Constructed wetlands for removal of metals from contaminated effluents

Studying the role of plants, microorganisms, and substrate

Pau Porras i Socias

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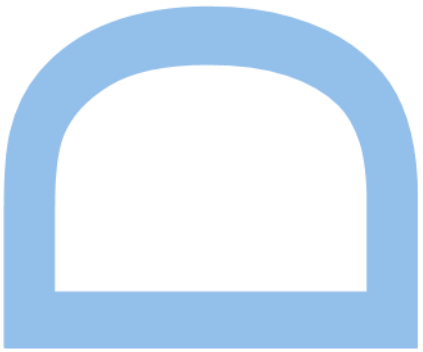

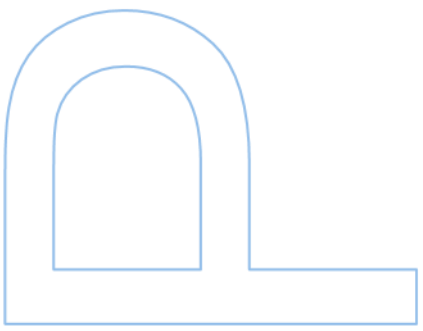

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The Supervisor,

Porto, 16 / 10 / 2024

Dedicated to Júlia, my family and friends

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Resumo

A digestão anaeróbia está a expandir globalmente para gerir a elevada geração de resíduos orgânicos e responder às crescentes demandas energéticas. No entanto, persistem desafios na valorização do digerido devido a constrangimentos regulatórios e potenciais riscos de poluição ambiental. A reutilização de água usada é imperativa para uma gestão eficaz dos recursos hídricos e para fazer face à crescente pressão sobre a água. A implementação de soluções baseadas na natureza, como as fito-ETAR, para o tratamento de águas residuais industriais para posterior reuso, pode promover uma bioeconomia circular. Esta tese explora a integração das fito-ETAR como um passo de polimento no tratamento da fração líquida do digerido para uma potencial reutilização na irrigação. O foco está na remoção de metais, antibióticos e genes de resistência a antibióticos, e na produção de biomassa para recuperação de energia. Foi testada uma configuração inovadora usando *Sparganium erectum* para fitorremediação com quatro composições líquidas de digestato (adulteradas com oxitetraciclina, sulfadiazina, ou ofloxacina, ou sem antibióticos). Sistemas à escala laboratorial demonstraram elevadas eficiências de remoção de matéria orgânica, iões de amónio e fosfato, metais, antibióticos, genes de resistência a antibióticos e potenciais patógenos, mostrando o seu potencial no tratamento da fração líquida do digestato. Apesar da dosagem de antibióticos, nas concentrações testadas, não foi observado efeito nas eficiências de remoção. No entanto, após três meses de dosagem de antibióticos, as comunidades microbianas do substrato do leito radicular e da endosfera das raízes mostraram diferenças significativas em comparação com o controlo, especialmente nos sistemas que tratavam sulfadiazina e ofloxacina. Além disso, foram investigadas estratégias para melhorar a produção de metano a partir da biomassa de *S. erectum* das fito-ETAR, através de três métodos de pré-tratamento, armazenamento, amónia aquosa e tratamentos hidrotérmicos. Cada pré-tratamento resultou em alterações distintas na morfologia da biomassa e nas propriedades físico-químicas, com efeitos mais notórios na amónia aquosa e nos tratamentos hidrotérmicos. Toda a biomassa pré-tratada demonstrou melhorias nas produções de metano, com a carbonização hidrotermal a 200°C durante 60 min a mostrar o aumento mais substancial. No entanto, enquanto o armazenamento a seco surge como a opção mais promissora, a implementação da amónia aquosa e dos tratamentos hidrotérmicos requerem mais otimização para a viabilidade económica. No geral, o acoplamento de fito-ETAR com digestão anaeróbia contribui para o fecho do ciclo de vida dos produtos de digestão anaeróbia e promove

os princípios da economia circular, facilitando a sinergia entre a utilização de recursos e a proteção ambiental. Por fim, a tese propõe ainda duas atividades STEM de divulgação educativa para motivar os alunos e promover uma aprendizagem significativa, envolvendo-os com as disciplinas ambientais, contribuindo para expandir a economia circular para além da Academia.

Palavras-chave: Digestão anaeróbia, fito-ETAR, digerido, metais, antibióticos, economia circular, *Sparganium erectum*, comunidades microbianas, pré-tratamento, valorização de biomassa, atividades de divulgação, STEM.

Abstract

Anaerobic digestion biotechnologies are globally expanding to manage the high organic waste generation and address rising energy demands. However, challenges remain in valorising digestate due to regulatory constraints and potential environmental pollution risks. Reclaimed water reuse is imperative for effective water resource management to address the increasing pressure on freshwater. The implementation of nature-based solutions, such as constructed wetlands, for the treatment of industrial wastewater for subsequent irrigation purposes can promote a circular bioeconomy. This thesis explores integrating constructed wetlands as a post-treatment of anaerobic digestion to reuse the liquid fraction of the digestate for irrigation. The focus is on removing metals, antibiotics, and antibiotic resistance genes, and producing biomass for energy recovery. A novel configuration using *Sparganium erectum* for phytoremediation was tested with four liquid digestate compositions (spiked with oxytetracycline, sulfadiazine, or ofloxacin, or without antibiotic spiking). Lab-scale systems demonstrated high removal efficiencies of organic matter, ammonium and phosphate ions, metals, antibiotics, antibiotic resistance genes, and potential pathogens, showing its potential in liquid fraction of the digestate treatment. Despite the antibiotics dosing, at the tested concentrations, no effect on the removal efficiencies was observed. However, after three months of antibiotic dosing, the microbial communities of the rood bed substrate, and the endosphere of the roots, showed significant differences as compared with the control, especially systems treating sulfadiazine and ofloxacin. Additionally, strategies to enhance the methane production from *S. erectum* biomass, harvested from constructed wetlands systems, were investigated through three pretreatment methods, storage, aqueous ammonia soaking, and hydrothermal treatments. Each pretreatment altered biomass properties, with aqueous ammonia soaking and hydrothermal treatments having notorious effects in biomass morphology and physicochemical properties. All pretreated biomass improved methane yields, with hydrothermal carbonisation at 200°C for 60 minutes showing the most substantial increase. Moreover, dry storage emerged as the most feasible option, while aqueous ammonia soaking and hydrothermal treatments require further optimisation for economic feasibility. Overall, coupling constructed wetlands with anaerobic digestion contributes to closing the loop of AD products' lifecycles, and promotes circular economy principles, facilitating synergy between resource utilisation and environmental conservation. Finally, the thesis also proposes two educational outreach STEM activities to motivate students and promote meaningful learning,

engaging them with environmental disciplines, contributing to expand circular economy beyond Academia.

Keywords: Anaerobic digestion, constructed wetlands, digestate, metals, antibiotics, circular economy, *Sparganium erectum*, microbial communities, pretreatment, biomass valorisation, dissemination activities, STEM.

Resum

La digestió anaeròbia està expandint-se globalment per gestionar la elevada generació de residus orgànics i fer front a la creixent demanda energètica. No obstant això, continuen existint desafiaments en la valorització del digestat a causa de les restriccions normatives i els possibles riscos de contaminació ambiental. La reutilització de l'aigua és imprescindible per a una gestió efectiva dels recursos hídrics davant la creixent pressió sobre l'aigua dolça. La implementació de solucions basades en la natura, com els aiguamolls construïts, per al tractament d'aigües residuals industrials per a la seva posterior utilització en reg pot promoure una bioeconomia circular. Aquesta tesi explora la integració de aiguamolls construïts com a tractament posterior de la digestió anaeròbia per reutilitzar la fracció líquida del digestat per a reg. L'atenció se centra en l'eliminació de metalls, antibiòtics i gens de resistència a antibiòtics, i en la producció de biomassa per a la recuperació d'energia. Es va provar una configuració innovadora utilitzant *Sparganium erectum* per a la fitorremediació amb quatre composicions líquides de digestat (amb oxitetraciclina, sulfadiazina, ofloxacina, o sense antibiòtics). Els sistemes a escala de laboratori van demostrar altes eficiències en l'eliminació de matèria orgànica, ions d'amoni i fosfat, metalls, antibiòtics, gens de resistència a antibiòtics i patògens potencials, mostrant el seu potencial en el tractament de la fracció líquida del digestat. Tot i la dosificació d'antibiòtics, en les concentracions provades, no es va observar cap efecte en les eficiències d'eliminació. No obstant això, després de tres mesos de dosificació d'antibiòtics, les comunitats microbianes del substrat del llit radicular i de l'endosfera de les arrels van mostrar diferències significatives en comparació amb el control, especialment en els sistemes que tractaven sulfadiazina i ofloxacina. A més, es van investigar estratègies per millorar la producció de metà a partir de la biomassa de *S. erectum*, recol·lectada dels sistemes de aiguamolls construïts artificials, mitjançant tres mètodes de pretractament: emmagatzematge, remull en amoníac aquós i tractaments hidrotermals. Cada pretractament va alterar les propietats de la biomassa, amb remull en amoníac aquós i tractaments hidrotermals tenint efectes notoris en la morfologia i les propietats fisicoquímiques de la biomassa. Tota la biomassa pretractada va millorar els rendiments de metà, amb la carbonització hidrotermal a 200 °C durant 60 minuts mostrant l'augment més substancial. A més, l'emmagatzematge en sec va resultar ser l'opció més viable, mentre que el remull en amoníac aquós i els tractaments hidrotermals requereixen una major optimització per a la viabilitat econòmica. En general, combinar aiguamolls construïts i digestió anaeròbia contribueix a tancar el cicle de vida dels productes de la digestió anaeròbia i promou els principis

de l'economia circular, facilitant la sinergia entre l'ús de recursos i la conservació ambiental. Finalment, la tesi també proposa dues activitats de divulgació educativa STEM per motivar els estudiants promovent aprenentatges significatius, despertant inquietuds en disciplines ambientals. Així doncs, hi ha una contribució a expandir l'economia circular més enllà de l'Acadèmia.

Mots claus: Digestió anaeròbica, aiguamolls construïts, digestat, metalls, antibiòtics, economia circular, *Sparganium erectum*, comunitats microbianes, pretractaments, valorització de biomassa, activitats divulgatives, STEM.

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List of Abbreviations

Anammox:	Anaerobic ammonia oxidation
AAS:	Aqueous ammonia soaking pretreatment at room temperature for 4 days
AAST:	Aqueous ammonia soaking pretreatment at 51 °C for 27 h
AD:	Anaerobic digestion
ADF:	Acid detergent fiber
ADL:	Acid detergent lignin
ANOSIM:	Analysis of similarity
ANOVA:	Analysis of variance
ARGs:	Antibiotic resistance genes
ASV:	Amplicon sequence variant
BMP:	Biochemical methane potential
C:	Control constructed wetlands systems treating the liquid fraction of the digestate
COD:	Chemical oxygen demand
Ct:	Control reactor with water, bicarbonate, and inoculum
CWs:	Constructed wetlands
DM:	Dry matter
DS:	Dry storage pretreatment
EC:	Electrical conductivity
EPA:	Environmental Portuguese Agency
EU:	European Union
FB:	Untreated fresh biomass
HR-AD:	Heterotrophic nitrification and aerobic denitrification
HRT:	Hydraulic retention time
HT:	Hydrothermal pretreatment at 120 °C for 120 min
HTC:	Hydrothermal carbonisation pretreatment at 200 °C for 60 min
HTC-L:	Liquid or centrate phase of hydrothermal carbonisation pretreatment at 200 °C for 60 min
LECA:	Light-expanded clay aggregates
LFD:	Liquid fraction of the digestate
LOD:	Limit of detection
MGES:	Mobile genetic elements
MSW:	Municipal solid waste

NBS: Nature-based solutions
OF: Constructed wetlands systems treating the liquid fraction of the digestate spiked with ofloxacin
OX: Systems treating the liquid fraction of the digestate spiked with oxytetracycline
PERMANOVA: Permutational multivariate analysis of variance
PGPR: Plant growth-promoting rhizobacteria
SD: Systems treating the liquid fraction of the digestate spiked with sulfadiazine
SDG: Sustainable development goals
SPE: Solid phase extraction
TKN: Total kjeldahl nitrogen
TN: Total nitrogen
TP: Total phosphorus
TS: Total solids
VFA: Volatile fatty acids
VS: Volatile solids
WHO: World Health Organisation
WS: Wet storage pretreatment

Chapter 1. Introduction

1. Background

1.1. Towards sustainable development and circular economy

The past decade has been marked by a multitude of crises presenting serious challenges including climate change, dwindling natural resources, conflict, ongoing effects of the COVID-19 pandemic, and both global economies and population development requiring increased food, energy, and other resources. Global leaders met in the United Nations Sustainable Development Summit in 2015 and declared that “the future of humanity and our planet lies in our hands” (United Nations, 2023). However, we are nowadays at the midpoint of Sustainable Development Goals (SDG) and an assessment of their progress revealed that 37 % are in stagnation or regression and 48 % are moderately or severely off track (United Nations, 2023). Consequently, to address these global challenges effectively, we require comprehensive, cross-sectoral actions involving governments, policymakers, corporations, local associations, educational institutions, individuals, and other stakeholders. Circular economy, which embodies a regenerative economic system seeking to replace the “end of life” concept with reducing, alternatively reusing, recycling, and recovering materials across the supply chain, merits increased attention as it offers solutions to these challenges (Kirchherr et al., 2023). Circular economy aims to uphold value maintenance and sustainable development, thereby enhancing environmental quality, promoting economic development, and ensuring social equity for present and future generations. It is enabled by an alliance of stakeholders and their technological innovations and capabilities (Kirchherr et al., 2023).

In 2023, the global production of municipal solid waste (MSW) reached 2.3 billion tons, with approximately 38% accounting for uncontrolled waste, which continues to be dumped in terrestrial and aquatic environments (United Nations Environment Programme, 2024). This unregulated disposal contributes to many adverse health and environmental effects (Maalouf et al., 2020), besides an estimated MSW management costs up to 252.3 billion US$ in 2020 (United Nations Environment Programme, 2024). To promote sustainability in MSW management, the European Union (EU) approved the Waste Framework Directive 2008/98/EC aiming to prevent waste generation with the “end of waste” criteria, promoting recycling and source separation, recovering resources, and minimising the landfill disposal of biodegradable waste (European Commission,

2023). A common practice for the sustainable management of MSW involves waste-to-energy processes, which harness heat and electricity from waste. These methods include thermal technologies like incineration, gasification, and pyrolysis, as well as physical methods such as refused-derived fuel, and biochemical conversion technologies, namely anaerobic digestion (AD), microbial fuel cells, or landfill gas capture (Kaur et al., 2021).

1.2. Harnessing anaerobic digestion

Among all the existing options for converting organic waste into valuable resources, a well-established and effective technology for recovering energy and nutrients is anaerobic digestion (AD), producing biogas, a renewable energy source, and digestate (Wainaina et al., 2020). Several upgrading methodologies such as co-digestion, for example, involving the biodegradation of diverse wastes in a reactor, provide economic benefits by enhancing biogas yield and treating various substrates in a single facility (Abad et al., 2019). AD potentially contributes to fulfilling all 17 SDGs directly or indirectly addressing not only the environmental-based SDGs (7, 6, 13, 14, and 15) by reducing fossil fuel reliance and decreasing water bodies, soil and air pollution, but also contributing to the economic (1, 7, 8, 9, and 12) through the development of green industries, local businesses, jobs in waste management and fertiliser trading, and social-based ones (1, 2, 3, 4 5, 10, 11, 12, 16, and 17) such as poverty reduction through affordable fertilizer production and alleviating education and gender inequalities by providing reliable energy, and ultimately addressing international conflicts caused by energy and environmental crises (Piadeh et al., 2024). Therefore, AD stands out as an established technology tackling many global sustainability challenges present across the world.

In the EU, around 180 million tons of digestate are produced each year, with around 28% being after the biomethanisation of organic fraction of mixed MSW and of sewage sludge (Kovačić et al., 2022). The digestate is usually separated into clarified or “liquid” and concentrated or “solid” fractions, with the former (comprising 80-90 % of the total mass of raw digestate) being rich in N and K, whereas the latter has high C and P concentrations (Drosg et al., 2015). The separation in two fractions enables more efficient management, simplifying subsequent processing, storage, and transport (Sfetsas et al., 2022). The solid fraction, partially stabilised and no longer undergoing rapid decomposition, is usually applied directly in agricultural land as a biofertilizer or soil conditioner, while a common practice for the liquid fraction of the digestate (LFD) is to

be sent to a wastewater treatment plant (Kovačić et al., 2022). This widely used approach to digestate management results in underestimating the digestate's potential value.

Resource recovery has emerged as a crucial factor contributing to sustainable development and circular economy (Wainaina et al., 2020). Beyond the energy recovery potential of AD through biogas production, the liquid and solid products of AD hold great agricultural and economic potential (Piadeh et al., 2024). In recent years, digestate has transitioned from being perceived as waste to being acknowledged as a valuable product, fully exploiting its benefits (W. Wang et al., 2023). Figure 1.1 shows the outline of different options for digestate valorisation. To valorise the solid fraction, AD can be coupled with hydrothermal carbonisation (HTC), pyrolysis, gasification, or bioethanol fermentation of the solid to produce biochar, hydrochar, biofuels or bioethanol (W. Wang et al., 2023).

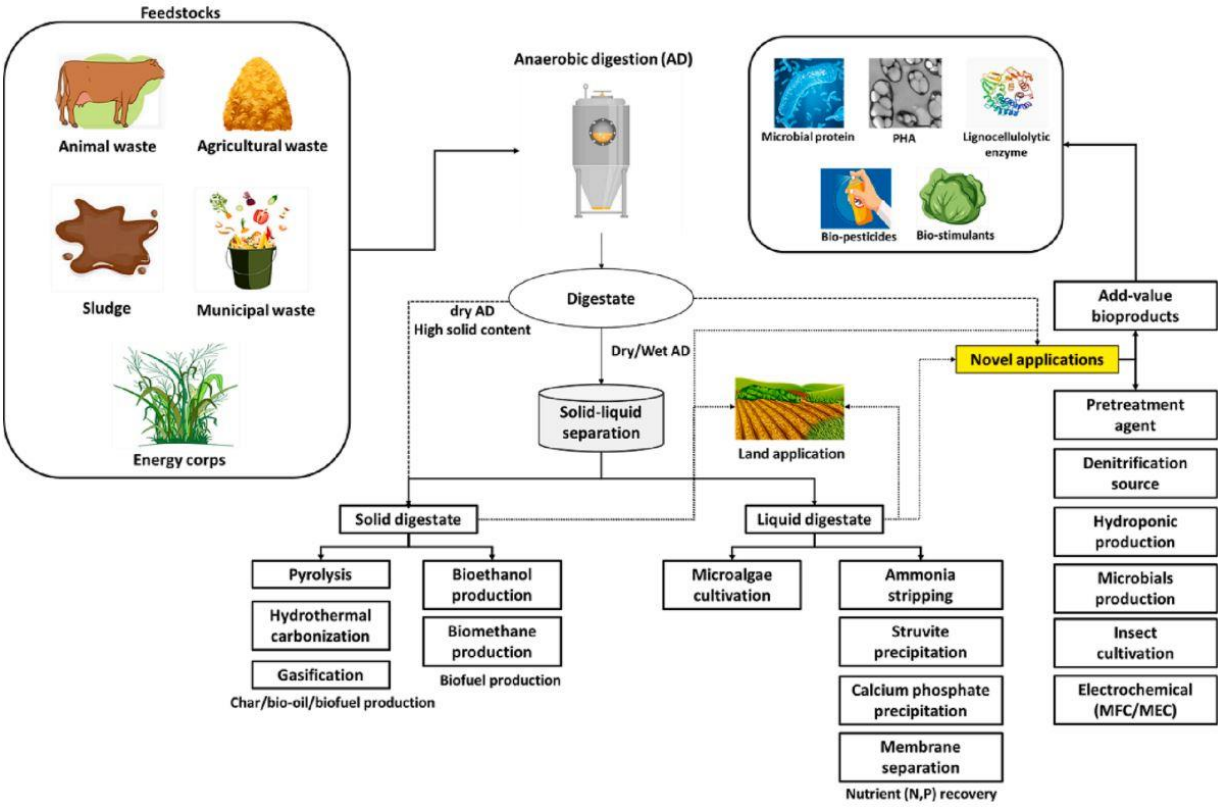


Figure 1.1 Valorisation routes of digestate. Retrieved from Wang et al. (2023).

