	54	814.2			
pH						
Parameter	**SS**	**DF**	**MS**	**F (DFn, DFd)**	**P value**	**Geisser-Greenhouse's epsilon**
Time x Plant treatment	4.653	27	0.1723	F (27, 72) = 1.538	P=0.0765	
Time	2.475	9	0.2751	F (1.745, 13.96) = 2.454	P=0.1267	0.1939
Plant treatment	9.873	3	3.291	F (3, 8) = 17.79	P=0.0007	
Subject	1.48	8	0.1849	F (8, 72) = 1.650	P=0.1259	
Residual	8.07	72	0.1121			
ORP						
Parameter	**SS**	**DF**	**MS**	**F (DFn, DFd)**	**P value**	**Geisser-Greenhouse's epsilon**
Time x Plant treatment	16682	16	1043	F (16, 48) = 1.573	P=0.1136	

Time	71484	8	8936	F (3.256, 19.54) = 13.48	P<0.0001	0.407
Plant treatment	209	2	104.5	F (2, 6) = 0.02807	P=0.9724	
Subject	22336	6	3723	F (6, 48) = 5.617	P=0.0002	
Residual	31812	48	662.8			

Table B6. Cyanotoxin concentrations(ng/g) (std deviation, n=3) in the substrates. Sp indicates TW with *S.erectum*, Ci indicates TW with *C.indica*, and CS indicates TW with both *S.erectum* and *C.indica*.

ng/g	**MC-LR**	**CYN**
	Sand	
Sp	1.31 ($3.4E^{-05}$)	2.57 ($1.2E^{-03}$)
Ci	0.39 ($1.7E^{-05}$)	0.33 ($1.4E^{-05}$)
CS	0.42 ($4.1E^{-06}$)	0.27 ($1.4E^{-04}$)
	Rhizo-substrate	
Sp	0.34 ($2.6E^{-04}$)	3.8 ($2.7E^{-03}$)
Ci	0.19 ($1E^{-06}$)	0.65 ($5.2E^{-04}$)
CS	0.20 ($7E^{-07}$)	1.1 ($4.3E^{-04}$)

a)

b)

c)

d)

e)

f)

g)

h)

i)

Figure B2. Pictures of TW systems at Weeks 1, 5, 8, and 10. a) and b) shows healthy looking plants in week 1; c), d), and e) show plants in week 5 where *S.erectum* displays droopy leaves and signs of blight; f) and g) show plants in week 8 with further signs of blight in *S.erectum*; h) and i) show plants in week 10 with *S.erectum* looking further diseased.