Conversely, there are other novel methodologies to valorise the LFD. For instance, struvite precipitation for N and P recovery allowed to recover more than 87 % of P from digestate from swine manure (T. Zhang et al., 2020), and the addition of Ca, enhanced P recovery as calcium phosphate granules (Schott et al., 2022). Moreover, stripping-scrubbing is another promising technology to recover N, producing ammonium sulphate

having fertiliser value (Jin et al., 2022). Coupling membrane filtration and chemical precipitation also helps to simultaneously recover N and P (Piash et al., 2022). Finally, microalgae cultivation with LFD as a culture medium presents a promising approach to produce biofuel and biochemical products (Chong et al., 2022).

Table 1.1. European legislation on minimum standards for fertiliser products application. Retrieved from Sfetsas et al. (2022).

Parameter	**DM**	**OC**	**N**	**P_2O_5**	**K_2O**	**SoN**	**Cd**	**Cr**	**Hg**	**Ni**	**Pb**	**As**
Units			**%**				mg/kg DM					
SOF_{one}	solid	> 15	> 2.5	> 2	> 2	–	< 1.5	< 2	< 1	< 50	< 120	< 40
SOF_{multi}	solid	> 15	> 1	> 1	> 1	> 4	< 1.5	< 2	< 1	< 50	< 120	< 40
LOF_{one}	liquid	> 5	> 2	> 1	> 2	–	< 1.5	< 2	< 1	< 50	< 120	< 40
LOF_{multi}	liquid	> 5	> 1	> 1	> 1	> 3	< 1.5	< 2	< 1	< 50	< 120	< 40
SI	> 20	> 7.5	–	–	–	–	< 2	< 2	< 1	< 50	< 120	< 40

DM: dry matter, OC: organic carbon, N: nitrogen, SoN: sum of nutrients, SOF_{one}: solid organic fertiliser that contains only one of the nutrients (N, P_2O_5, K_2O), SOF_{multi}: solid organic fertiliser that contains all the nutrients (N, P_2O_5, K_2O), LOF_{one}: liquid organic fertiliser that contains only one of the nutrients (N, P_2O_5, K_2O), LOF_{multi}: liquid organic fertiliser that contains all the nutrients (N, P_2O_5, K_2O), SI: soil improver.

However, to valorise the digestate ensuring its quality, the new Fertilising Products Regulation 2019/1009/EC limits its use depending on the limits presented in Table 1.1, and on AD feedstock. Nevertheless, digestates from wastewater treatment sludge and mixed MSW are excluded from the European market of nutrient recycling products because its high concentration of contaminants such as metals, organic micropollutants and pathogens (Environmental Protection Agency, 2021). Residual antibiotics in LFD can promote the propagation and transmission of antibiotic resistance genes (ARGs), posing an enormous threat when applied in fields or released into the environment (Gurmessa et al., 2020). The proliferation of ARGs can also be triggered by metals present in LFD, exerting selective pressure and spreading them via horizontal gene transfer among microbial communities through mobile genetic elements (MGEs), namely integrons (Carr et al., 2021; Shi et al., 2022). Furthermore, the presence of antibiotic resistance bacteria (ARB) in plants can contribute to the transfer of resistant strains in human hosts (Andleeb et al., 2019). The growing impact of antibiotic-resistant strains to natural ecosystems demands urgent action, including the implementation of effective programs to increase awareness about ARGs or the minimisation of the dissemination

and incidence of antibiotics and non-antibiotic chemicals (Andleeb et al., 2019). Consequently, other alternatives must be contemplated for MSW digestates, especially the LFD, that cannot be valorised and commercialised in the European market. These alternatives encompass hydroponics, irrigation, and environmental release, but LFD treatment is needed before release to meet legislation (Sfetsas et al., 2022).

1.3. Implementation of Nature-Based Solutions for sustainable liquid digestate management

The SDG 6 underscores the need to ensure the sustainable management and availability of water and sanitation, as both the pressure on water bodies and global freshwater use increase annually (Ritchie & Roser, 2018). Integrated, resource-oriented, and distributed wastewater collection systems are essential to face global needs and SDGs (Masi et al., 2018). Nature-Based Solutions (NBS) offer cost-effective approaches in aiding in these water management objectives and enable the transition towards circularity (Nika et al., 2020). NBS are solutions inspired by natural processes to provide environmental, social, and economic benefits while enhancing resilience, integrating diverse natural elements locally adapted through resource-efficient interventions (European Commission, 2020a). Investment in NBS development is needed to address the European Green Deal and the 2030 SDG Agenda (European Commission, 2020a). Implementing NBS in wastewater treatment creates systems mimicking natural ecosystems functioning with minimal dependence on mechanical and technical components.

Constructed wetlands (CWs) are ecologically engineered systems that function as NBS for water and wastewater management. They consist of assemblages of substrates, vegetation and associated microorganisms that remove contaminants from wastewater, improving its quality (Ferreira et al., 2023). CWs have low operation and maintenance requirements and show consistent performance to fluctuations in inputs, being primary, secondary, or tertiary treatment of a wide range of wastewater (Dotro et al., 2017). The multiple removal mechanisms of pollutants are illustrated in Figure 1.2 and combine physical, chemical, and biological processes. Each pollutant type has various simultaneous removal processes shown in Table 1.2.

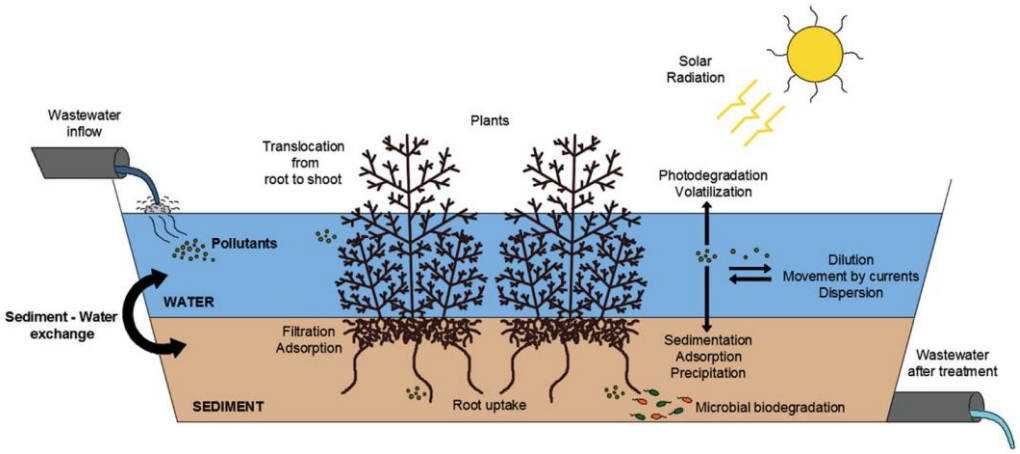


Figure 1.2 General mechanisms for the removal of pollutants in constructed wetlands. Retrieved from Gorito et al. (2017).

Substrates are important components in CWs, normally constituting the largest volume within systems. They physically support plants and microorganisms carrying biological processes (C. Yang et al., 2022). The substrate type plays an important role in microbiome diversity and in pollutant removal mainly through filtration and adsorption (Kataki et al., 2021; Long et al., 2016). Plants in CWs also have crucial functions as their roots and rhizomes serve as attachment sites for microbial biofilms, enhancing the biological activity of the systems (Gagnon et al., 2007). Vegetation also diffuses the flow, reducing hydraulic short-circuiting, and releases oxygen and organic carbon compounds to the root matrix. This creates the presence of large redox gradients supporting a diverse consortium of microorganisms, including aerobic, facultative, and obligate anaerobic microorganisms (Dotro et al., 2017). Microorganisms can form biofilms, coordinated microbial structures producing extracellular polymeric substances that thrive in extreme conditions, namely nutrient scarcity, and antimicrobial and other pollutants exposure (Kataki et al., 2021). They contribute to important biogeochemical processes such as nutrient cycling, energy flow, ecosystem stability, and pollutant removal, especially nitrogen and organic contaminants. For example, Methanotrophs, *Nitrosomonas* and *Pseudomonas* dominate the oxygen-rich zones and are involved in organic pollutants degradation and carbon and nitrogen cycling, while methanogenic and sulphur-reducing bacteria dominate the anaerobic zones participate in organic matter decomposition, anaerobic biodegradation processes, sulphur detoxification and cycling, among others (Rajan et al., 2019).

Table 1.2 Removal mechanisms of various pollutants in constructed wetlands. Adapted from (Dotro et al., 2017).

Pollutant	Removal mechanism	Reference
Organic matter	Sedimentation, filtration, and biological degradation	Vymazal (2014)
Nitrogen	Nitrification and denitrification processes, plant uptake, volatilisation, adsorption, ammonification, and anaerobic ammonium oxidation (anammox)	Malyan et al. (2021)
Phosphorus	Adsorption, microbial and plant uptake, complexation, mineralisation, sedimentation, and precipitation	Malyan et al. (2021)
Metals	Sedimentation, filtration, complexation, adsorption, microbial sorption, and plant uptake processes	Malyan et al. (2021)
Antibiotics	Adsorption, plant uptake, microbial degradation or bioaccumulation, volatilisation, photodegradation and precipitation.	M. Lv et al. (2022)
Pathogens	Sedimentation, filtration, adsorption, natural die-off, and predation	Dotro et al. (2017)

Vertical sub-surface flow CWs are characterised by wastewater entering from the top and exiting from the bottom, flowing vertically through the bed substrate. This flow design promotes aerobic conditions, resulting in more efficient removal of organics and nitrification processes (Parde et al., 2021). Along the CWs performance treating wastewater, the vegetation, tolerating the concentration of pollutants carried by wastewater, grows while it accumulates metals and some organic contaminants in their tissues (Brisson & Chazarenc, 2009). Macrophytes are vascular plants that typically complete their life cycle by the end of autumn. To maintain effective pollutant removal in CWs systems over time, proper management of macrophytes is essential (Vymazal, 2020). Seasonal harvesting, ideally 2 or 3 harvest per year to enhance nutrient removal, is one of the most common approaches (Verhofstad et al., 2017). Different resource recovery approaches for the harvested biomass include co-composting for nutrient recovery (Kouki et al., 2016), as well as direct combustion or AD for energy recovery (Avellán & Gremillion, 2019).

Considering the resource-oriented and circular economy paradigm, CWs potentially offer various applications and purposes including water reuse, nutrient recovery, energy production, or ecosystem services namely cooling, wildlife habitat, landscaping, and recreation (Masi et al., 2018). Therefore, in this thesis, it is important to consider not only

the potential of CWs for LFD treatment to reuse it as a reclaimed water source for irrigation, but also employ these systems as biomass production biofactories capable of generating biogas or biomethane (Figure 1.3).

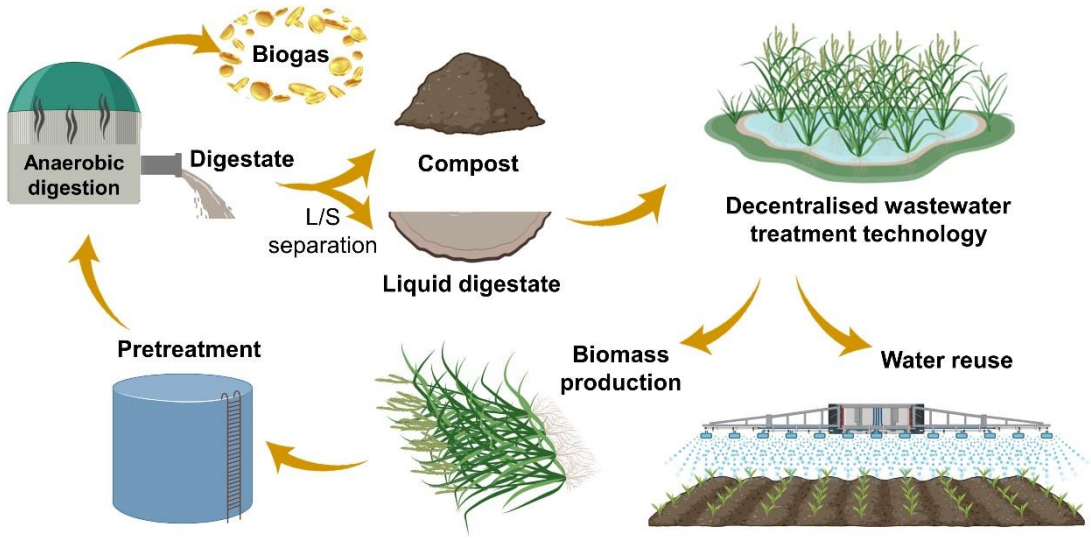


Figure 1.3 Circular approach of the integration of CWs with AD technologies explored in the thesis.

1.4. Spreading the word: society engagement matters!

Science has a crucial role in responding to global challenges through research and development of innovative technologies. Many researchers are driven by passion and curiosity to explore new insights in a specific field, while contributing to society by solving specific problems and advancing knowledge. Public engagement in science establishes connections between research institutions and the general public, reinforcing trust and recognition between them, enhancing the researchers' skill sets, and leading society to science literacy (National Coordinating Centre for Public Engagement, 2024). Numerous researchers are enthusiastic to be involved in public engagement, viewing it as a moral responsibility, though not necessarily obligatory. However, the main obstacle lies in the perception that public engagement is not inherent to the research job (The Royal Society, 2005). In Academia, the priorities are research, the number of publications and the number of citations, rather than educational or public outreach, as these factors serve as competitive incentives for research funding and as quantitative metrics to measure a researcher’s performance (Edwards & Roy, 2017). Overcoming the main obstacles to public engagement in science, namely lack of time, money, and training, can be achieved

by participating in established programs where researchers are not responsible for event development or organisation (Concannon & Grenon, 2016).

As previously mentioned, to tackle global challenges effectively, extensive actions across multiple sectors are needed involving a broader range of stakeholders. Sharing science with policy makers is crucial for evidence-based policy making, fostering collaboration between researchers and policy makers to address complex societal challenges (Choi et al., 2005). However, it should not be the sole focus. Disseminating knowledge to a broader public and building their trust, allows consumers, patients, parents, students, voters, and others to clarify beliefs, inspire, revise opinions, articulate values, and make informed decisions and actions (National Academies of Sciences Engineering and Medicine, 2017). Therefore, this thesis also explores the importance of public engagement outreach activities concerning both AD and NBS, emphasising their value beyond the scientific community.

2. Knowledge gaps addressed in this thesis

This thesis will explore some of these applications, specifically the use of CWs as a low-cost AD polishing stage for safe water reuse, and as a plant biomass producer to convert it into biofuels. This approach integrating AD and NBS will contribute to close the loop of AD, fostering the principles of circularity.

This thesis aims to address the following knowledge gaps:

- While some attention has previously focused on the LFD treatment in CWs capabilities to remove nutrients, no studies have focused on the removal of other contaminants namely metals, antibiotics, and antibiotic resistance genes (ARGs) in this complex matrix for reclaiming water.
- In addition, little research to date exists on the use of *Sparganium erectum* in planted CWs, for nutrient and metal removal. Data on its growth and metal accumulation and translocation in these systems will provide valuable insights for understanding the efficacy of this common species in northern hemisphere natural wetlands and watercourses.
- Most studies primarily examine the performance of CWs in pollutant removal, yet there is a need for further investigation into the microbial ecosystem function within CWs and its dynamics during contaminant removing processes. In addition, up until now, the root-associated microbiome of *S. erectum* has been not characterised.

- There is a paucity of information on different pre-treatments to enhance the methane yield of harvested bur-reeds (*S. erectum*) from CWs or water weed control.
- Novel activities to disseminate the principles of AD and CWs to students or other citizens, emphasising not just the scientific content but also the fostering of soft skills and motivation, are currently missing.

3. Research aims

The first aim of this study was to investigate a novel configuration of vertical subsurface flow CWs on a microcosm scale to simultaneously remove various types of pollutants from LFD for its potential irrigation reuse. The specific objectives to achieve this aim were to:

- To assess the removals of organic matter and nutrients, and pH stabilisation of the systems over time.
- To determine the removal of metals, antibiotics (oxytetracycline, sulfadiazine, and ofloxacin), mobile genetic elements (MGEs, *intI1*), ARGs (*tetA, tetW, sul1*, and *qnrS*), and potential pathogens.
- To characterise the shifts in microbial community structure between influent and effluent.
- To evaluate the response of root-bed substrate and root microbiome to LFD contaminated with metals and possible interference of antibiotics.
- To analyse *S. erectum* acclimation and growth in CWs treating LFD and its capacity to accumulate metals in different tissues.

The second aim of this dissertation was to evaluate the efficiency of different pre-treatments with available resources in biogas plants to enhance the valorisation potential of harvested *S. erectum*. The specific objectives to accomplish this aim were to:

- Perform three different pre-treatments on bur reeds, namely storage, aqueous ammonia soaking and hydrothermal pre-treatments, each under two different testing conditions.
- Assess the efficiency of the pre-treatments through physicochemical and morphological analysis and through biochemical methane potential (BMP) tests.

- Characterise the active prokaryotic communities during the methane production exponential phase of untreated and pre-treated biomass and the methane production kinetics.
- Evaluate the cost-effectiveness of the pre-treatments compared to untreated plants through a preliminary techno-economic assessment.

Finally, the third objective of this thesis was to develop innovative and engaging activities to showcase the importance and principles of CWs and AD to the general public, particularly targeting young secondary education students.

4. Hypothesis

The hypothesis for these objectives were:

1. The novel CWs configuration with sand, LECA, gravel, and *S. erectum* will reduce significantly the organic matter, nutrients, metals, antibiotics and ARGs concentration from LFD, making the treated effluent suitable for irrigation purposes. Antibiotics might have an effect on the microbial communities of the root-bed substrate and root microbiome, potentially leading to variations on the pollutant removal levels.

2. The three different pretreatments (storage, aqueous ammonia soaking, and hydrothermal pre-treatments) will significantly alter the physicochemical properties of *S. erectum*, improving its degradability and hence, increasing the biogas yield of this plant. The cost and the efficiency of the pretreatment will vary, being the alkali and the hydrothermal pretreatment the most effective and costly.

3. Innovative educational activities will increase understanding of CWs and AD among young secondary education students, leading to greater interest and engagement in environmental science subjects.

5. Structure of the thesis

The thesis is structured into six chapters as follows.

Chapter 2 investigates the potential of vertical subsurface flow constructed wetlands to simultaneously remove emerging contaminants, organic matter, and nutrients for LFD safe reuse in irrigation, addressing the first aim of the thesis.

Chapter 3 also covers the first aim of the thesis, specifically focusing on the fate of metals within CWs compartments and the responses of microbial communities in the substrate

and the roots to LDF with metals. Whereas the second chapter describes the influent and the effluent, this third chapter elucidates the processes within CWs systems.

Chapter 4 explores the effect of storage, aqueous ammonia soaking and hydrothermal pre-treatments in AD performance of *S. erectum*, focusing on the second aim of the thesis.

Chapter 5 presents an activity, Wetlands Wonders, designed along the doctoral degree to disseminate in an innovative and pedagogic perspective the importance and the basis of CWs.

Finally, Chapter 6 discusses the conclusions from the thesis and the recommendations for future research.

The Appendices section covers on the one hand the figures and tables that are supplementary data of the experiments (A), and on the other hand, the materials of Wetlands Wonders (B) and another educational activity on AD that was designed along with two colleagues, Kris Silveira and Raffaello Mattiussi (C).

6. Contribution of existing knowledge

6.1. Journal Papers

Porras-Socias, P., Tomasino, M. P., Fernandes, J. P., De Menezes, A. B., Fernández, B., Collins, G., Alves, M. J., Castro, R., Gomes, C. R., Almeida, C. M. R., and Mucha, A. P. (2024). Removal of metals and emergent contaminants from liquid digestates in constructed wetlands for agricultural reuse. *Frontiers in Microbiology, 15:* 1388895.