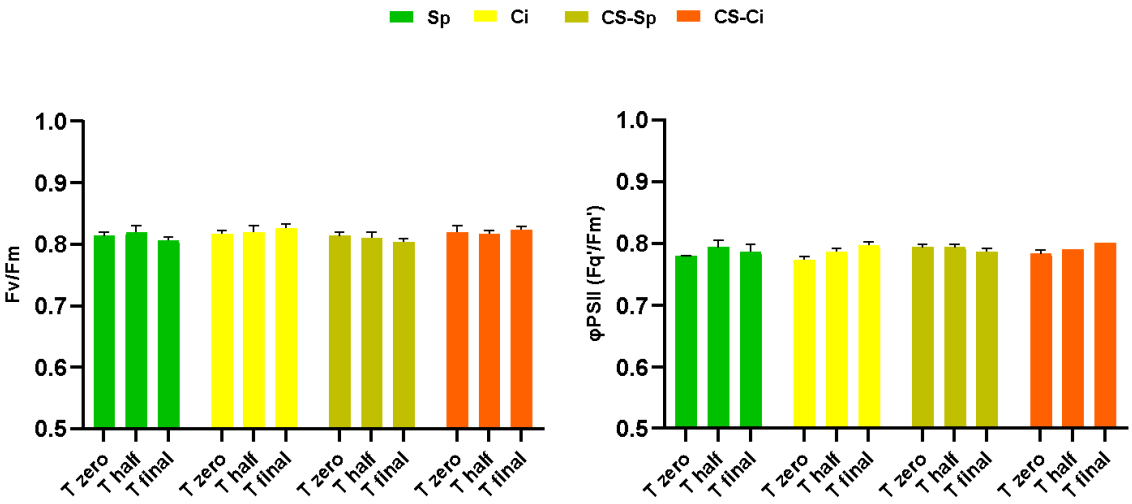


Figure B3. Maximum quantum yield (Fv/Fm) and effective quantum yield (ΦPSII) of PSII of plant leaves. T-zero indicates before cyanotoxin exposure, T-half indicates week 5 (middle) of the experiment and T-final indicates week 10 (end) of the experiment. Error bars represent standard deviation (n=6). Sp indicates TW with *S.erectum*, Ci indicates TW with *C.indica*, CS-Sp indicates *S.erectum* from the polyculture TW, and CS-Ci indicates *C.indica* from the polyculture TW.

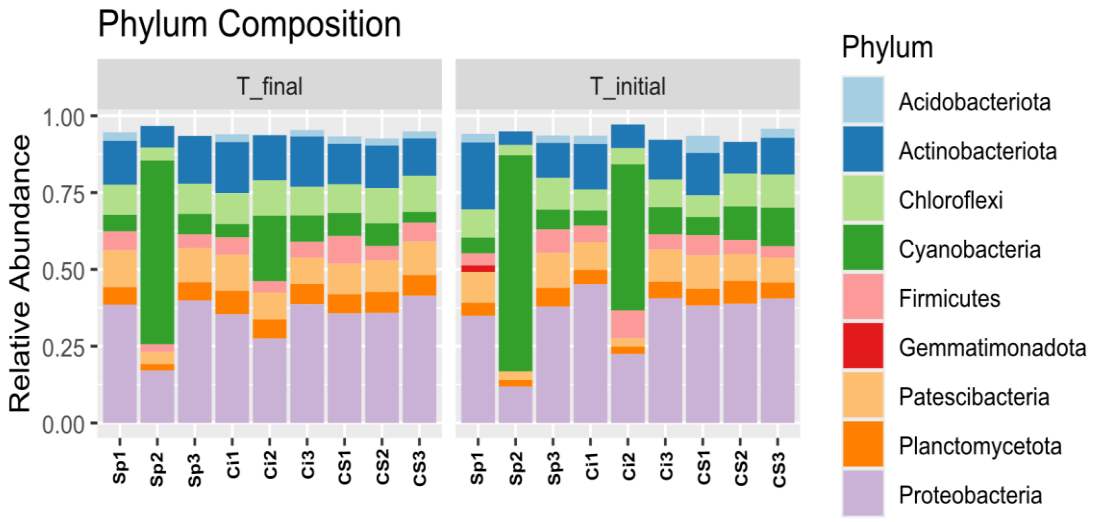


Figure B4. Relatively abundant phyla in sand samples of TW systems at the end of the experiment (T_final) and the beginning of the experiment (T_initial) (>2 % threshold). Sp indicates TW with *S.erectum*, Ci indicates TW with *C.indica*, and CS indicates TW with both *S.erectum* and *C.indica*.