Porras-Socias, P., Fernandes, J. P., Tomasino, M. P., De Menezes, A. B., Fernández, B., Collins, G., Gomes, C. R., Almeida, C. M. R., and Mucha, A. P. (*in prep*). Responses of constructed wetlands substrate microbiome and *Sparganium erectum* root-associated prokaryotic communities to liquid digestate contaminated with metals and possible interference of antibiotics presence.

Porras-Socias, P., Guivernau, M., De Menezes, A. B., Gomes, C. R., Almeida, C. M. R., and Mucha, A. P., and Fernández, B., (*in prep*). Effect of storage, aqueous ammonia soaking and hydrothermal pre-treatments in anaerobic digestion performance of *Sparganium erectum*

Porras-Socias, P., Almeida, C. M. R., Mucha, A. P., De Menezes, A. B., and Fernández, B., (*in prep*). Wetlands Wonders: a constructivist-based game to improve motivation and learning outcomes in environmental engineering education. *FEMS letters.*

Porras-Socias, P., Mattiussi, R., Silveira, K. A., and O'Flaherty, V. (*in prep*). Methan-o-poly: A giant collaborative game to 'digest' the microbiology of green biogas production. *Journal of Microbiology and Biology Education.*

6.2. Conference presentations

6.2.1. Oral communications

Authors: Pau Porras-Socias, Maria Paola Tomasino, Joana P. Fernandes, Gavin Collins, Alexandre B. de Menezes, Carlos R. Gomes, C. Marisa R. Almeida, Ana Paula Mucha
Title: Removal of ARGs from anaerobic digestion effluents in constructed wetlands for agricultural reuse.
Scientific Meeting: Centre for One Health Annual Conference 2022
Dates & place: November 3rd-4th, Online.

Authors: Pau Porras-Socias, Maria Paola Tomasino, Joana P. Fernandes, Gavin Collins, Alexandre B. de Menezes, Carlos R. Gomes, C. Marisa R. Almeida, Ana Paula Mucha
Title: Microbial community responses in constructed wetlands treating liquid digestate contaminated with metals and antibiotics
Scientific Meeting: IMAB23-International Congress on Metal-microbe applications for circular economy
Dates & place: April 19th -21st, Porto, Portugal

6.2.2. Poster presentations

Authors: Pau Porras-Socias, Carlos R. Gomes, C. Marisa R. Almeida, and Ana Paula Mucha.
Title: Constructed wetlands for removal of metals, antibiotics, and antibiotic resistance from anaerobic digestion effluent.
Scientific Meeting: Blue Think Conference, CIIMAR.
Dates & place: 23rd-24th September 2021, Matosinhos, Portugal.

Authors: Pau Porras-Socias, Alexandre B. de Menezes, Gavin Collins, Carlos R. Gomes, C. Marisa R. Almeida, and Ana Paula Mucha.

Title: Anaerobic digestion effluent treatment in constructed wetlands for agricultural reuse.
Scientific Meeting: 6th IWA International Conference on eco-Technologies for Wastewater Treatment.
Dates & place: 26th-29th June 2023, Girona, Spain.

Authors: Pau Porras-Socias, Alexandre B. de Menezes, Gavin Collins, Belén Fernández, Carlos R. Gomes, C. Marisa R. Almeida, and Ana Paula Mucha.
Title: The role of *Sparganium erectum* in constructed wetlands treating anaerobic digestion effluents.
Scientific Meeting: 10th International Symposium on Wetland Pollutant Dynamics and Control.
Dates & place: 10th-14th September 2023, Bruges, Belgium.

6.3. Public engagement activities

Event: *Noite Europeia dos investigadores 2021* event in Porto.
Activity: *Estação de Tratamento de Águas Residuais baseada na natureza: FitoETAR*
Dates & place: 24th September 2021, Porto, Portugal.

Event: *Noche Europea de l@s investigador@s 2021* event in Seville.
Activity: *Proyecto M2ex – ¿Qué son los humedales construídos?*
Dates & place: 24th September 2021, Seville, Spain.

Event: Galway's Science and Technology Festival.
Activity: From Trash to Cash
Dates & place: November 2022, Galway, Ireland.

Event: Science is Wonderful! Marie Sklodowska-Curie Actions
Activity: Methanopoly
Dates & place: 15th-18th March, 2023, Brussels, Belgium.

Education institution: Primary and secondary school Sol Ixent
Activity: Wetland Wonders
Dates & place: October 2023, Mataró, Spain.

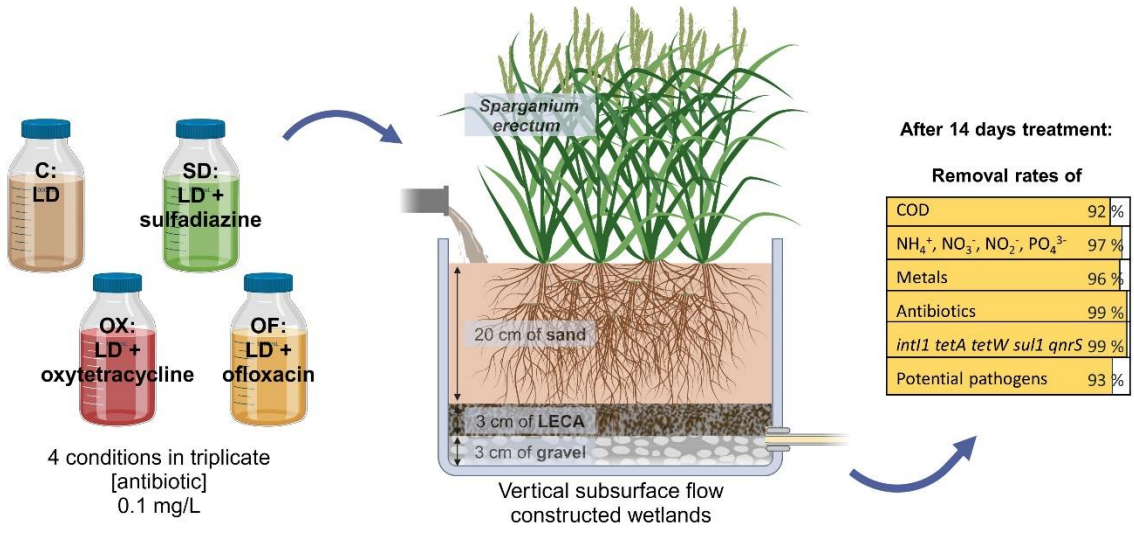
Education institution and participants: Undergraduate students from the degree in Biology at the Faculty of Sciences of the University of Porto.
Activity: Methanopoly
Dates & place: November 2023, Porto, Portugal.

Education institution and participants: Master students from the degree in Ecology and Environment, and in Biotechnology Applications and Synthetic Biology at the Faculty of Sciences of the University of Porto.

Activity: Methanopoly

Dates & place: March 2024, Porto, Portugal.

Chapter 2. Removal of metals and emergent contaminants from liquid digestates in constructed wetlands for agricultural reuse



This chapter is based on the publication (published):

Porras-Socias, P., Tomasino, M. P., Fernandes, J. P., De Menezes, A. B., Fernández, B., Collins, G., Alves, M. J., Castro, R., Gomes, C. R., Almeida, A. M. R. & Mucha, A. P. (2024). Removal of metals and emergent contaminants from liquid digestates in constructed wetlands for agricultural reuse. *Frontiers in Microbiology*, *15*: 1388895. https://doi.org/10.3389/fmicb.2024.1388895

1. Abstract

Given the increasing pressure on water bodies, it is imperative to explore sustainable methodologies for wastewater treatment and reuse. The simultaneous presence of múltiples contaminants in complex wastewater, such as the liquid effluents from biogas plants, can compromise biological treatment effectiveness for reclaiming water. Vertical subsurface flow constructed wetlands were established as a low-cost decentralised wastewater treatment technologies to treat the liquid fraction of digestate from municipal organic waste with metals, antibiotics, and antibiotic resistance genes, to allow its reuse in irrigation. Twelve lab-scale planted constructed wetlands were assembled with gravel, light expanded clay aggregate and sand, testing four different treating conditions (liquid digestate spiked with oxytetracycline, sulfadiazine, or ofloxacin, at 100 µg/ L, or without dosing) during three months. Physicochemical parameters (pH, chemical oxygen demand (COD), nutrients, metals, and antibiotics), the microbial communities dynamics (through 16S high-throughput sequencing) and antibiotic resistance genes removal (qPCR) were monitored in influents and effluents. Systems removed 85.8 -96.9 % of organic matter (as COD), over 98.1 % of ammonium and phosphate ions, and 69.3 -99.4 % of nitrate and nitrite ions, with no significant differences between the presence or absence of antibiotics. Removal of Fe, Mn, Zn, Cu, Pb and Cr exceeded 82 % in all treatment cycles. The treatment also removed oxytetracycline, sulfadiazine and ofloxacin over 99 %, and decreased *intl1, tetA, tetW, sul1,* and *qnrS* gene copies. Nonetheless, after 3 months of ofloxacin dosing, *qnrS* gene started being detected. Removal processes relied on high HRT (14 days) and various mechanisms including sorption, biodegradation, and precipitation. Microbial community diversity in liquid digestate changed significantly after treatment in constructed wetlands, with a decrease in the initial *Firmicutes* dominance, but with no clear effect of antibiotics on the microbial community structure. The relative abundance of *Streptococcus* and *Clostridium* decreased in over 85 % and 94 %, respectively. Results suggest that vertical subsurface flow constructed wetlands were a suitable technology for treating the liquid digestate to reuse it in irrigation agricultural systems, contributing to the circular bioeconomy concept. However, a more profound understanding of effective wastewater treatment strategies is needed to avoid antibiotic resistance genes dissemination.

Keywords: Constructed wetlands, metals, antibiotics, antibiotic resistance genes, anaerobic digestion effluent, *Sparganium erectum.*

2. Introduction

The transition towards circular economy is one of the challenges of the 21st century. In 2018, in response to the Circular Economy Action Plan, the European Commission created the Bioeconomy Strategy addressing, in part, the sustainable management of organic waste (European Commission, 2022b). About 60 million out of 138 million tons of municipal and industrial organic waste were valorised in the European Union in 2019 (Gilbert and Siebert, 2022), but the European Commission targets to increase the reusing strategies by 2035 (European Environment Agency, 2020). Anaerobic digestion is the most promising valorisation process of organic waste, generating bioenergy products and digestate. Digestate is a complex matrix of biosolids rich in organic matter, macro, and micronutrients, which makes it a potentially excellent fertiliser (European Environment Agency, 2020). However, most biogas plant practices prioritise the improvement of biogas production over the digestate management (Logan and Visvanathan, 2019).The management options of the digestate include in most cases the separation of the liquid and solid fractions, for the subsequent composting of the solid fraction for its use as a soil amendment (Wang et al., 2023). Processes to valorise the liquid fraction of the digestate (LFD) include membrane filtration, struvite precipitation, ammonia stripping, and microalgae cultivation. However, challenges such as high power consumption and maintenance costs of the processes, and early-development stages technologies, hinder its reuse and hence, LFD are frequently disposed in centralised wastewater treatment facilities (Lamolinara et al., 2022).

The LFD contains over 90 % of water and represents above 80 % of the total digestate weight. It comprises a high concentration of organic matter, soluble ions, namely ammonium, potassium and phosphate ions, and humic substances, (Akhiar et al., 2017). Unfortunately, as many feedstocks contain chemical and biological pollutants that are not efficiently degraded during the anaerobic digestion process, LFD can harbour more complex pollutants namely metals (Dragicevic et al., 2018), organic contaminants, pathogens (Bloem et al., 2017), and other micropollutants (Venegas et al., 2021). For example, veterinary antibiotics, namely oxytetracycline, sulfadiazine and ofloxacin, among many others, have low removal rates during anaerobic digestion and are persistently found in LFD of reactors treating livestock manure in concentrations between 3.8 and 940 µg/L (Yang et al., 2022; Gurmessa et al., 2020). After assessing the risk of oxytetracycline, sulfadiazine and ofloxacin in water systems, these compounds were classified as high-risk (Ilyas et al., 2020). Antibiotics, in combination with metals, exert selective pressure on microorganisms and mobile genetic elements (MGEs) can

promote the horizontal gene transfer of antibiotic resistance genes (ARGs) (Wolak et al., 2023). Although ARGs can present 51 % reduction along anaerobic digestion, many different ARGs and their associated antibiotic-resistant bacteria are persistent in the digestate (Goulas et al., 2020).

Water is a limited valuable resource, and as many other parts of the world, the European Union is suffering from growing pressure on water resources resulting from variable availability, climate change and poor water quality. Consequently, reusing reclaimed water is a crucial practice for efficient water resource management, ensuring a predictable water supply and reducing freshwater consumption (Chen et al., 2021). The agricultural sector is particularly interested in the reuse of the LFD for irrigation purposes in the fields, as a non-conventional water reuse source (Chen et al., 2021), since 71.7 % of the total water withdrawal is used in agriculture (FAO, 2023). The Water Reuse Regulation establishes minimum water quality standards for the safe reuse of treated wastewater in agricultural irrigation (European Commission, 2020, 2022a). Hence, effective treatment of LFD is crucial before its reuse to avoid risks to the groundwaters and human health associated to the release of the LFD contaminants into the environment. Conventional wastewater treatments include a primary treatment to remove large solid particles, a secondary one involving biological processes, and a tertiary one to ameliorate the water quality (Al-Hazmi et al., 2023). Recently, more sustainable, and cost-effective wastewater treatment techniques have been studied and developed, namely photocatalysis (Baaloudj et al., 2021), adsorption filtration, coagulation (Badawi et al., 2023), and microalgae-based systems (Badawi et al., 2022). Additionally, there is growing interest in combining low-cost nature-based solutions with traditional infrastructure for very efficient treatment methods (Cross et al., 2021).

Constructed wetlands (CWs) are nature-based solutions that effectively treat wastewater through different physical, chemical, and biological reactions (Gorito et al., 2017). Previous works reported that CWs could successfully remove metals (Dias et al., 2020) and antibiotics in some wastewater types (Ilyas et al., 2020), being an efficient and fit-for-purpose treatment technology, However, a lingering risk remains as our understanding of CWs' effectiveness in eliminating ARGs is still limited (Ilyas et al., 2020). Moreover, although CWs can be an option to reduce the organic matter and nutrient loads from LFD (Comino et al., 2013; Guo et al., 2016; Maucieri et al., 2016; Wu et al., 2016), no research has been published on the removal of contaminants of emerging concern from this complex matrix of biosolids. CWs could fail to remove simultaneously a wide range of contaminants. To the best of our knowledge, this is the

first study to focus on the potential of CWs to treat LFD for agricultural reuse, considering both chemical and biological contaminants, including metals, antibiotics, ARGs and potential pathogens.

This study aims to evaluate the potential of a new configuration of vertical subsurface flow CWs, at a microcosm scale, to remove simultaneously different types of pollutants from the LFD to allow its use in irrigation. Firstly, removals of organic matter and nutrients, and pH stabilisation were assessed to confirm that CWs were treating the high load of organic matter, nitrogen, and phosphorus of the LFD. Secondly, the concentration of metals, antibiotics (oxytetracycline, sulfadiazine, and ofloxacin) and MGEs (*intI1*) and ARGs (*tetA, tetW, sul1* and *qnrS*) was analysed in CWs influents and effluents to evaluate if the effluent was safe for water reuse. Third, microbial communities in the influent and effluent were characterised to monitor population shifts and structure and assess the removal of potentially pathogenic microorganisms.

3. Materials and methods

3.1. CWs assembly and acclimation

Twelve laboratory scale vertical subsurface flow CWs systems were assembled on 12th July 2021, each in 0.4 x 0.3 x 0.3 m^3 plastic containers with a bottom layer of gravel (3 cm), a second layer of light expanded clay aggregate (LECA, 3 cm) and a top layer of sand (20 cm) in which *Sparganium erectum* plants were transplanted (Figure 2.1). To our knowledge, it is the first time this plant species is used in this CWs design. *S. erectum* were harvested in the Ribeira da Certagem, Lavra, Portugal (N 41º15'31.252"; W 8º43'24.924") on 11th July 2021, and were rinsed with abundant deionised water in the lab before the assembly. Each microcosm had between 3 and 4 individual plants, with a total fresh weight of around 700 g. The containers were wrapped with aluminium foil to prevent the photodegradation of compounds in the substrate and were placed in a greenhouse in the gardens of the Faculty of Sciences of the University of Porto (Portugal), under a natural light/dark regime with a temperature fluctuation between 14.0 °C and 44.1 °C in July 2021, and 2.0 °C and 36.5 °C in November 2021.

The systems simulated vertical subsurface flow CWs with the influent being poured on the surface and drained through the substrate layers of the systems. For the acclimation of the systems, each microcosm was saturated with 5.4 L of Hoagland nutrient solution. The effluent was daily recirculated and every two/three days, the nutrient solution was

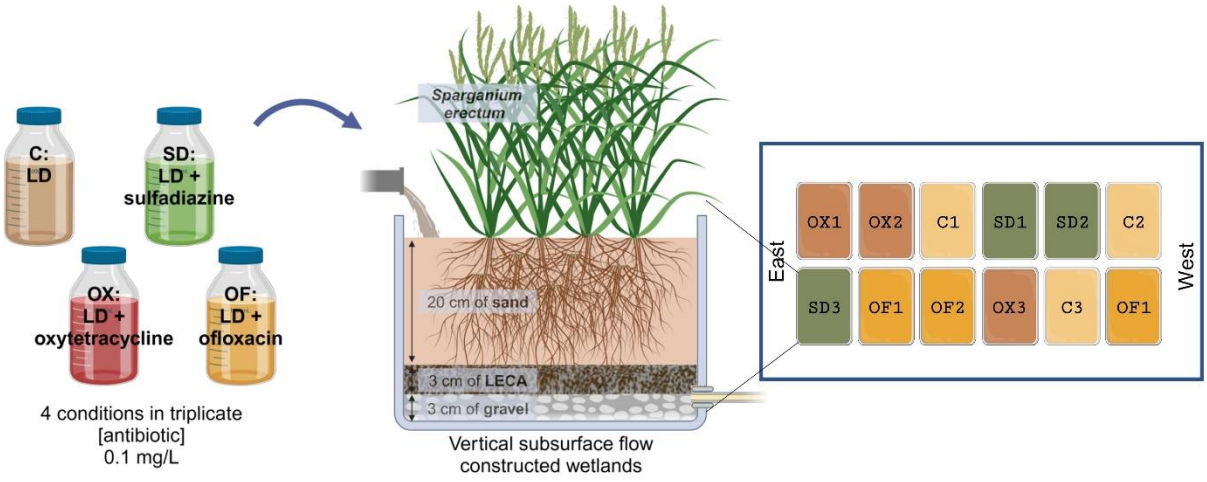


Figure 2.1 Diagram of vertical subsurface flow CWs microcosms experiments. At the left, the four LFD to be treated in the systems (with or without antibiotics), in the middle the different layers of substrate and the CWs' vegetation and in the right the different system distribution in the greenhouse. Created with BioRender with the publication licence nº SE267I8KKZ.

replaced with a new one. After two weeks, the solution was drained from CWs, and a three-step adaptation process started by adding LFD to the systems. Firstly, 1 L of a 1/10 dilution (v/v) with deionised water of the LFD was added to each system, recirculated for seven days, and then removed. Secondly, new fresh LFD was added to each system, this time, 1 L of 1/4 dilution (v/v), being recirculated for 14 days, and removed. Thirdly, 1 L of 1/2 dilution (v/v) of the LFD was added to microcosms, recirculating it for 14 days, before removing it all. Deionised water was added to saturate the systems whenever necessary (filling the systems just below the surface to compensate evapotranspiration).

3.2. CWs experiments

The LFD was collected from TratoLixo, Mafra, Portugal (N 38°56'14.435"; W 9°17'5.3916"), a full-scale anaerobic digestion plant treating the organic fraction of municipal solid waste, processing 65,000 tons of organic waste annually from an intervention area with 100,000 inhabitants. LFD collection was performed every 14 days. The physicochemical characterisation of the LFD was performed by the biogas plant company, except for chemical oxygen demand (COD) analysis.