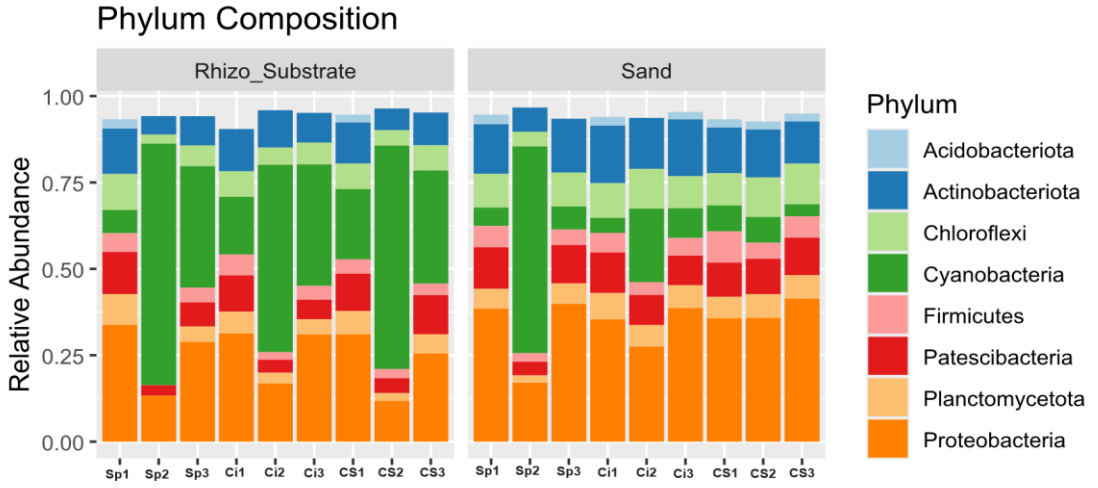


Figure B5. Relatively abundant phyla in rhizo-substrate and sand of TW systems at Tf (>2% threshold). Sp indicates TW with *S.erectum*, Ci indicates TW with *C.indica*, and CS indicates TW with both *S.erectum* and *C.indica*.

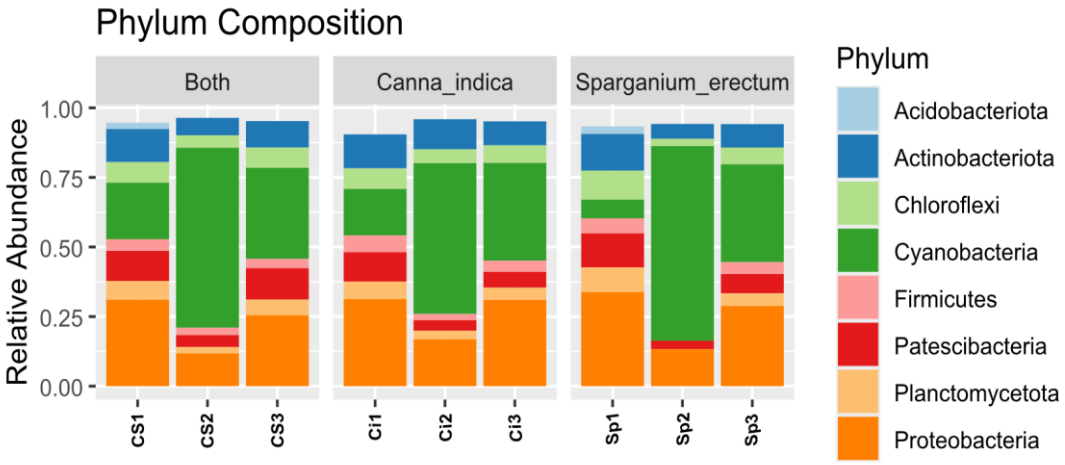


Figure B6. Relatively abundant phyla in rhizo-substrate of TW systems at Tf (>2% threshold). Sp indicates TW with *S.erectum*, Ci indicates TW with *C.indica*, and CS indicates TW with both *S.erectum* and *C.indica*.

Table B7. PERMANOVA tests using the dataset after rarefication of the ASVs and filtering, denoising, merging, and non-chimeric sequence identification of all samples

PERMANOVA Tests using Bray distances (999 permutations); for all samples						
	Df	**Sum Of Sqs**	**R^2**	**F**	**Pr (>F)**	
Model (Plant)	**2**	**0.602**	**0.076**	**0.9845**	**0.422**	
Residual	24	7.336	0.924			
Total	26	7.938	1.000			
Model (Matrix/Substrate)	**1**	**0.991**	**0.125**	**3.5681**	**0.001**	***
Residual	25	6.946	0.875			
Total	26	7.938	1.000			
Model (Time)	**1**	**0.847**	**0.107**	**2.9847**	**0.003**	**
Residual	25	7.091	0.893			
Total	26	7.938	1.000			

Table B8. PERMANOVA tests using the dataset after rarefication of the ASVs and filtering, denoising, merging, and non-chimeric sequence identification of samples at Tf

PERMANOVA Tests using Bray distances (999 permutations); for samples at Tf (after cyanotoxin exposure)						
	Df	**Sum Of Sqs**	**R^2**	**F**	**Pr (>F)**	
Model (Matrix)	1	0.6305	0.13579	2.5139	0.002	**
Residual	16	4.0128	0.86421			
Total	17	4.6433	1			
	Df	**Sum Of Sqs**	**R^2**	**F**	**Pr (>F)**	
Model (Plant)	2	0.6355	0.13687	1.1893	0.169	
Residual	15	4.0078	0.86313			
Total	17	4.6433	1			

Table B9. PERMANOVA tests using the dataset after rarefication of the ASVs and filtering, denoising, merging, and non-chimeric sequence identification of rhizo-substrate samples

PERMANOVA Tests using Bray distances (999 permutations) ; for rhizo-substrate samples						
	Df	**SumOfSqs**	**R^2**	**F**	**Pr(>F)**	
Model (Plant)	2	0.80489	0.41428	2.1219	0.004	**
Residual	6	1.13795	0.58572			
Total	8	1.94284	1			

Table B10. PERMANOVA tests using the dataset after rarefication of the ASVs and filtering, denoising, merging, and non-chimeric sequence identification of sand samples

PERMANOVA Tests using Bray distances (999 permutations); for sand samples						
	Df	SumOfSqs	R^2	F	Pr(>F)	
Model (Time)	1	0.4839	0.09287	1.638	0.047	*
Residual	16	4.7269	0.90713			
Total	17	5.2109	1			
	Df	SumOfSqs	R^2	F	Pr(>F)	
Model (Plant)	2	0.7412	0.14223	1.2436	0.133	
Residual	15	4.4697	0.85777			
Total	17	5.2109	1			

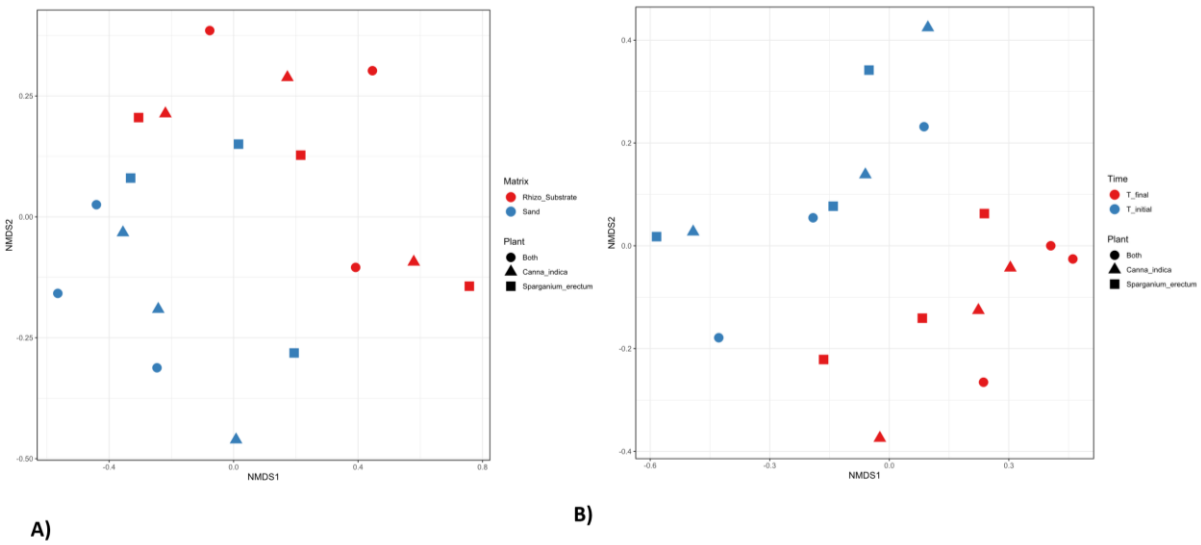


Figure B7. NMDS analysis using Bray-Curtis distances of A) samples at Tf grouping plants and matrices, B) sand samples grouping different plant compositions and time (T0 and Tf)