Before adding the LFD to the systems, a homogenised 1/4 dilution (v/v) of the collected effluent with deionised water was prepared and allowed to stand overnight at room temperature to allow solids to settle. This dilution was chosen to avoid clogging of the CWs systems due to the high amount of dissolved solids, considering the acclimation results. The supernatant was then transferred to a clean vessel, where it was spiked with oxytetracycline, sulfadiazine, and ofloxacin methanolic solutions, or without dosing (control) to have a total of four different LFD to be treated in parallel (Figure 2.1). C systems were treating 1 L of LFD without antibiotic spiking. OX, SD and OF CWs were the systems treating the LDF with 100 µg/L of oxytetracycline, sulfadiazine and ofloxacin, respectively. The antibiotic concentration selected is an average of concentrations found in LFD (Yang et al., 2022). C, OX, SD and OF systems were set up all in triplicates, distributed randomly within the greenhouse, and were always treating 1 L of one of the four LFD types.

The LFD was recirculated over 14 days, then removed from the systems and replaced with fresh LFD, simulating the cumulative effect of full-scale CWs with a hydraulic retention time (HRT) of 14 days. In total, six 14-day cycles were performed between 1st September and 25th November 2021.

3.3. Samples collection and preservation

Samples of influent and effluent per CW, treatment and cycle were collected. Influent was sampled just before pouring it in the CWs for analysis of different parameters namely, pH, organic matter (estimated through COD), nutrients (ammonium, phosphate, nitrate, and nitrite ions), and metals (Fe, Mn, Zn, Ni, Cr, Cu, Pb). After each two-week treatment cycle, all the effluents from each CWs were collected in dark glass flasks, to protect them from light. After homogenisation, different aliquots were collected to analyse the different parameters.

Influent samples for metals analysis were stored at -20 °C, whereas fresh CWs effluent was acidified with 1% (v/v) nitric acid after collection and kept at 4 °C until direct analysis.

Samples for pH and COD were collected and immediately analysed. To analyse nutrients, aliquots of freshly collected influent and effluent samples were filtered through nitrate cellulose filters (0.45 mm) and kept at -20 °C until analysis.

For the analysis of the antibiotic compounds, CWs effluents were filtered through glass fibre filters and concentrated by solid phase extraction (SPE) with Oasis HBL 3 cc (60 mg) Extraction Cartridges (Waters Corporation, Milford, MA, USA) immediately after sample collection using a vacuum manifold system (Supelco, Spain) coupled with a vacuum pump. SPE cartridges were eluted with a 96/4 (v/v) methanol/formic acid solution, adapting the methodology optimised by Cavenati et al. (2012). SPE extracts were kept at -20 °C until analysis.

For the microbial community characterisation and qPCR analysis, fresh CWs effluent samples of only the second, the fourth and the sixth treatment cycles were immediately filtered through Sterivex™ filter units with a pore size of 0.22 mm (Merck Millipore, Portugal), in duplicate, for 3 h until the filters were clogged. These sampling times were chosen to evaluate the monthly evolution of the communities. The filtered volume was on average 30 mL per sample (ranging from 7 mL to 42 mL). The inlet and outlet of these filter units were covered with parafilm, after removing the remaining liquid, and the Sterivex™ were kept in sterile plastic bags at –80 °C. The initial LFD (influent) of the second, fourth and sixth cycles was also stored at –80 °C right after the sampling.

3.4. Physicochemical analysis

pH was measured with a Crison micro pH 2002 with a Crison pH electrode in freshly collected samples. The COD content was determined using kits HI93754B-25 MR for a range 0 – 1500 mg O_2/L and HI93754A-25 LR for a range 0 – 150 mg O_2/L and the

absorbance of the samples was read in a HI83214 Multiparameter Bench Photometer (Hanna Instruments, Portugal). The concentration of ammonium, nitrite and phosphate ions was analysed following the protocol described by Dias et al., (2020). The limit of detection (LOD) of ammonium, phosphate, nitrite, and nitrate ions was 0.05, 0.05, 0.01 and 0.1 mg/L, respectively.

For metal determinations, samples (2.8 g per sample) were digested in a high-pressure microwave system (Ethos, Millestone, Sorisole, Italy) with 1 mL of nitric acid and 5 mL of 30 % of a hydrogen peroxide solution in microwave Teflon vessels. The microwave digestion program was: 5 min at 250 W, 5 min at 400 W, 5 min at 500 W and 10 min at 0 W, following a previously optimised lab protocol (Almeida et al., 2017b). Then, concentration of metals in microwave extracts and in the acidified CWs effluents were analysed by atomic absorption spectrophotometry with flame atomisation (Aanalyst 200AA spectrometer, PerkinElmer Inc., Waltham, MA, USA) for Fe, Mn, Zn, and Cu, and with electrothermal atomisation (Atomic Absorption Spectrometer PinAAcle 900Z with Furnace Autosampler AS900, PerkinElmer Inc., Waltham, MA, USA) for Ni, Cr, and Pb, using external calibrations prepared with aqueous standard solutions for metal quantification. The LOD of Fe, Mn, and Cu were 0.1 mg/L, the one of Zn was 0.025 mg/L, whereas for Ni and Pb, LOD were 5 µg/L, and for Cr, 10 µg/L.

Before organic contaminant analysis, SPE extracts of CW effluents were evaporated until dryness and re-suspended in 200 µL of a 70/30 (v/v) methanol/water solution. The concentrations of oxytetracycline, sulfadiazine and ofloxacin antibiotics were analysed by high-performance liquid chromatography (HPLC; Beckman Coulter Inc., Brea, CA, USA), adapting a previously optimised laboratory procedure (Cavenati et al., 2012). This equipment was coupled with a diode array detector (module 128) set up at 298 nm and an automatic sampler (module 508) and the antibiotics were separated in a 150 × 4.6 mm C18 Luna column (Phenomenex, UK). The LOD for oxytetracycline, sulfadiazine and ofloxacin were 0.8 µg/L.

3.5. Microbial community analysis

Microbial communities from CW influent and effluent samples were characterised using a 16S rRNA gene sequencing approach.

For the CWs effluents, liquid samples were collected, and DNA was extracted from Sterivex™ filters with the DNeasy PowerWater Sterivex Kit (QIAGEN Inc., Venlo, Netherlands). In the case of CWs influents, DNA was extracted from 0.5 g of LFD with the DNeasy PowerSoil Pro Kit (QIAGEN Inc., Venlo, Netherlands) following the

manufacturer's instructions and treating the samples as solid ones. DNA concentration and purity were determined by spectrophotometric analysis (NanoDrop ND-2000 and Qubit 4 Fluorometer, Invitrogen, MA, USA).

The prokaryotic community of influent and effluent samples along the different CWs treatment cycles was characterised by sequencing the V4 region of the *16S rRNA* gene targeting for both bacteria and archaea communities. For that, the V4 region of the *16S rRNA* gene was amplified using the primer pair 515FB (GTGYCAGCMGCCGCGGTAA) and 806RB (GGACTACNVGGGTWTCTAAT), according to the Earth Microbiome Project protocols. Sequencing of the amplicons was carried out on an Illumina MiSeq sequencer with the V3 chemistry (Illumina, San Diego, CA, USA) in Genoinseq, Biocant – Biotechnology Park (Cantanhede, Portugal).

The raw reads were pre-processed with PRINSEQ-Lite v0.20.4 that excluded reads shorter than 100 bp and an average quality lower than Q25 in a 5 bp window. The residual adapter sequences were removed with AdapterRemoval v 2.1.5. Then, all sequences were processed using R Software (v 4.1.2; R Core Team, 2021) in the DADA2 pipeline v 1.20.0 to filter, clean, dereplicate the sequences, infer amplicon sequence variants (ASVs) on forward and reverse reads, merge pair-end reads, and remove chimeras. The taxonomic assignment of the ASVs was performed with Silva v138 database using the Naïve Bayes classifier method (Quast et al., 2013; Yilmaz et al., 2014).

3.6. Quantification of ARGs

The abundance of five target genes (*intI1, tetA, tetW, sul1* and *qnrS*) encoding class 1 integron-integrase and resistance to oxytetracycline, sulfadiazine and ofloxacin were quantified through real-time qPCR. pGEM Easy with tetracycline-resistant genes and pNORM1 containing the other target genes were extracted from *Escherichia coli* strain CM865 and *E. coli* JM109, respectively with the QIAprep Spin Miniprep Kit (QIAGEN Inc., Venlo, Netherlands). The concentration of the purified plasmid was quantified by Qubit 4 Fluorometer (Invitrogen, MA, USA).

Plasmids were digested with FastDigest BamHI (Thermo Scientific, MA, USA) for 15 minutes at 37 °C and the linearised products were purified with the PCR purification kit (QIAGEN Inc., Venlo, Netherlands). The eluted DNA was quantified with Qubit and these products were used to do serial dilutions of the target genes from 10^8 to 10^1 number of copies/µL standard curves to generate the standard curves, being 10^1 copies/µL the LOD. 1 mL aliquots of standards were prepared with the equation 2.1.

$$\frac{nº\ of\ copies}{\mu L} = \frac{concentration\ of\ DNA\left(\frac{g}{\mu L}\right) \times Avogadro\ constant\ (\frac{nº\ of\ copies}{mol})}{amplicon\ size \times molecular\ weigh\ of\ 1\ bp\ in\ dsDNA\left(\frac{g}{mol}\right)} \quad \text{(Eq. 2.1)}$$

The qPCR analysis was performed in 96-well plates. Primer sequences, amplicon size and qPCR conditions of the different target genes are shown in Table 2.1. Each reaction was run in triplicate for DNA samples from the influent and effluents of CWs on a LightCycler 480 II platform (F. Hoffmann-La Roche AG, Basel, Switzerland). The reaction volume was 20 µL and consisted of 10 µL of LightCycler 480 Sybr Green I Master (F. Hoffmann-La Roche AG, Basel, Switzerland), 0.5 µL of both primers at 10 µM, 1 µL DNA template standardised at 20 ng/µL and 8 µL of nuclease-free water. The qPCR reactions were as described in Table 2.1 followed by the melting curve step with temperature ramping from 60 to 95 °C to confirm the specificity of the amplicon.

To normalise the data, absolute abundances were represented by the number of gene copies within 1 mL of effluent or 1 g of influent samples. The relative abundances of ARGs were also calculated by dividing the number of copies of the target gene by the number of copies of 16S rRNA.

3.7. Data analysis and statistics

Each condition in CWs was tested independently in three microcosm systems in the same greenhouse and all the chemical analyses were also performed in triplicates. Means and standard deviations were calculated.

A Shapiro-Wilk test with $p > 0.05$ was carried out to confirm the normality of the dataset. A two-way analysis of variance (ANOVA) was used. Alternatively, Kruskal-Wallis one-way anova on ranks test was performed when the normality test was violated. A multiple comparison Tukey test was run to determine differences that were statistically significant between treatments and 14-days CWs treatment cycles for a 95 % of confidence level with Sigmaplot software v 14.0. The removal efficiencies of the pollutants were calculated according to the equation 2.2.

$$Removal\ efficiency\ (\%) = \frac{\text{Cin} - \text{Cout}}{\text{Cin}} \times 100 \quad \text{(Eq.2.2)}$$

where C_{in} and C_{out} are the concentrations of the target pollutant entering and leaving the different systems, respectively. Whenever the compound was not detected in the CWs effluent, the removal efficiency was calculated considering the value of the LOD of the respective analytical methodology for C_{out}.

Table 2.1 qPCR primers and conditions used in this study

Target genes	Primer sequence (5'to 3')	References	Mechanism	Amplicon size (bp)	qPCR conditions
tetA	F: GCTACATCCTGCTTGCCTTC R: CATAGATCGCCGTGAAGAGG	Ng et al., 2001	Efflux	210	95 ºC - 10 min (1 cycle); 95 ºC - 30 seg, 60 ºC - 60 seg (40 cycles)
tetW	F: GAGAGCCTGCTATATGCCAGC R: GGGCGTATCCACAATGTTAAC	Aminov et al., 2001	Degradation enzyme	168	95 ºC - 10 min (1 cycle); 95 ºC - 30 seg, 60 ºC - 60 seg (40 cycles)
sul1	sul1-FW: CGCACCGGAAACATCGCTGCAC sul1-RV: TGAAGTTCCGCCGCAAGGCTCG	Pei et al., 2006	Protection	162	95 ºC - 5 min (1 cycle); 95 ºC - 10 seg, 60 ºC - 30 seg (35 cycles)
qnrS	qnrSrtF11: GACGTGCTAACTTGCGTG qnrSrtR11 TGGCATTGTTGGAAACTT	Marti & Balcázar, 2013	Protection	118	95 ºC - 5 min (1 cycle); 95 ºC - 15 seg, 60 ºC - 1 min (45 cycles)
intI1	IntI1LC5: GATCGGTCGAATGCGTGT IntI1LC1: GCCTTGATGTTACCCGAGAG	Barraud et al., 2010	Class 1 integrase protein	196	95 ºC - 10 min (1 cycle); 95 ºC - 15 seg, 60 ºC - 1 min (45 cycles)
V3 region 16S rRNA	331F: TCCTACGGGAGGCAGCAGT 518R: ATTACCGCGGCTGCTGG	Nadkarni et al., 2002		195	95 °C - 10 min (1 cycle); 95 °C - 15 s, 60 °C - 1 min (45 cycles)

All bioinformatic analysis were performed with R software (v 4.1.2; R Core Team, 2021) and plotted with MicrobiomeAnalyst 2.0 (Lu et al., 2023). The number of raw reads from influent and effluent DNA samples ranged between 49400 and 137947 reads and after processing through the DADA2 pipeline the number of sequences decreased to between 34920 and 97368, per sample (Table 2.2). The alpha and beta diversity analysis were run with phyloseq package v 1.38.0 rarefying the number of ASV to 34920 reads (the lowest number). On the one hand, the alpha diversity indexes analysed were the observed ASVs, Shannon and Simpson indices at a featured level. On the other hand, beta diversity was studied with a non-metric multidimensional scaling using the Bray-Curtis index on the rarefied data followed by total sum scaling and removal of singletons. Dissimilarities between the ASVs distribution were examined with a permutational multivariate analysis of variance (permanova) with 999 permutations and with an analysis of similarity (anosim) with the vegan package v 2.5.2. Besides, the taxonomic composition of the microbial communities was performed at a phylum and genus level, also with the phyloseq package v 1.38.0. Genera associated with potential pathogenic bacteria were listed according to the 10 bacterial genera housing most pathogen species list (Bartlett et al., 2022), and then, its relative abundance in the CWs influent and effluent was calculated.

Table 2.2 Number of reads of Illumina libraries the beginning and the end of DADA2 pipeline of the influent and effluent samples of the second, fourth and sixth treatment cycles. C, OX, SD and OF indicate the four different liquid digestates to be treated (with or without antibiotics).

Cycle		**2nd**		**4th**		**6th**	
Dada2 pipeline		**Input**	**Output**	**Input**	**Output**	**Input**	**Output**
Influent	1	93629	76202	64971	53308	60649	49961
	2	99251	80125	72538	60595	53375	44319
Effluent	C1	94818	63676	72622	53613	72439	53284
	C2	82870	52535	57200	40247	85953	66406
	C3	137947	97368	76385	57160	116214	92452
	OX1	70060	45205	78794	57319	96813	77543
	OX2	91713	62175	54521	38018	90872	67618
	OX3	94447	58507	49400	37736	119973	94364
	SD1	82599	55474	57129	41440	88797	68457
	SD2	127431	78684	88961	66676	101367	82028
	SD3	66469	43605	52875	34920	104898	84894
	OF1	121236	90726	68574	50003	112247	90788
	OF2	115008	79543	61811	47500	97730	80688
	OF3	83783	63127	66660	45167	81281	65152

4. Results and discussion

4.1. Initial characterisation

The six LFDs collected in the biogas plant to be treated in CWs exhibited very stable physicochemical characteristics despite being collected over a four-month period with seasonal shifts and operational adjustments of the biogas plant (Table 2.3). These effluents presented an average total solids content of 9.6 %, density of 0.95 g/cm^3, pH of 8.15, electrical conductivity of 34 mS/cm, and COD of 73 g/L, values consistent with previous reports (Akhiar et al., 2017). The concentrations of metals and nutrients in these LFDs are presented in Table 2.4. Ammonium, nitrate, nitrite, and phosphate ions amount in LFDs were, on average, 2220, 89, 2.7 mg N/L, and 156 mg P/L, respectively. Moreover, iron levels exceeded the recommended concentration for irrigation water by more than 150 times, while the concentration of zinc, copper, manganese, and chromium was over 20 times the limits in the guidelines (World Health Organisation, 2006). Consequently, the collected LFD did not meet the minimum quality standards of the Water Reuse Regulation (European Commission, 2020b), and exceeded the irrigation guidelines set by WHO for all the measured metals, except for lead (World Health Organisation, 2006), and by APA for total nitrogen and phosphorus (Ministério da Economia, 2015). Hence, proper treatment of LFD was needed to meet all these standards.

Table 2.3 Average physicochemical parameters (± standard deviation, n = 6) of the liquid fraction of digestate (during the six 14-day cycles) before treatment.

Parameter	**Value**
Total solids (TS)	9.6 ± 0.4 %
Volatile solids	51 ± 1 % TS
Inert particles <0.5 mm	20 ± 4 % TS
Density	0.95 ± 0.04 g/cm^3
Temperature	23 ± 3 ºC
pH	8.15 ± 0.06
Conductivity	34 ± 1 mS/cm
COD	73 ± 4 g O_2/L

Table 2.4 Average concentration (± standard deviation, n = 18) and standard deviation of metals and nutrients measured in the six initial liquid fractions of digestate (LFD; analysis in triplicate) and water quality standards guidelines of World Health Organisation (WHO) and Environmental Portuguese Agency (APA) for recommended metal and nutrient levels in irrigation with reused water (World Health Organisation, 2006; Ministério da Economia, 2015).

Metal	**Concentration in LFD (mg/L)**	**WHO metal level (mg/L)**
Fe	769 ± 93	5.0
Zn	40 ± 3	2.0
Cu	11 ± 1	0.2
Mn	7.4 ± 0.8	0.2
Pb	4.6 ± 0.9	5.0
Cr	2.7 ± 0.7	0.1
Ni	2.1 ± 0.3	0.2
Nutrient	**Concentration in LFD (mg N/L or mg P/L)**	**APA nutrient level (mg N/L or mg P/L)**
NH_4^+-N	2220 ± 119	10
NO_3^--N	89 ± 17	TN = 15
NO_2^--N	2.7 ± 1.5	TN = 15
PO_4^{3-}-P	157 ± 36	TP = 5

4.2. CWs treatment efficiency

4.2.1. Physicochemical parameters

The pH variation and the COD removal percentage are presented in Figure 2.2A. On the one hand, during CWs treatment, pH decreased from above 8 to a range between 7.1 (in the sixth cycle) and 8.0 (in the fifth cycle), with significant variations among the effluents of different cycles. No significant differences were observed within each cycle among treatments, except in the fifth cycle. On the other hand, CWs microcosms showed effective COD removal, ranging from 82 to 98 %, always below 2 g O_2/L in CWs effluent (Table A.1). Although no significant differences were found among treatments within any cycle, significant variations were observed between cycles. These removal rates were in line with other studies of two hybrid pilot CWs treating digestate from a digester fed with livestock waste, where the percentages of reduction of COD were 88 and 89.2 % (Comino et al., 2013; Maucieri et al., 2016). However, other studies treating LFD with CWs presented lower removal rates of COD, between 52 and 73 % (Wu et al., 2016;

Zhou et al., 2020). The higher removal rates observed in the present case could be attributed to the low organic loading and flow rate, specifically 8.87 g COD/m^3·d and 2.57 L/d, respectively. In vertical subsurface flow CWs, the removal of organic matter is caused by physical, chemical, and biological processes. Physical processes such as filtration and sedimentation, occurring in the substrate layers, are primarily responsible for the retention of particulate organic matter that is hydrolysed into humic-like substances, generating soluble organic matter. This soluble organic matter is oxidised and degraded by aerobic microbial metabolism (García et al., 2010).

Furthermore, the concentration of ammonium and phosphate ions in CWs effluents were below 10 mg N/L in cycles 1, 2, 3, and 6, and below 1.0 mg P/L in all cycles and treatments, respectively (Table A.2). Results of nutrient removal from the LFD are shown in Figure 2.2B-E. CWs removed over 98 % of ammonium and phosphate, 69 % of nitrate, and 90 % of nitrite ions, with no significant differences among treatments, except in the fifth cycle where significant differences were observed between both C and OX, with OF treatment. Similar results have been obtained in other studies treating LFD in CWs (Comino et al., 2013; Nakamura et al., 2017), and in studies, previously published by the authors, treating wastewater spiked with antibiotics (Almeida et al., 2017b; Santos et al., 2019), indicating consistent nutrient removal across different experimental conditions, independently of the presence-absence of tested antibiotics in the influent.

The mechanisms involved in nutrient removal encompass a combination of mechanical and biogeochemical processes, including sedimentation, adsorption, volatilisation, chemical precipitation, nitrification-denitrification, plant, and microbial uptake, and rhizofiltration (Kamilya et al., 2022). On the one hand, phosphate ions are removed primarily through sorption, sedimentation, and plant uptake (Vymazal, 2007). On the other hand, nitrogen removal is mainly driven by microorganisms through ammonification, nitrification, and denitrification (Wang et al., 2022b). Vertical subsurface flow CWs, characterised by higher oxygen capacity, exhibit enhanced removal of ammonium ions compared to horizontal subsurface flow CWs (Kamilya et al., 2022; Vymazal, 2007). In the present study, despite the daily recirculation to promote aerobic conditions, there are still some anoxic zones in the systems that could favour conditions for facultative anaerobes to facilitate denitrification processes, thus explaining the variability in nitrate and nitrite concentrations observed among systems. However, from the third cycle onwards, there was a tendency for a slight decrease on nitrate and nitrite removal for all the systems, being the removal rates of nitrates in OF systems the lowest. Previous studies have reported that 10 µg/L of ofloxacin reduced the nitrate removal

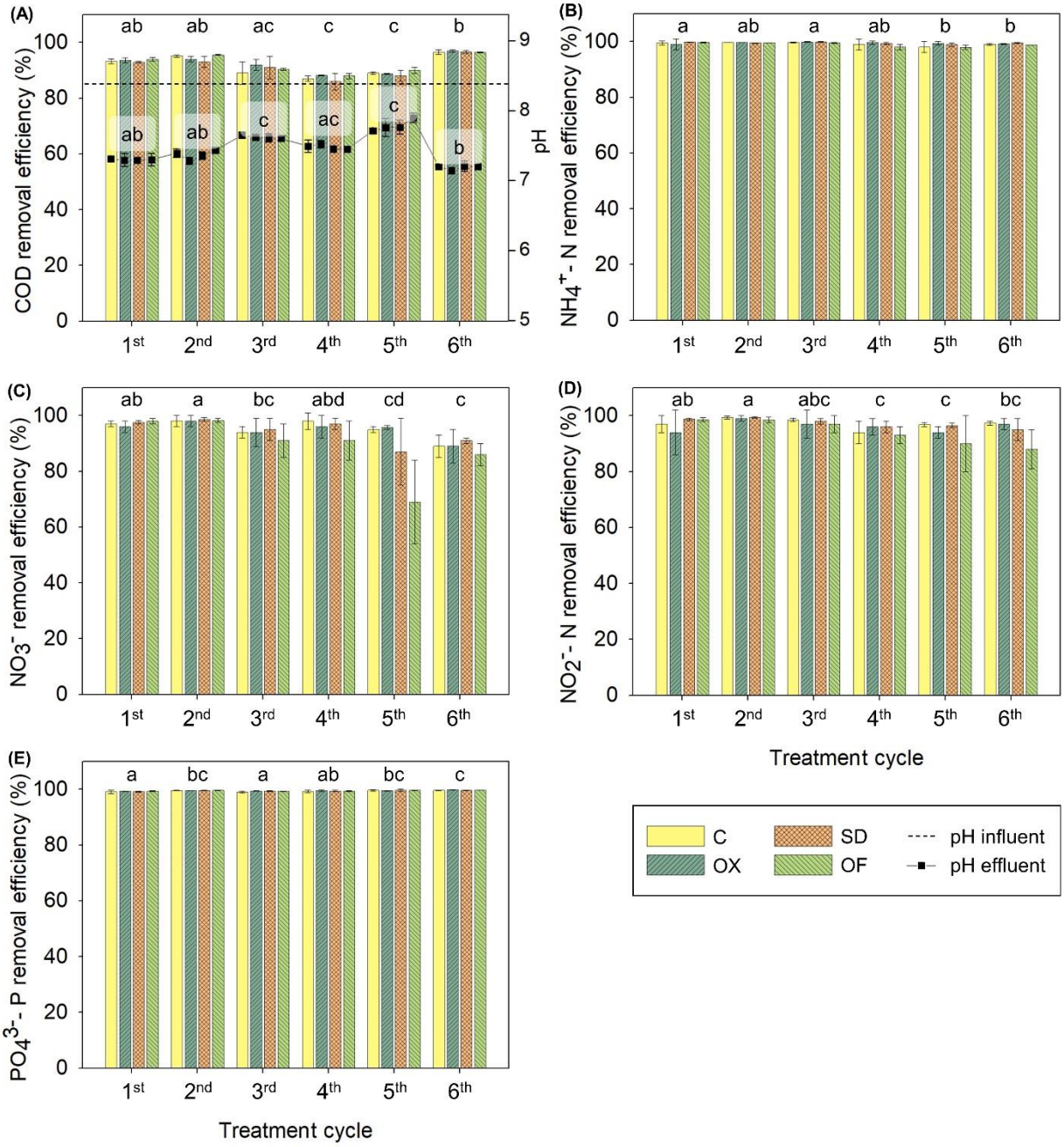


Figure 2.2 Average removal percentages of organic matter (A), ammonium (B), nitrate (C), nitrite (D) and phosphate (E) ions in CWs treating in parallel the 4 different LFD during six 14-days treatment cycles. In A, the dashed line indicates the average pH of the six influents and the dots represent the average pH of the effluents for each treatment. There were no significant differences in pH, COD or nutrient concentration between the different LFD except in the 5th cycle where OF treatment presented significant differences with both C and OX treatment. Only statistical data regarding the treatment cycles is presented and the same letters indicate that subsets are not significantly different at $P < 0.05$ by ANOVA on ranks.

denitrifying bacteria activity (Tong et al., 2019; Zhang et al., 2022).

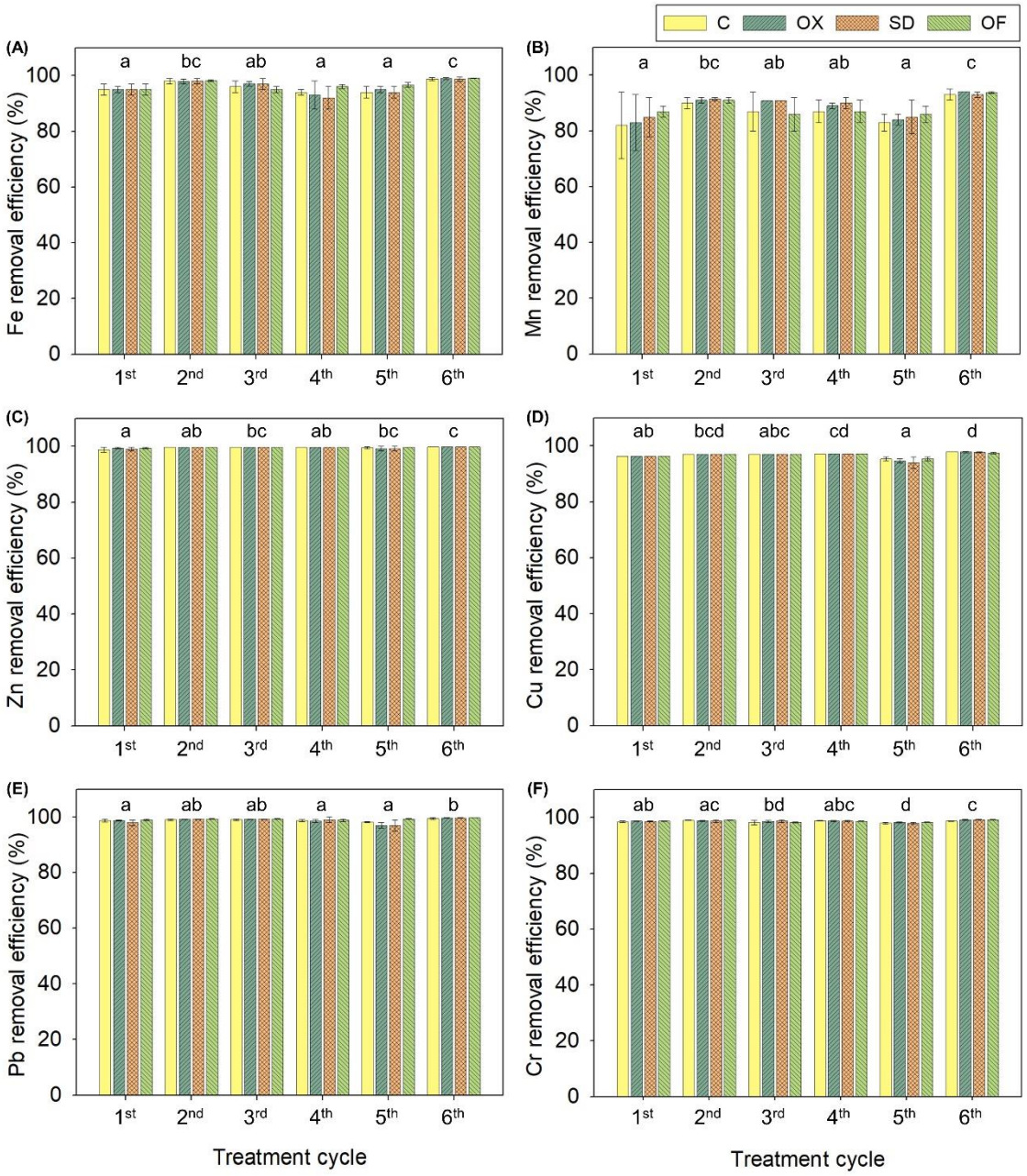


Figure 2.3 Average removal percentages of metals in CWs treating in parallel the 4 different LFDs during six 14-days treatment cycles: iron (A), manganese (B), zinc (C), copper (D), lead (E), and chromium (F). The same letters indicate that the cycles subsets are not significantly different at $P < 0.05$ by ANOVA on ranks (there were no significant differences between LFD treatments).

In addition, in this study, CWs showed also high removal efficiency of metals (Figure 2.3). Over 94 % of zinc, copper, lead, and chromium were removed in all cycles from all LFD after CWs treatment. Regarding the removal of iron and manganese, the second and sixth cycles exhibited the highest rates, over 98 % and 90 %, respectively, while in the other cycles, the removals were slightly lower ranging between 92 and 97 % for iron, and between 82 and 91 % for manganese. No significant differences among treatments were observed, except for copper and lead, where its removals in the fifth cycle presented differences. Overall, the concentrations of manganese, zinc, copper, lead, and chromium in the effluent were in all cases below the concentrations of the WHO guidelines for irrigation water, the highest concentrations being 0.2, 0.07, 0.09, 0.006, and 0.009 mg/L, respectively (Table A.3). However, the concentration of iron exceeded the recommended limits in the third, fourth and fifth cycles reaching concentrations up to 9 mg/L. High removals of metals in CWs have been published in systems treating pig industry effluents, with removals over 85 % for iron, zinc and copper and slightly lower removals of manganese too (Almeida et al., 2017a). The main processes contributing to the removal of metals from effluents are sedimentation, filtration, adsorption, (co-)precipitation, plant ad/absorption and microbial immobilisation (García et al., 2010; Yu et al., 2022).

4.2.2. Antibiotics

In CWs effluents, the concentration of oxytetracycline, sulfadiazine and ofloxacin was below the LOD in all cycles and treatments, indicating a removal efficiency above 99 %. These high antibiotic removals are in accordance with previous studies evaluating the performance of CWs removing veterinary antibiotics from livestock wastewater (Almeida et al., 2017a; Carvalho et al., 2013; Santos et al., 2019). These high removals can be attributed to several factors: the vertical subsurface flow configuration facilitating the chemical oxidation and the growth of aerobic communities; a high HRT promoting biodegradation of antibiotics by microbial communities; the combination of different substrates (sand, LECA and gravel) promoting filtration and adsorption of molecules with different chemical properties. Also, the presence of *S. erectum* might enhance plant uptake, microbial growth, and adsorption too (Y. He et al., 2021; M. Lv et al., 2022). However, biodegradation can be the principal removal mechanism of oxytetracycline, sulfadiazine and ofloxacin, driven by various microorganisms and different metabolic pathways (J. Ma et al., 2022). Other complementary removal pathways could be *S.*

erectum uptake by water transport and passive absorption for oxytetracycline (because of its low octanol-water partition coefficient), methylation and oxidation for sulfadiazine, and formation of complexes with dissolved organic matter for ofloxacin, further degraded by the carbon metabolism and denitrifiers (Lv et al., 2022; Liu et al., 2023).

Although antibiotics can alter microbial communities and have toxic effects to both microorganisms and plants in CWs (Ohore et al., 2022), that was not the case for the present study. At the tested antibiotic concentration and CWs conditions, there were no significant differences in the removal of COD, ammonium and phosphate ions and metals. These findings indicate that the presence of antibiotics did not negatively affect the performance of CWs systems. The fact that there were generally no discernible changes in the concentrations of the many parameters examined over time suggests that the system remained functional throughout the three months of the experiments. Following the removal kinetics proposed by A et al. (2021), the amount of antibiotics could have been rapidly adsorbed and removed from the effluent (in 1 day approximatively), explaining the absence of significant differences between treatments.

4.3. Prokaryotic community diversity and composition

4.3.1. Diversity analysis

The prokaryotic community diversity within CWs influent and effluent samples is summarised by analysing the richness with the observed ASVs, and other diversity indexes, namely Simpson and Shannon indexes (Figure 2.4A). Around 400 different ASVs were detected in influent samples whereas 1150 on average were observed in the effluent (Figure 2.4A). Thus, samples after the treatment in CWs exhibited higher richness in microbial community (almost 3 times more) than the LFD before the treatment.

However, both influent and effluent samples presented Simpson index values close to 1, indicating low species evenness, where few groups dominated the community. Figure 2.4A shows that communities in the effluent were less even than in the influent.

Regarding Shannon index, which considers both the richness and evenness of ASVs within a sample, there was a significant increase in diversity in the effluent compared with the digestate samples before treatment (Figure 2.4A).

The beta-diversity analysis of the LFD before and after the treatment in CWs was performed through a non-metric multidimensional scaling using the Bray-Curtis index. Figure 2.4B showed significant dissimilarities between the community structures of the

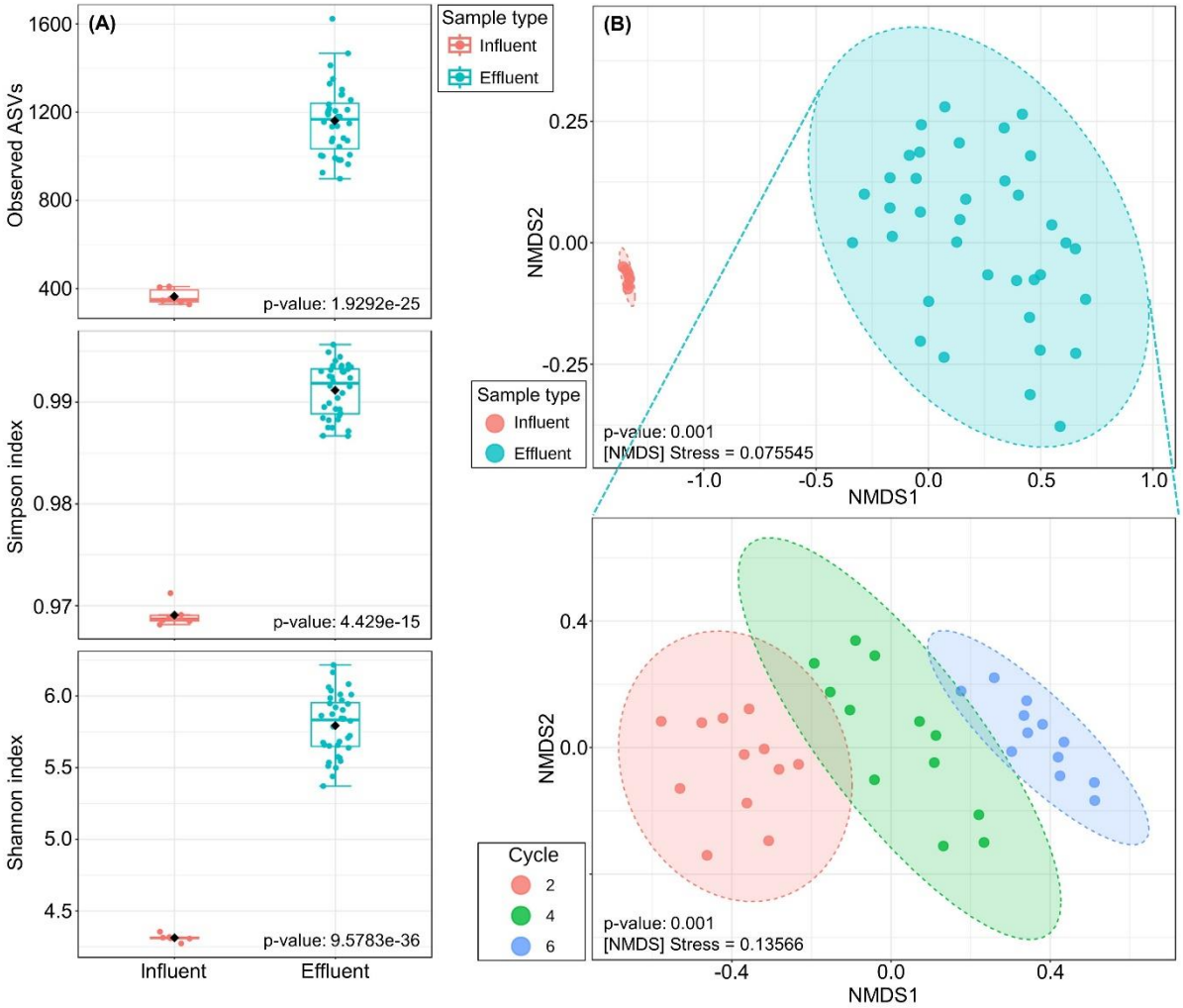


Figure 2.4 (A) Box plots of three alpha diversity indexes, observed ASVs, Simpson index, and Shannon index, in the LFD before and after the 14-days treatment in CWs of the second, fourth and sixth CWs treatment cycles. The bottom, centre, and top of each box correspond to the 25th, 50th, and 75th percentiles, respectively and error bars show the 95% confidence range. (B) Non-metric multidimensional scaling plots with Bray-Curtis index of all the CWs samples, including both influent and effluents samples (on the top), and of the effluent samples of the 2nd, 4th, and 6th cycle (on the bottom).

CWs influent and the effluent samples, with no distinctions among the different treating LFD conditions (C, OX, SD and OF). The stress value of 0.076 confirmed the good representation in the plot of the distance matrix of influent and effluent data. When focusing on the diversity between samples after the treatment in CWs, effluent samples of the same treatment cycle were clustered together. Significant differences were,

however, observed among communities of the second, fourth and sixth CWs treatment cycles, showing distinctions in the communities along time (Figure 2.4B).

4.3.2. Taxonomic composition

CWs influent, LDF, showed a consistent microbial community over time, with 16-18 phyla identified, while in CWs effluent 34 phyla were counted on average. Figure 2.5A presents the 10 prokaryotic phyla with the highest abundance, all belonging to the bacterial domain. Before CWs treatment, the LFDs population was dominated by *Firmicutes*, accounting for 53 - 66 % of the community. This phylum is frequently detected in digestate and other livestock effluents (Bôto et al., 2023; Koniuszewska et al., 2021; Pan et al., 2023). *Cloacimonadota* and *Bacteroidota*, with relative abundances of 9 and 8 % on average, respectively, were the following phyla more abundant, also reported previously in digestates (Blasco et al., 2022; Koniuszewska et al., 2021; Pan et al., 2023). On the one hand, *Firmicutes* and *Bacteroidota* are well-known for their ability to break down volatile fatty acids (VFA), and they can tolerate variations in temperature, pH, and oxygen levels. Additionally, they are characterised by their potential for hydrolysis and hydrogenogenic acidogenesis. *Bacteroidota* are not only involved in protein degradation but also produce lytic enzymes and acetic acid and are commonly found in anaerobic digestion processes using various substrates (Koniuszewska et al., 2021). On the other hand, *Cloacimonadota* bacteria, commonly found in engineered and wastewater systems, exhibit acetogenic and fermentative metabolism. They contribute to the carbon and energy cycling processes and are involved in the degradation of lipids and long-chain VFA (Johnson and Hug, 2022).