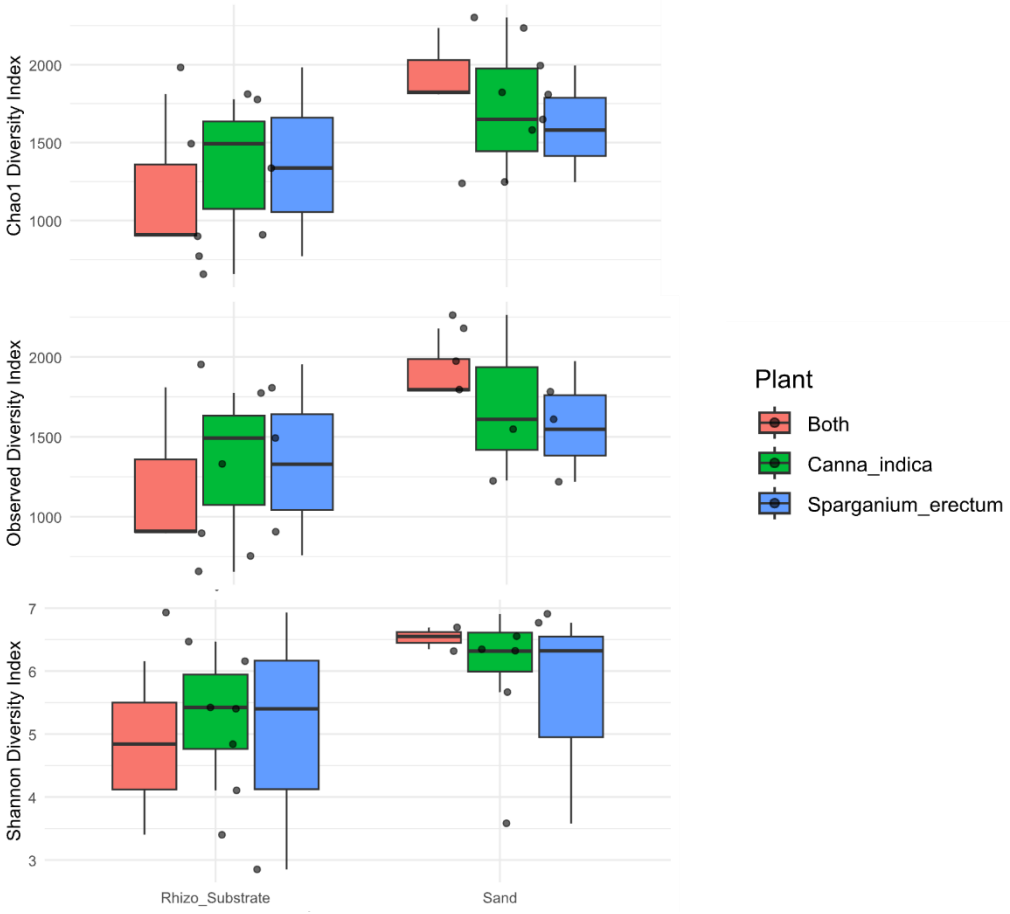


Figure B8. Alpha diversity indices (Chao1, Observed and Shannon) of samples at Tf, grouped with different TW plant compositions and substrate matrices.

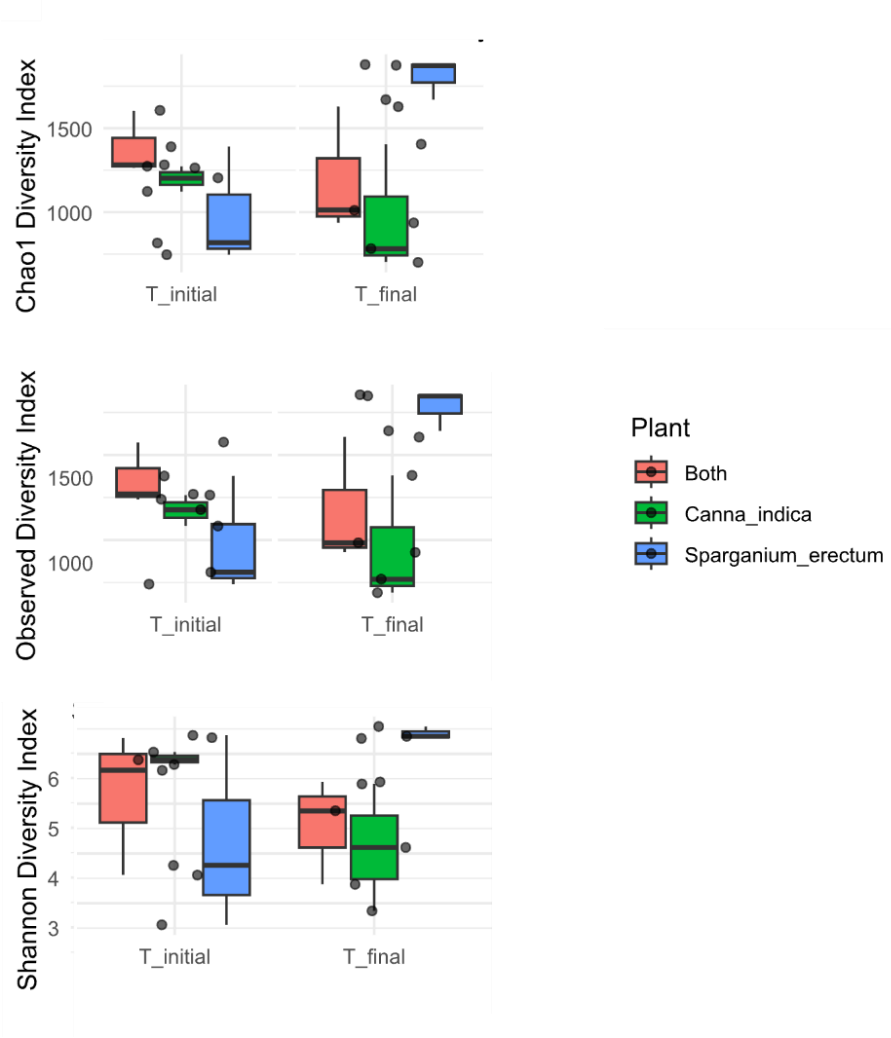


Figure B9. Alpha diversity indices (Chao1, Observed and Shannon) of only sand samples, grouped with different TW plant compositions and time; T_initial indicates T0 and T_final indicates Tf.