Different trends in relative abundances were noted between the CWs influent and effluent bacterial communities, with no discernible pattern between the different treatments (C, OX, SD and OF) in each cycle (Figure 2.5A). After the treatment in CWs, *Patescibacteria* became the most abundant phylum (between 22 and 59 %), and while no studies have been found, to the best of our knowledge, that specifically identify *Patescibacteria* as the dominant phylum in CWs effluents, its presence has been reported in the episphere of *Vallisneria natans* leaves in CWs treating water contaminated with erythromycin and in the rhizosphere of *Iris pseudacorus* in CWs treating wastewater spiked with enrofloxacin (Chen et al., 2023; Ramdat et al., 2022). *Patescibacteria*, formerly referred to as Candidate Phyla Radiation, represents a superphylum characterised by being obligate fermenters and playing important roles in subsurface carbon and nitrogen cycling. These microorganisms are commonly found in groundwater environments with a preference for oxic conditions and planktonic growth

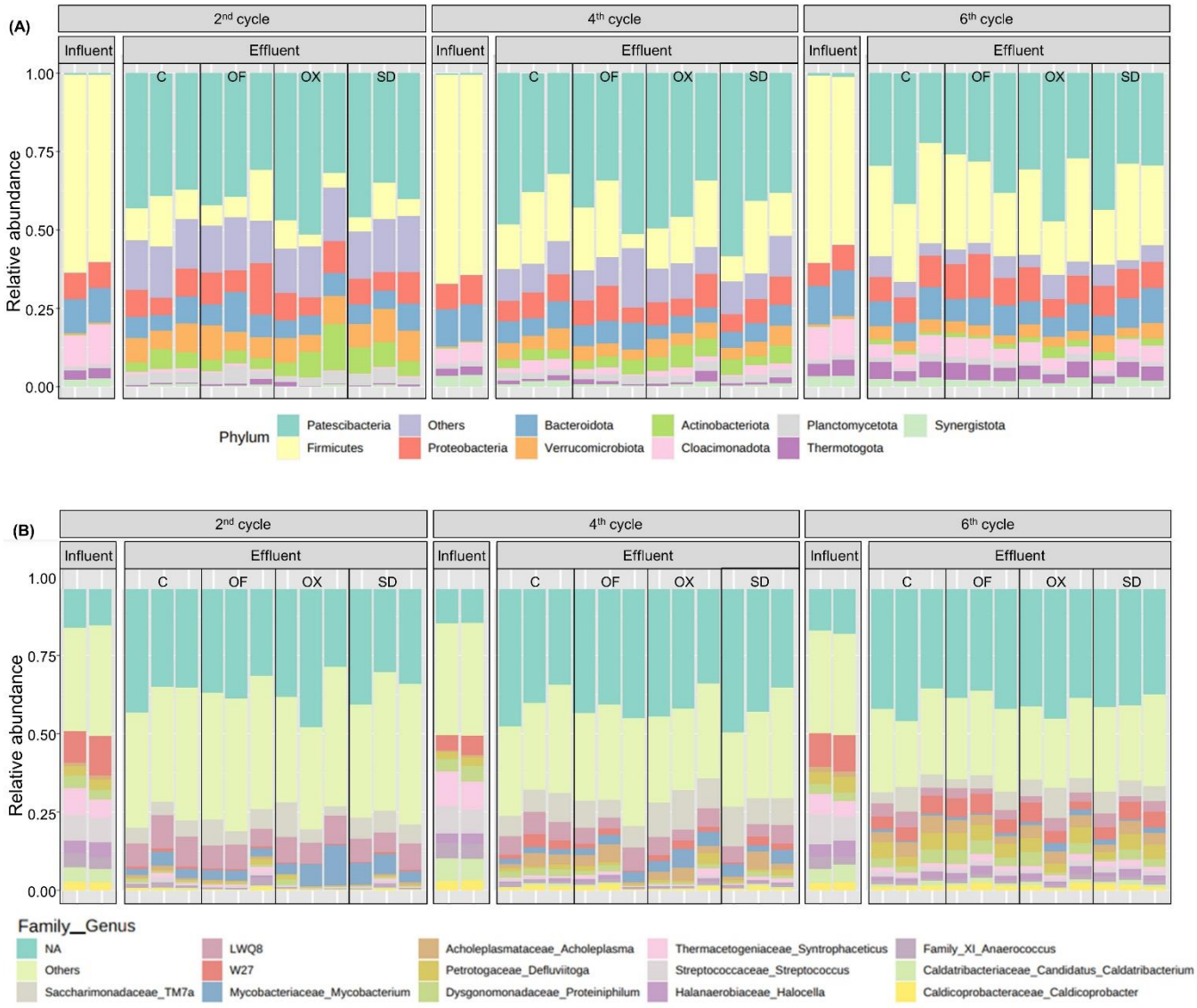


Figure 2.5 Taxonomic profile of prokaryotes in the influent and effluent of CWs based on the most relatively abundant at the phylum level (A, top 10 phyla), and at the family and genus level (B, top 14 genera). Each black box shows the replicates.

(Danczak et al., 2017; Gios et al., 2023). In this study, this abundant phylum was followed by *Firmicutes, Proteobacteria, Bacteroidota*, and *Verrucomicrobiota. Proteobacteria*, widely detected in CWs substrates and effluents, comprises the main functional microorganisms involved in the removal of organic matter, nitrogen, and antibiotics from various types of wastewaters (Bôto et al., 2023; Wang et al., 2022b).

Along CWs treatment cycles, a diminishing trend was observed for *Patescibacteria, Proteobacteria, Verrucomicrobiota* and *Actinobacteria* in the effluents. On the contrary, *Firmicutes'* relative abundance in samples of the last cycle (17 – 33 %) increased in comparison with the second one (3 – 17 %), and *Bacteroidota, Cloacimonadota* and

Thermotogota showed a similar rising trend. The increase of *Firmicutes* and *Bacteroidota* in the effluent over time suggested a potential loss of CWs removal capacity of microorganisms from the influent or an enhancement of their growth conditions within the systems. These two phyla, commonly detected in CWs, are also related with the removal of pollutants (Wang et al., 2022b).

At the genus level, prokaryotic communities of the CWs influent and effluent presented different taxonomic profiles (Figure 2.5B). In both influent and effluent samples, non-assigned taxa and less abundant ASVs (other than the 14 most abundant genera) were predominant in the community. *Streptococcus* and *Syntrophaceticus* were the genera in CWs influent with the highest abundance (around 8 %). In addition, 6 additional bacterial genera presented a relatively even distribution in the LFD before treatment in CWs: *Candidatus* Caldatribacterium (5.7 %), *Halocella* (4.2 %), *Anaerococcus* (4.1 %), *Proteiniphilum* (3.7 %), *Defluviitoga* (3.3 %), and *Fastidiosipila* (3.0 %). These genera are commonly found in anaerobic digestors as carbohydrates degraders, sugar fermenters, proteolytic bacteria, or syntrophic acetate-oxidising bacteria (Lim et al., 2020; Kostopoulou et al., 2023).

However, in the effluent there were fewer dominant genera, namely *Mycobacterium* (1.9 – 12.9 %) and *TM7a* (2.8 – 10.9 %) after the first month of the experiment, and *TM7a* (2.9 – 9.6 %), *Acholeplasma* (2.4 – 5.5 %), *Defluviitoga* (2.3 – 5.4 %), and *Proteiniphilum* (0.7 – 4.7 %) after the third month. *Mycobacterium* was found to be involved in denitrification and in the co-metabolism of organic matter and antibiotics in CWs, and another sulfonamide, sulfamethoxazole, promoted the growth of this bacteria such (Qu et al., 2022). In this study, the relative abundance of *TM7a* and non-assigned bacteria from LWQ8 family, both belonging to the Saccharimonadales order, also increased notoriously after the treatment in CWs. Previous studies have also reported the presence of *TM7a* in CWs effluents, ranging from 2.8 to 10.9 % of the community (Cheng, et al., 2022a). Saccharimonadales, related to the carbon and nitrogen cycles, were proposed as potential bioindicators of elevated phosphorus levels and were found to be predominant in environments with high organic content. These bacteria exhibit synergistic interactions with genera associated to nitrification and denitrification (Wang et al., 2022a). Hence, the effluent microbiome showed higher abundance of genera than the influent, resulting in a bacterial diversification that potentially contributed to higher pollutant removal, enhanced different metabolic pathways, and increased stability in the ecosystem, as previously observed (Choi et al., 2022; Bôto et al.,2023).

Moreover, numerous genera detected in the effluent are related to functional microorganisms with a crucial role in the removal of nitrogen (Saccharimonadales, *Candidatus* Nitrotoga*, Candidatus* Omnitrophus*, Denitratisoma*, *Gemmobacter*, *Thermomonas*), phosphorus (Rhodobacteraceae, Anaerolineaceae, *Dechloromonas, Acinetobacter*, and *Brevundimonas*), metals (*Desulfovibrio, Geobacter, Sideroxydans, Hydrogenophaga,* and *Chryseobacterium*) and antibiotics (Wang et al., 2022a). Certain bacterial genera detected in effluents of OX systems namely Comamonadaceae bacteria, *Dechloromonas*, *Thiobacillus* and *Mycobacterium* were reported to be involved in oxytetracycline degradation. *Bacillus*, *Geobacter*, and unclassified Gemmatimonadaceae bacteria, detected in low abundances in SD systems effluents, were involved in sulfadiazine degradation. *Rhizobacter* and *Bacteroides*, detected in OF systems effluents, were associated with ofloxacin degradation (Chen et al., 2019b, 2022; Wang et al., 2022b).

4.4. Removal of potential pathogens and ARGs

Although anaerobic digestion (especially at thermophilic conditions) is effective in reducing most of the pathogens from livestock and other organic waste, residual pathogenic bacteria are still present in LFD posing a risk when reusing these effluents in agriculture. Pathogens commonly found in digestates are coliform bacteria, *Salmonella, Staphylococcus aureus, Mycobacterium paratuberculosis*, and *Streptococcus*. *Streptococcus faecalis* is an indicator of the sanitation efficiency of digestates because is one of the most resilient organisms in anaerobic digestion processes compared to other hazardous bacteria, viruses, and parasites (Seadi et al., 2010). In this study, *Streptococcus* and *Clostridium* (sensu stricto 1, 8 and 15) were the potential pathogenic genera detected in LFD, the former in high abundance (Table A.4). The relative abundance of *Streptococcus* decreased with CWs treatment, with removal percentages averaging 94%, 96%, and 85% in the second, fourth, and sixth treatment cycles, respectively (Figure 2.6), suggesting a persistence of this potential pathogen ranging from 6% to 15%. A similar tendency was observed for *Clostridium* with removals of 97%, 99% and 94 % on average in these treatment cycles, suggesting a persistence of this potential pathogen ranging from 1% to 6%. Although associated with pathogenic bacteria, both genera are also involved with essential metabolic pathways in anaerobic digestion processes. *Streptococcus* can be strictly fermenters producing VFA, ethanol, H_2 and CO_2 and *Clostridium* can contribute to biomass breakdown, participate in acetogenesis, and produce various extracellular enzymes that degrade biopolymers, leading to improved methane production (Yao et al., 2019; Zhang et al., 2017).

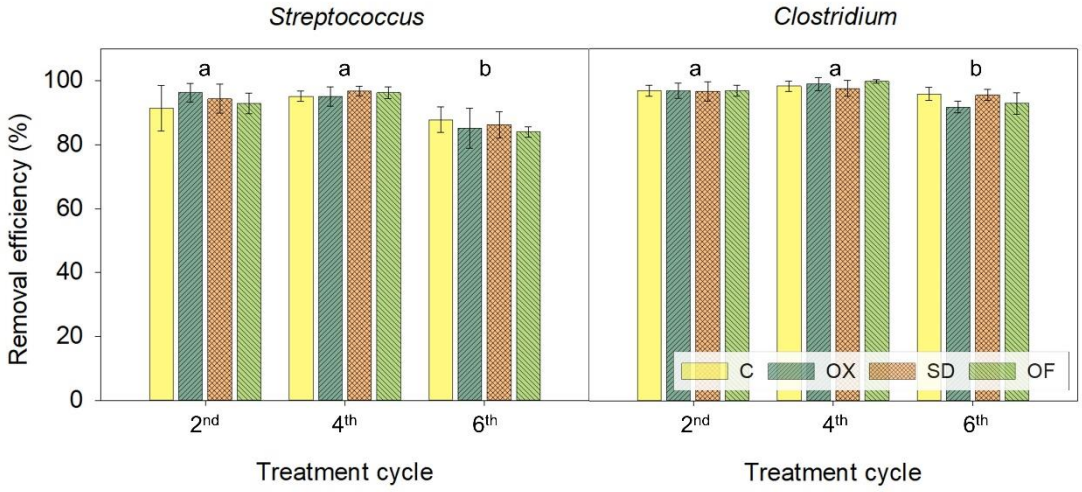


Figure 2.6 Average removal percentages of *Streptococcus* and *Clostridium* genera in CWs treating in parallel the 4 different LFDs during the second, the fourth and the sixth 14-day treatment cycles. The same letters indicate that the cycles subsets are not significantly different at $P < 0.05$ by two-way ANOVA (there were no significant differences between LFD treatments).

Other contaminants with growing concern in wastewater treatment processes are ARGs. Despite the removal of ARGs and MGEs during anaerobic digestion, these genes can remain in the LFD (Gurmessa et al., 2020). The potential transfer of ARGs to bacterial pathogens poses a significant global public health issue. However, the present study reveals a notable reduction of ARGs absolute abundances (10 to 1,000 times) after LFD treatment in CWs, in line with the substantial decrease of the total bacteria marker (*16S RNA*), from 7 x10^{10} in the influent to around 1.8 x10^{8}. This indicates a huge reduction of bacteria in the effluent (Figure 2.7). *intI1* genes concentration in the influent was 6.7 x10^{5} copies/mL on average, which further decreased to 7.0 x10^{4} copies/mL, on average, after the treatment. Also, *intI1* relative abundance among the bacterial community was almost negligible (Figure 2.8), suggesting poor potential for MGEs transference. The measured ARGs were also in a very low abundance in the microbial community of the CWs influent. More specifically, the resistance genes to oxytetracycline (*tetA* and *tetW*) were notably reduced 100 and 1,000 times (from 5.6 x10^{5} to 9.8 x10^{3}, and from 8.7 x10^{7} to 6.8 x10^{4} copies/mL), respectively, on average, in line with prior research (Huang et al., 2017). Nonetheless, the removal rates of these genes exhibited a decreasing trend across successive cycles. Additionally, *sul1* gene, encoding resistance to sulfadiazine, was reduced along CWs treatment from 4.3 x10^{6} to 6.9 x10^{4} copies/mL on average. Finally, *qnrS* gene abundance, encoding resistance to ofloxacin, was below the LOD in the

influent and in the effluent of the second and fourth cycles. In the sixth cycle, only

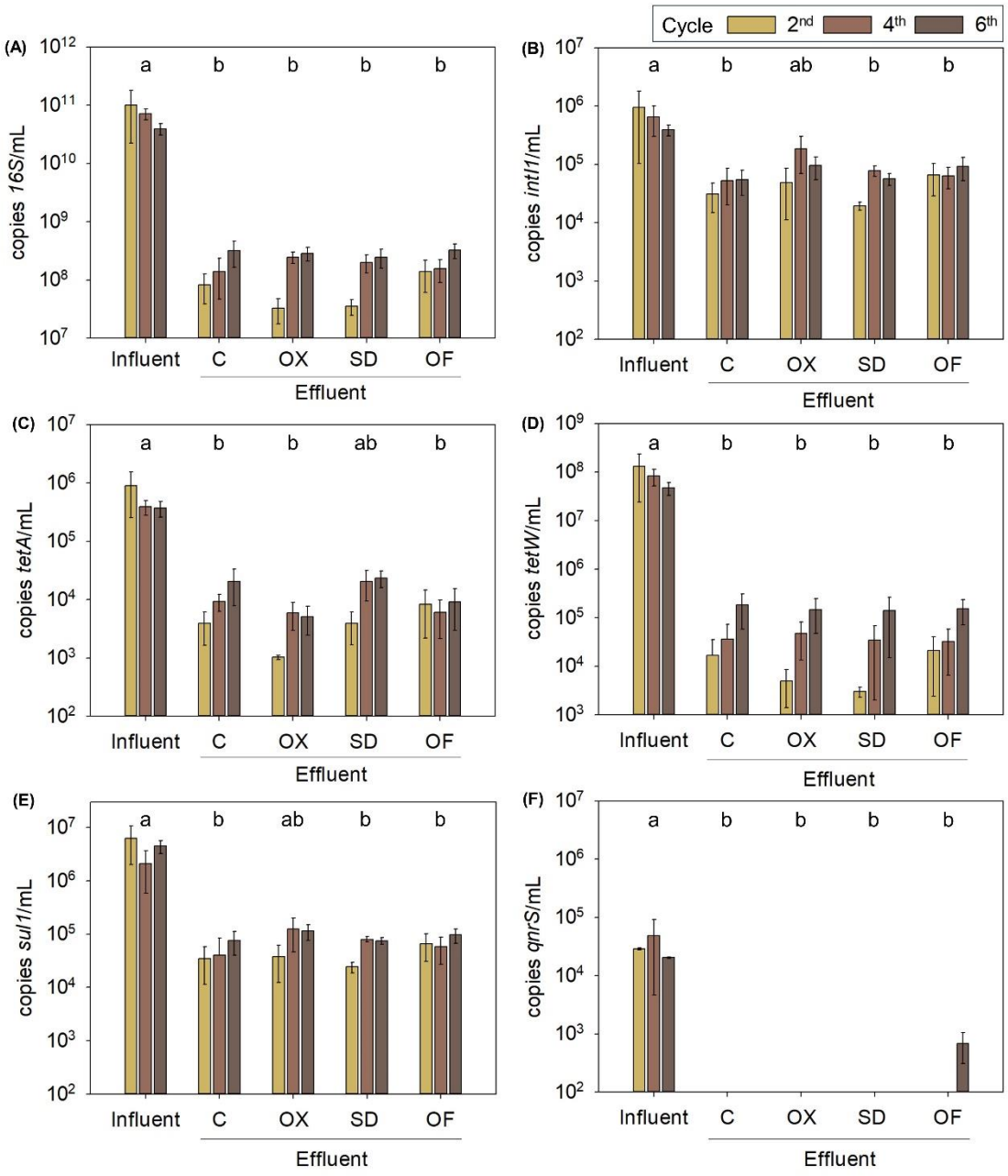


Figure 2.7 Absolute abundance of V3 region of *16S rRNA* (A), *intI1* (B), *tetA* (C), *tetW* (D), *sul1* (E), and *qnrS* (F) genes in LFD before (influent) and after (effluent) the treatment in CWs. In the legend, numbers 2nd, 4th and 6th indicate the number of the two-week treatment cycle. The letters correspond to the subsets that are not significantly different at $P < 0.05$ by ANOVA on ranks.

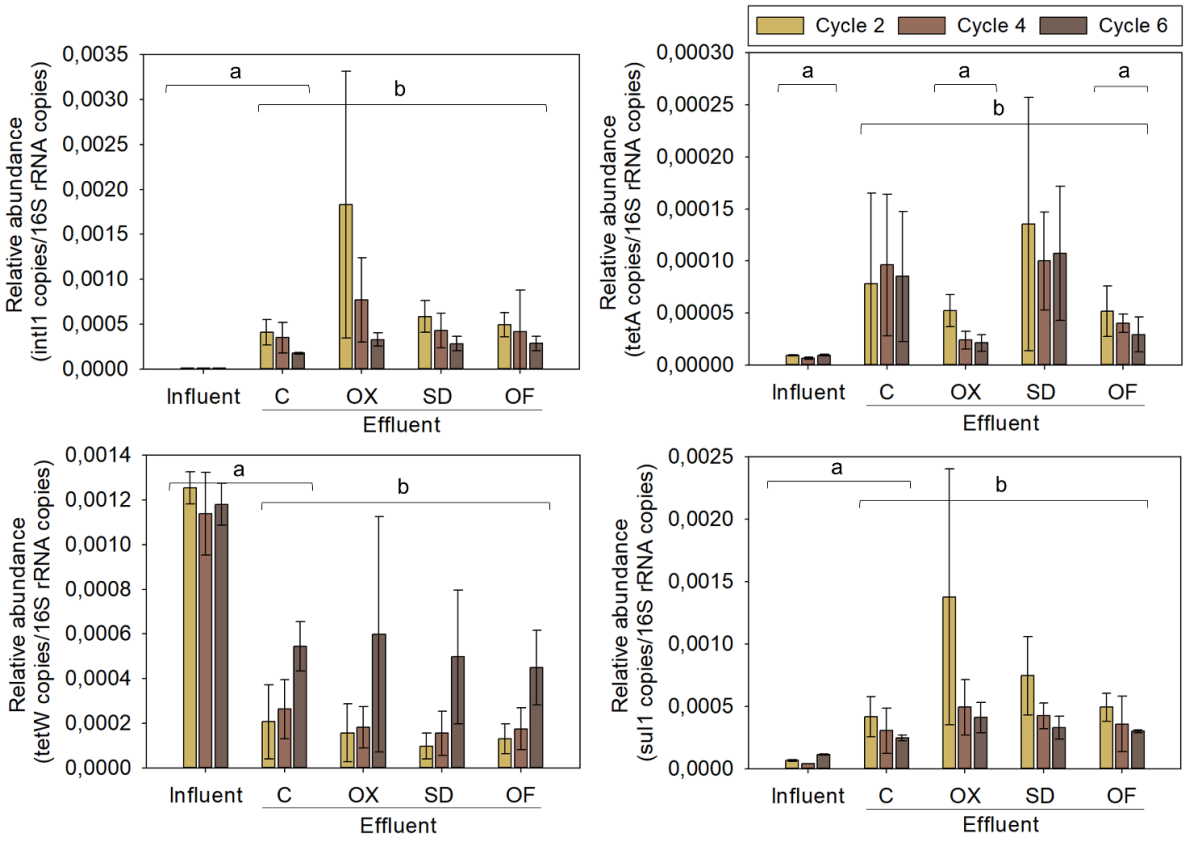


Figure 2.8 Relative abundance of ARGs in LFD before (influent, n = 6) and after (effluent, n = 36) treatment in CWs microcosms experiments. In the legend, 2, 4 and 6 indicate the number of the 14-days treatment cycle when the samples were collected. C, OX, SD, and OF correspond to the different treatment conditions in CWs. The letters correspond to the subsets that are not significantly different at $P < 0.05$ by ANOVA on ranks.

systems treating LFD spiked with ofloxacin showed a concentration of *qnrS* gene copies above the LOD, reaching 6.2 x10^2 copies/mL. The increase in *qnrS* was also observed in Sun and Zheng (2023).

ARGs could have been eliminated mainly through plant uptake, die-off of bacterial hosts or sorption to organic matter (Sabri et al., 2021). The low ARGs discharge in CWs potentially minimised the transference of ARGs to pathogens. However, previous studies have shown that ARGs relative abundance can increase in CWs treating wastewater with antibiotics (Ohore et al., 2022). In fact, this was also observed in the present study, as the relative abundance of *intI1, tetA* and *sul1* increased in CWs effluents compared to CWs influent with no significant differences between the presence or absence of antibiotics (Figure 2.8), suggesting that the selective pressure of antibiotics was not the only mechanism promoting this increase. Metals and high HRT (considering 14 days as

a high HRT) are two factors that could have induced ARGs proliferation (Ohore et al., 2022). An option to reduce the ARGs dissemination in the environment in the long term could be to combine the systems with advanced treatment technologies such as advanced oxidation processes or membrane filtration (Monsalves et al., 2022).

Nevertheless, the removal of pathogens and bacteria with ARGs in CWs is driven by a combination of many factors. Sedimentation has been shown to effectively remove *Streptococcus* and other bacteria with high settling velocity (Wu et al., 2016). Moreover, these removals were related to high removals of COD removals of due to the attachment of bacteria in retained organic matter particles. Other processes that could have happened are mechanical filtration in sand, adsorption mainly in LECA and plant roots, and natural die-off because of inactivation processes such as predation and starvation (Wu et al., 2016).

Overall, the high removal of all pollutants obtained could be attributed to the high HRT (14 days), as many studies reported that the residence time of treatment significantly impacts the removal of pollutants (T. Yuan et al., 2022). This study makes a valuable contribution to the application of vertical subsurface flow CWs with gravel, LECA and sand, planted with *S. erectum* as a decentralised wastewater treatment technology to treat LFD with metals and antibiotics for water reuse purposes.

Depending on the CWs' load, systems are expected to last over two decades, up to 20 years or more (Dotro et al., 2017). Previous long-term research showed that despite initial high concentrations of metals in the influent, CWs maintained high removal efficiencies, with metals often deposited in sediments in a non-bioavailable form (Knox et al., 2021) or accumulated by plants. Similarly, long-term studies reported high nutrient removal percentages in CWs (Nilsson et al., 2020). When the vegetation management techniques are appropriate, like seasonally harvesting, and eventually removal of accumulated solids, the removal efficiencies of CWs can be maintained over the years (Vymazal, 2020). Regarding scalability, although some previous pilot-plant studies showed similar removals as those achieved in lab-scale previous works, when scaling up the CW prototypes, deviations in efficiencies could be observed (Saúco et al., 2021). Hence, future research should focus on evaluating the potential of CWs to remove metals, antibiotics and ARGs from LFD at a pilot scale in the long term, optimising the operational parameters, adapted to the digestate volume produced annually by the biogas plant. Additionally, further studies testing the dimensions, the systems' shape and the hydraulic characteristics and configuration are necessary to prevent potential clogging problems or the dissemination of pollutants.

5. Conclusion

The present work studied the performance of CWs to treat the liquid effluents of anaerobic digesters, to allow its reuse in irrigation, taking into account both chemical and biological contaminants, including potential pathogens and ARGs. The results showed that CWs removed COD, ammonium, nitrates, nitrites, and phosphate ions at rates over 86, 98, 69, 90 and 98 %, respectively. The systems reduced the metal levels between 88.2 and 99.5 % for Fe, between 68.8 and 94.0 % for Mn, over 97.8 % for Zn, over 92.4 % for Cu, over 95.9 % for Pb and over 97.3 % for Cr, with no significant differences between the four treatments (LFD spiked with oxytetracycline, with sulfadiazine, or with ofloxacin or without dosing).

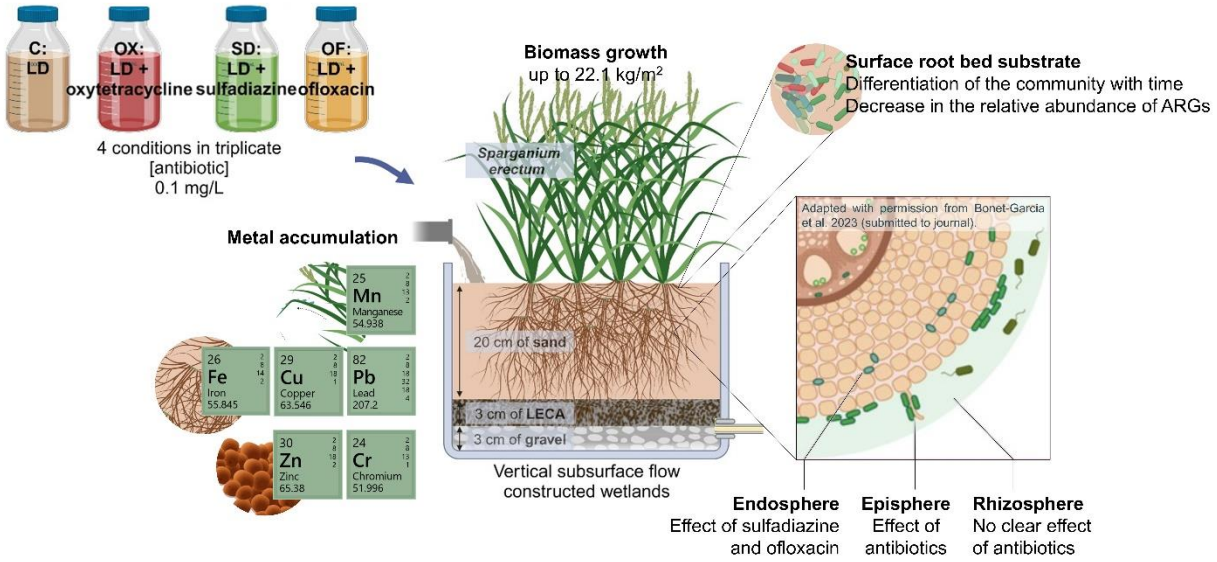
After the treatment in CWs, concentrations of oxytetracycline, sulfadiazine and ofloxacin were below the detection limit in all systems, indicating successful removal. For most of the ARG analysed (*intl1*, *tetA*, *tetW* and *sul1*), the absolute abundance decreased after the treatment of LFD in CWs. However, a slight increase in the relative abundance of some these ARG (*intl1*, *tetA*, and *sul1*) was observed, with a tendency to diminish over time.

Moreover, prokaryotic communities presented higher diversity after the treatment in CWs with significant differences between the community structures of the CWs influent and the effluent samples. Although no significant changes in the community were detected between treatments (presence or absence of antibiotics in the influent), there was a clear differentiation in the effluent's communities over time. Removal of the potential pathogenic genera were observed, above 85 % for *Streptococcus* and 94 % for *Clostridium.* Overall, CWs are a suitable alternative to valorise the liquid effluents of anaerobic digesters, allowing its reuse in irrigation, closing the loop under a circular bioeconomy model, contributing to sustainable development goals of the 2030 Agenda (SDG6, SDG7, SDG11). However, the dissemination of ARGs in the environment remains a grand challenge that needs further understanding and management for their proper removal, and wastewater treatment solutions must consider this aspect to mitigate potential risks.

Author Contributions:

PP-S: Conceptualisation, Methodology, Investigation, Validation, Formal analysis, Data curation, Writing - original draft, Visualisation; MPT: Resources, Writing - review & editing; JPF: Methodology, Resources; GC: Conceptualisation, Project administration, Funding acquisition; ABM: Validation, Writing - review & editing, Supervision; BF: Writing - review & editing, Supervision; MJA**:** Resources; RC: Resources; CRG: Resources, Writing - review & editing, Supervision; CMRA**:** Conceptualization, Methodology, Validation, Resources, Writing - review & editing, Supervision, Project administration, Funding acquisition; APM: Conceptualisation, Methodology, Validation, Resources, Writing - review & editing, Supervision, Project administration, Funding acquisition

Chapter 3. Plant–microbiome interaction in constructed wetlands treating liquid digestate – fate of metals and interference of antibiotics



This chapter is based on the manuscript that is forthcoming in publication:

Pau Porras-Socias, Joana P. Fernandes, Maria Paola Tomasino, Alexandre B. De Menezes, Gavin Collins, Belén Fernández, Carlos R. Gomes, C. Marisa R. Almeida and Ana Paula Mucha. **“**Plant–microbiome interaction in constructed wetlands treating liquid digestate – fate of metals and interference of antibiotics”

1. Abstract

Sparganium erectum, a macrophyte with phytoremediation potential, could play an important role in pollutant removal and constructed wetlands performance. However, this plant species is not common in constructed wetlands. This study aimed to understand the role of plant-microbiome association in vertical subsurface flow constructed wetlands treating liquid digestate contaminated with metals and a possible interference of antibiotics. For 86 days, systems planted with *S. erectum* filled with gravel, light expanded clay aggregate and sand, treated four different liquid digestates in parallel (control (no antibiotic spiking), spiked with oxytetracycline or sulfadiazine or ofloxacin, each at 100 µg/L). *S. erectum* presented good adaptability to the treating conditions and high biomass yield during the experiment. At the end of the experiment, Fe, Cu, and Pb were mainly present in plant roots, Mn was translocated into the plant leaves, and Zn, and Cr were predominantly captured in the clay layer. The characterisation of the microbial community revealed a temporal differentiation of the communities in surface sand layer (root bed substrate), with a decrease in the relative abundance of *intI1*, *tetA*, *tetW*, and *sul1.* The sand microbiome was dominated by *Proteobacteria*, *Actinobacteriota, Planctomycetota, Firmicutes* and *Bacteroidota*. In addition, regarding the microbial communities in *S. erectum* roots, the rhizosphere, episphere and endosphere presented significant differences in their structure. While adding antibiotics to the liquid digestate had no clear effect on the rhizosphere microbiome, sulfadiazine and ofloxacin produced significant changes in the endosphere community and the three antibiotics altered the taxonomic profile of the episphere. Therefore, this study provides valuable insights into the contribution of different constructed wetlands compartments to the contaminant removal processes during the treatment of the liquid digestate in constructed wetlands.

Enhancing the understanding of microbial communities' roles in the ecological processes of constructed wetlands will facilitate their optimization as a decentralised wastewater treatment solution in biogas plants. This includes assessing the suitability of *S. erectum* as an effective macrophyte candidate.

Keywords: Constructed wetlands, *Sparganium erectum*, metals, antibiotics, microbial communities.

2. Introduction

The centralisation of wastewater treatment has been the preferred option since the mid-nineteenth century in metropolitan areas and has expanded in rural areas as well. In these systems, wastewater is collected and transported through a large network of pipelines to a central facility requiring high operational, maintenance, and capital costs (Pasciucco et al., 2022). The centralised wastewater flows collected from many different sources (municipalities, industry, agriculture…), result in a complex, heterogeneous sewage containing a wide range of contaminants at varying concentrations (Krzeminski et al., 2019). Over the years, contaminants of emerging concern, including pharmaceuticals, personal care products, flame retardants, plasticizers, metals, and antimicrobial resistances (including antibiotic resistance bacteria (ARB) and antibiotic resistance genes (ARGs)), have been released in aquatic systems from wastewater treatment plants, posing a challenge in designing new effective wastewater treatment processes (A. I. Shah et al., 2020). Additionally, beyond preserving the water quality in receiving bodies, wastewater treatment should aim to recover resources, namely energy, nutrients, and water, aligning with the Sustainable Development Goals of the United Nations and adopting a circular economy approach (Torre et al., 2021). A solution could be to combine centralised and decentralised systems, designing appropriate on-site/off-site wastewater management (Pasciucco et al., 2022). Decentralised wastewater systems are focused on the treatment, disposal and collection minimisation of wastewater, which is very suitable for rural clusters, industrial facilities or small communities. These small-scale wastewater treatments are adapted to each wastewater type and source, and efficiently reclaim resources and minimise contamination with a sustainable approach (Pundlik et al., 2022). A promising decentralised technology due to its low-costliness, potential effectiveness, and sustainability is constructed wetlands (Fernández del Castillo et al., 2022). Constructed wetlands (CWs) are engineered systems designed to treat wastewater by mimicking natural wetlands, removing contaminants through a combination of physical, chemical, and biological reactions (Dotro et al., 2017). The systems are shallow ponds filled with substrate and wetland vegetation, promoting the growth of microbial communities, that drive the removal of organic matter, nutrients, pathogens, and other contaminants from different wastewater types (Kataki et al., 2021).

Over the past decade, the global biogas industry expanded by more than 90 %, with plans for further growth in the future, as it is a promising alternative to fossil fuels (Abanades et al., 2022). Today, although the biorefinery concept in anaerobic digestion

plants has attracted attention, the liquid fraction of the digestate (LFD) often remains an unattended ammonium-rich effluent with a high-value potential, treated with physico-chemical methods and sent to wastewater treatment (Chozhavendhan et al., 2023). It has widely been reported that anaerobic digestion effluents harbour different contaminants, namely metals, as well as other contaminants depending on waste source, such as antibiotics, ARB and ARGs (Gurmessa et al., 2020; Venegas et al., 2021), Therefore, CWs could be a good low-installation and maintenance costs secondary treatment technology to be applied on-site, at biogas plants, to treat the LFD (Maucieri et al., 2016), and promote water reuse, but CWs must be specifically designed to remove these contaminants from the complex matrix. The main removal processes of these contaminants in CWs are sorption, microbial biodegradation, plant uptake, precipitation, hydrolysis and photo-degradation, responding differently to the influent composition, environmental parameters and operational conditions (García et al., 2010; Yan et al., 2022).

Macrophytes are crucial in CWs systems, actively participating in the nutrient cycle within the system and in the removal of pollutants from wastewater. Their function in CWs is primarily indirect by transporting oxygen to the roots and rhizomes and releasing it to the rhizosphere, creating not only an aerobic environment but also support for microorganisms to grow, secreting exudates that enhance microbial processes, improving sedimentation and filtering, and insulating the substrate from low temperatures (Kataki et al., 2021). The selection of the macrophyte species is essential for effectively removing contaminants from wastewater (Kamilya et al., 2022). The requirements that plant species have to meet are the ecological adaptability and fast propagation, establishment and growth, the tolerance of local conditions (climate, diseases) and high concentration of nutrients and other pollutants, and the capacity to remove contaminants through direct or indirect processes (Scholz, 2024). *Sparganium erectum* are common monocots from the Typhales order and Sparganiaceae family found in emerged and submerged forms in aquatic environments of the north hemisphere (North America, Europe, North Africa, and Asia) and Australia (Gottsberger, 2020). Although the use of this species has gone unnoticed in CWs, previous studies reported the potential of *S. erectum* to uptake metals, having interesting applications in CWs for phytoremediation (Parzych, 2016; Senze et al., 2023). Employing uncommon macrophyte species in CWs could provide insights into how the plant interacts with the specific wastewater, its pollutants, and microbial communities, as each species has unique characteristics that affect pollutant removal efficiency (Kulshreshtha et al., 2022). By exploring new

candidate species, plants that are better suited for a specific wastewater treatment could potentially be identified, contributing to optimising CWs design.

Microorganisms have an important role in the CW removal processes of mainly nitrogen and organic compounds. The main biological reactions are biodegradation, nitrification, denitrification, nitrogen fixation and sulphate reduction (Moazzem et al., 2023). The microbial community composition is mainly determined by the wastewater type, the contaminants, the specific plant species used in the systems, the substrate type, and the hydraulic design (Moazzem et al., 2023; Vymazal et al., 2021). Microorganisms can grow in planktonic form or in biofilms, attached to plant roots or bed substrates, creating complex networks in an extracellular polymeric substances' matrix and very resilient communities against abiotic stresses and pathogens (Gebreyohannes et al., 2019; Kataki et al., 2021). Nonetheless, contaminants, namely antibiotics and metals, can have a negative impact on the CWs microbiome, by altering the community structure and function, and consequently contaminants removal efficiencies (Liu et al., 2020; Ohore et al., 2022). Most studies are focused on the performance of CWs in pollutant removal, while insights in CWs microbiome and its dynamics when removing contaminants still need further investigation. Identifying the composition and structure of the prokaryotic community over time allows to track the responses of the community to the influent and to characterise the key functional groups that are involved in biogeochemical processes and pollutant removal. This information could be valuable to improve CWs performance with management practices promoting the growth of beneficial bacteria for the system.

In addition, to our knowledge, no studies reporting the *S. erectum* (a potential excellent macrophyte species candidate in CWs) root microbiome have been published. Studying unexplored plant species in CWs contributes to understanding their interactions with microbial communities, elucidating pollutants removal mechanisms, improving CWs performance, and thus advancing research in phytoremediation in CWs, thereby fostering innovation in this field.