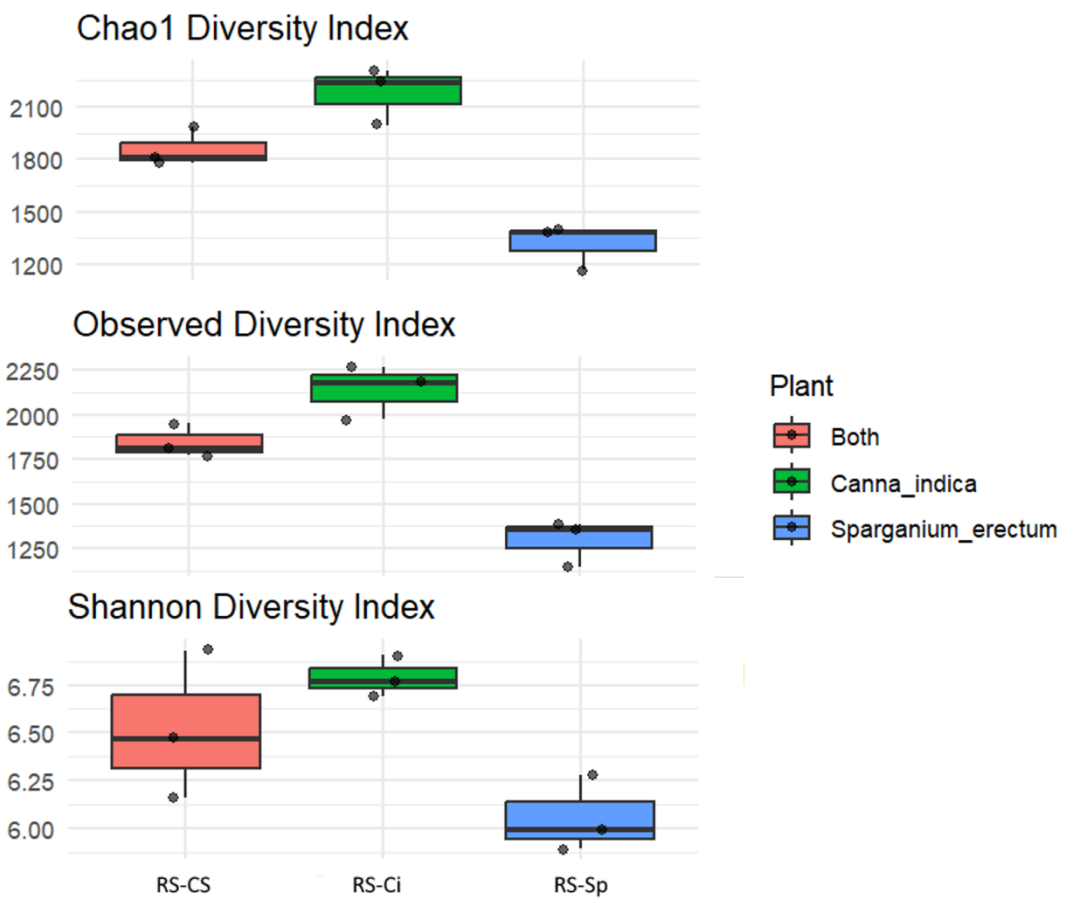


Figure B10. Alpha diversity indices (Chao1, Observed and Shannon) of RS (rhizo-substrate) samples, grouped with plants.

Appendix C. General Discussion and Future Perspectives

S1. Area Calculations for Scale-up

Area requirement calculations and area-based rate constants (k_a) were calculated using the P-k-C* model (Kadlec & Wallace, 2008), modified for background concentration (C*) equal to zero. Firstly, the k_a was calculated (equation S1) using the experimental conditions. Then the area for treating 1 m^3/day of water with an initial MC-LR and CYN concentration of 10 µg/L and different removal scenarios was calculated using equation S2, assuming the number of tanks in series (NTIS) as equal to 2, as the TW configuration is of VF with a more heterogeneous set-up of more than one, non-uniform substrate composition.

$$ka = -q\ x\ ln\ (Ce - Ci) \quad \text{...(Eq. S1)}$$

$$A = \frac{PQ\left[\left(\frac{Ci}{Ce}\right)^{\frac{1}{P}} - 1\right]}{ka} \quad \text{...(Eq. S2)}$$

Here, Ce= Cyanotoxin effluent concentration (µg/L); Ci: Cyanotoxin influent concentration (µg /L); ka: Area-based rate constant; P: Apparent number of tanks in series (NTIS= 2); q: HLR (m/year); Q: flow (m^3/year); A = area (m^2)

Table S1. Area-based first order rate constants results and area requirements for treating 1m^3 /day flow, initial concentration of 10 µg/L of MC-LR and CYN, calculated for desired effluent concentrations according to WHO recreational and drinking water GVs (Area $_{(rec)}$ and Area $_{(drinking)}$). The data shown here are of the TWs with different plant compositions

		k_a	Area $_{(rec)}$[1] (m^2)	Area $_{(drinking)}$[2] (m^2)
TW-Sp	*MC-LR*	0.98	0.67	4.41
	CYN	1.08	1.53	5.16
TW-Ci	*MC-LR*	0.98	0.67	4.41
	CYN	1.17	1.41	4.73
TW-CS	*MC-LR*	0.98	0.67	4.40
	CYN	1.17	1.41	4.73

[1] Recreational waters GV= 5.67 µg/L
[2] Drinking waters GV= 1 µg/L