The aim of the study was to understand the role of the plant-microbiome association and the effectiveness of *S. erectum* within vertical subsurface flow CWs treating LFD contaminated with metals to carry out the remediation functions. For that, the capacity of *S. erectum* to grow in LFD medium and to accumulate metals in different tissues was evaluated. Additionally, the study examined the response of root-bed substrate and plant roots microbiomes (rhizosphere, episphere and endosphere), in terms of microbial community dynamics and ARGs. A possible interference of antibiotics presence was also assessed.

3. Materials and methods

3.1. CWs assembly and operation

The previous chapter was focusing on the potential of CWs to remove pollutants from LFD, while the current chapter aimed to assess the role of the different CWs compartments, including the plants, microorganisms, and substrates, in the metal removal processes, as well as the response of the systems to a possible interference of antibiotics. Twelve vertical subsurface flow CWs microcosms were assembled in polypropylene containers each with 3-4 *Sparganium erectum* plants transplanted from Ribeira da Certagem, Lavra, Portugal, and three layers of substrate: gravel in the bottom (3 cm), then, light expanded clay aggregate (LECA, 3 cm) and quartz sand on the top (20 cm), the layer to where plants were transplanted. The containers, wrapped in aluminium foil to prevent photodegradation, were kept in greenhouse conditions under a natural light-dark regime (Figure 2.1). Details of the configuration and the setup were previously described in Chapter 2, section 3.1.

The vertical subsurface flow was emulated with the influent being poured onto the surface until all substrate layers were saturated, and the effluent drained through the bottom layer collecting the effluent in a flask. To acclimate the microcosms, Hoagland nutrient solution was daily recirculated in the systems for 14 days. Then, LFD at different dilutions (with deionised water) was added to the systems. The LFD was collected from a full-scale biogas plant that manages the organic fraction of mixed municipal solid waste. The whole acclimation period lasted 52 days as described in Porras-Socias et al. (2024).

The systems were treating in parallel LFD spiked or not with antibiotics at a final concentration of 100 µg/L. Four conditions were tested: LFD without antibiotics spiking, LFD spiked with oxytetracycline, LFD spiked with sulfadiazine and LFD spiked with ofloxacin, and CWs microcosms were named C, OX, SD and OF, respectively. These antibiotics were selected because they present low removal efficiencies during the anaerobic digestion process, persisting in the LDF in concentrations ranging between 3.8 and 940 $\mu g \cdot L^{-1}$ (Gurmessa et al., 2020), and because they were classified as high-risk compounds in water systems (Ilyas et al., 2020). C, OX, SD and OF microcosms were assembled in triplicates for each condition. Spiking with antibiotics was done to ensure significant levels of these contaminants in the LFD. The LFD contained already several metals at different relevant concentrations (Table 2.4).

Due to the high levels of solids in the collected digestate, the LFD added to the systems was diluted 1/4 (v/v) with deionised water. The experiments lasted 86 days where CWs were fed in batch mode with an HRT of 14 days.

3.2. Samples collection and preservation

Sand samples (root bed substrate) were collected from six randomly chosen points at 5 – 7 cm depth (roots depth) in each microcosm, wrapped in aluminium foil, homogenised, and then divided for metals analysis, and microbial community characterisation. Sand samples were collected when the systems were set up (Tsu), at the beginning (after acclimation) (T0), after one month (T1), after two months (T2) and at the end (T3) of the experiment (Figure S1). LECA was also sampled at Tsu and at T3 (collecting six pearls from six random points in each microcosm) crushing, mixing, and dividing for metal analysis. Regarding plant tissues, leaves were collected at Tsu, at T0, at T1, at T2, and at T3, whereas plant roots were only sampled at Tsu and T3 to determine metal concentrations and to characterise the roots microbiome. All collected samples were stored at -20 °C. To evaluate the plant growth, every two weeks, the third leave of 5 individuals per system was cut and its length and its fresh and dry weight were measured.

At the end of the experiments, immediately after the sampling, to characterise the rhizosphere of each microcosm, the sand stuck to plant roots was collected and stored at -20 ^{o}C. Also, per microcosms, three different roots from different individuals were soaked in a sterile 0.9% NaCl solution for 10 min with slight agitation. The solution was stored at -20 ^{o}C to further extract the episphere microbial community, growing at the surface of the roots. Subsequently, the roots were vigorously washed in a sterile 70 % ethanol solution for 3 min, then, in a 0.9 % NaClO solution for 3 min, in a 70 % ethanol solution for 20 min to sterilize the root surfaces, and finally rinsed five consecutive times with sterile deionised water (4 times for 2 min and the last time for 10 min). To confirm that the sterilization steps were effective, 100 mL of the last rinsing water was plated in tryptic soy agar. Roots were finally ground with a sterile mortar and pestle and 1 mL of sterile saline solution (0.9 % NaCl). The liquid homogenate was kept at -20 ^{o}C for DNA extraction of endosphere microbial community.

2.1. Physicochemical analysis

Trace metals were determined by atomic absorption spectrophotometry with flame atomisation (Aanalyst 200, PerkinElmer) for Fe, Mn, Zn, and Cu, and with electrothermal atomisation (PinAAcle 900Z, coupled to an AS900 autosampler, PerkinElmer) for Cr and Pb, following the procedures described and validated in Bonet-Garcia et al. (2023).

Briefly, substrate samples and plant tissues were dried at room temperature until reaching constant weight. Then, 0.5 g of sample were digested in an Ethos 1 high-pressure microwave system (Milestone Srl, Sorisole, Italy) with 1 mL of HNO_3 (69 %) and 5 mL of H_2O_2 (30 %) for plant tissues and 5 mL of HNO_3 (69 %) for substrates samples. The cooled digestion products were diluted with deionised water up to 15 mL and stored at 4 °C. The limits of detection (LOD) of Fe, Mn, and Cu were 0.1 µg/g, for Zn it was 0.025 µg/g, for Pb, 0.005 µg/g, and for Cr, 0.010 µg/g.

3.3. Microbial community analysis

DNA was extracted from sand samples (roots bed substrate), from the solution containing the microorganisms of the surface of the roots and from the ground root homogenate with the DNeasy PowerSoil Pro Kit (QIAGEN Inc., Venlo, Netherlands) following the manufacturer's instructions. To extract the DNA from the episphere community, in the first step of the extraction, 800 µL of sample, 2 zirconia/silica beads of 2.3 mm in diameter (BioSpec Products Inc., Bartlesville, OK, USA) and 800 µL of CD1 solution (from the kit) were added to the lysis tubes. The concentration and purity of the DNA extracts was checked by spectrophotometry (NanoDrop ND-2000 and Qubit 4 Fluorometer, Invitrogen, MA, USA). DNA samples were stored at -80 °C.

The microbiome of the superficial sand layer and the rhizosphere, episphere and endosphere of the roots was characterised through high throughput sequencing of the V4 region of the *16S rRNA* gene, marker gene for bacteria and archaea. The amplification of this gene was performed with the primer set 515FB (GTGYCAGCMGCCGCGGTAA) and 806RB (GGACTACNVGGGTWTCTAAT) (Walters et al., 2016). The amplicon libraries were sequenced using an Illumina MiSeq sequencer with the V3 chemistry (Illumina, San Diego, CA, USA) in Genoinseq (Cantanhede, Portugal).

The raw reads were processed according to the procedures described previously (Porras-Socias et al., 2024). The bioinformatic analysis was performed with the R Software v 4.1.2 (R Core Team, 2021), first using the DADA2 pipeline v 1.20.0 0 to do the upstream analysis, including parameters such as truncLen, maxEE, truncQ, maxN, trimLeft, trimRight and denoiser. ASVs were assigned using Silva v138 database with the Naïve Bayes classifier method (Quast et al., 2013; Yilmaz et al., 2014). Raw reads were reduced from 47394-121051 to 11586-79186.

The study of the diversity of microbial data was done with phyloseq v 1.38.0 and vegan v 2.5.2 packages. Alpha diversity was measured through three indices: observed ASVs

(a measure of richness in the samples), Shannon index (weighting equally the richness and the evenness) and Simpson index (also assessing richness and evenness even if the latter has greater weight) . The last two indices showed similar results and consequently only Shannon was presented. A Kruskal-Wallis test was conducted to identify significant differences among groups, followed by post-hoc pairwise comparisons using the Dunn's test ($p < 0.05$). Beta diversity, similarity of the prokaryotic composition across treatments or sample types, was calculated with the Bray-Curtis index and plotted using a non-metric multidimensional scaling as a distance-based ordination method. P values were calculated with a permutational multivariate analysis of variance (permanova) with 999 permutations, and the analysis was extended with pairwise permanova for detailed comparison between groups. A total sum scaling was used to standardise the counts in samples. ASV data was summarised at phylum and genus level, profiling most abundant taxa over 1 % and 2 %, respectively. Plots were performed using Sigmaplot v 14 and MicrobiomeAnalyst 2.0 (Lu et al., 2023).

3.4. Quantification of ARGs

Real-time quantitative PCR was conducted to quantify the abundance of a MGE, *intI1,* class 1 integron-integrase, associated with horizontal gene transfer of resistance gene cassettes, and four ARGs conferring resistance to oxytetracycline (*tetA, tetW*), sulfadiazine (*sul1*) and ofloxacin (*qnrS)* in roots bed substrate samples. To generate the standard curves, serial dilutions from 10^8 to 10^1 were prepared from the pGEM Easy with tetracycline resistance genes and pNORM1 containing the other target genes described in Porras-Socias et al. (2024). Genes were analysed with the qPCR conditions described in Table S1 following the methodology of Rocha et al. (2020). Reactions, with calibration curve efficiencies ranging from 93.5 to 105.1 %, were carried out on a LightCycler 480 II platform (F. Hoffmann-La Roche AG, Basel, Switzerland), with technical triplicates of samples from the superficial layer of the substrate. qPCR data was expressed in gene copy/g sand, and ARGs relative abundance was calculated to normalise results by dividing the number of copies of the target gene by the number of copies of 16S rRNA.

3.5. Data analysis and statistics

Three microcosms were tested for each condition of LFD to treat in the same vertical sub-surface flow CWs configuration. As mentioned previously, analyses were also conducted in triplicates. The samples' average and standard deviation values were calculated with Microsoft Excel 2019 and statistical tests were performed with Sigmaplot software v 14. First, a Shapiro-Wilk test was conducted to test the normal distribution of

the dataset with a 95 % confidence interval. Depending on the result, either a two-way analysis of variance (ANOVA) when the dataset followed a normal distribution or a Kruskal-Wallis one-way ANOVA on ranks was used. A multiple comparison Tukey test was conducted to detect statistically significant differences with p values < 0.05. Significant differences were tested between substrate samples over time and treatment and between plant samples over treatment and plant compartment.

The distribution of metals in the different compartments of CWs was calculated considering the concentration of all metals in LECA, sand, roots and leaves (in µg/g) and the volume and density of sand and LECA in the systems (in cm^3 and g/cm^3, respectively) and the mass of the roots and the leaves (g).

The bioconcentration factor and the translocation factor were calculated following the equations 3.1 and 3.2, respectively (Rezania et al., 2016).

$$\text{Bioconcentration factor} = \frac{\text{Concentration of metal in the roots (mg/kg dry weight)}}{\text{Concentration of metal in the influent (mg/L)}} \quad \text{(Eq. 3.1)}$$

$$\text{Translocation factor} = \frac{\text{Concentration of metal in the leaves (mg/kg dry weight)}}{\text{Concentration of metal in the roots (mg/kg dry weight)}} \quad \text{(Eq. 3.2)}$$

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4. Results and discussion

4.1. Metals fate in the different CWs compartments

In sustainable waste management, metal contamination continues to pose a global challenge, particularly with the expansion of industries such as mining, smelting, electroplating, and electronics. In this context, integrating CWs emerges as a solution to mitigate the harmful effects of metal pollution on ecosystems (Yu et al., 2022). Metals can be classified as either essential trace elements, serving metabolic functions in organisms (e.g. Fe, Mn, Zn, Cu, Cr), or nonessential (e.g. Pb). When exceeding the threshold limits, both can cause anomalies to living beings and even death (Khan et al., 2015). In a parallel study (Porras-Socias et al., 2024), the removal rates of metals from LFD in the CWs microcosms were evaluated, showing metals (Cu, Cr, Fe, Mn, Pb and Zn) removals over 82 %. These removal rates indicate that metals were being retained within the CW system. The main processes of metal removal from contaminated CW influents are adsorption, sedimentation, filtration, (co)precipitation, plant uptake and processes mediated by microorganisms within the system. Environmental conditions, especially pH and the redox potential, affect the reactions that occur in CWs (Yu et al., 2022).

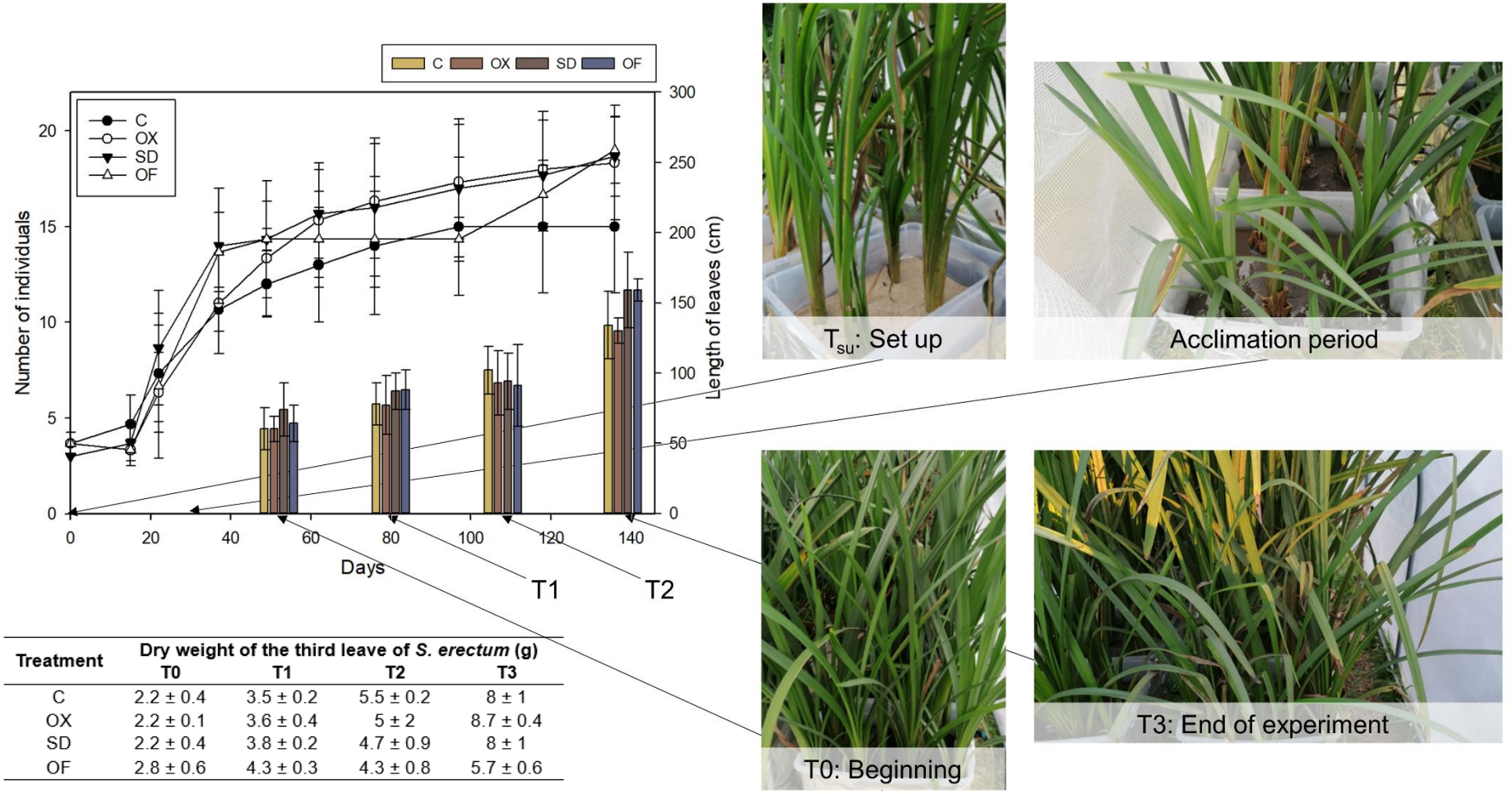
Table 3.1 shows the concentration of metals in CW substrates (LECA and sand) during their set up (Tsu) and at the end of the experiments (T3). For sand, results showed there were no significant differences ($p < 0.05$) in the levels of Fe, and Cu. However, a significant increase in the concentration of Mn and Zn was observed at T3 when compared with Tsu, while a significant decrease in Cr occurred. Pb was the only metal that presented significant differences between treatments in its concentration with lower levels in C and OX systems than in SD and OF systems, with Pb concentrations decreasing (in C and OX CW systems) or maintaining (SD and OF CW systems) over time. Although sand has a very low ion exchange capacity and therefore presents poor adsorption capacity for metals, this substrate has a strong interception ability, contributing to high COD removals (C. Yang et al., 2022). As metals form complexes with organic matter, good removal efficiencies of Fe, Mn, Cu, Cr and Pb have been previously reported in sand beds (Verma et al., 2017). In this study, in LECA, the concentration of Fe and Cu were identical, whereas those of Mn, Zn, Pb and Cr increased significantly over time, with no significant differences among treatments with or without antibiotics doping. LECA has a high cation-exchange capacity which leads to a good capacity to retain metal species (Mlih et al., 2020). Previous works also report an

increase in Zn and Cr in LECA of CWs treating landfill leachate, despite the low metal concentrations in the influent (Maine et al., 2022).

Table 3.1 Average concentration (± standard deviation, n = 3) of metals in the sand and LECA of CWs systems during their set up (Tsu) and at the end of the experiments (T3). C, OX, SD and OF indicate the different testing conditions.

Substrate	Time and treatment	Concentration of metal (µg metal/g substrate)					
		Fe	Mn	Zn	Cu	Pb	Cr
Sand	Tsu	688 ± 81	2.9 ± 0.2	2.6 ± 0.2	1.44 ± 0.07	1.1 ± 0.2	2.6 ± 0.6
	T3 C	544 ± 91	4.6 ± 0.5	6 ± 2	1.4 ± 0.1	0.6 ± 0.1	0.6 ± 0.1
	T3 OX	652 ± 208	4.2 ± 0.3	5.0 ± 0.5	1.50 ± 0.00	0.66 ± 0.05	0.5 ± 0.1
	T3 SD	727 ± 206	5 ± 1	6 ± 2	1.42 ± 0.07	1.2 ± 0.3	0.8 ± 0.3
	T3 OF	660 ± 121	6 ± 1	4.9 ± 0.5	1.5 ± 0.1	1.20 ± 0.08	0.7 ± 0.1
LECA	Tsu	21327 ± 757	70 ± 2	1.7 ± 0.2	26.8 ± 0.7	1.4 ± 0.4	18 ± 2
	T3 C	24694 ± 4201	137 ± 17	24 ± 2	24 ± 4	2.5 ± 0.4	33 ± 10
	T3 OX	24206 ± 1143	125 ± 8	23 ± 2	25 ± 5	2.23 ± 0.04	38 ± 4
	T3 SD	25288 ± 3784	130 ± 18	22 ± 4	23 ± 5	2.18 ± 0.05	35 ± 7
	T3 OF	26280 ± 2077	136 ± 7	25 ± 2	22 ± 2	2.6 ± 0.5	48 ± 6

In CWs, metals can also be removed by plants through adsorption to the root tissues, transport across membranes, translocation to the aerial parts, and accumulation into apoplasts, vacuoles, cell walls, or other structures (Mathur & Chauhan, 2020). The plant growth rate, the plant species, its bioconcentration capacity and its translocation capacity have a strong influence on the rate at which plants remove metals (Rezania et al., 2016). During the acclimation phase between 9 and 11 new individuals, on average, grew in each system (Figure 3.1), indicating that the plants were adapting successfully to the treatment conditions. After the 86 days of experiment, the number of plants individuals per microcosm (surface of 0.12 m^2) increased reaching 15-19 individuals, on average (~ 125 – 158 plants·m^{-2}). An increase in the biomass of each plant was also observed in terms of length, from 64 cm to 146 cm, and dry weight of leaves, from 2.4 g to 7.5 g per



Treatment	Dry weight of the third leave of *S. erectum* (g)			
	T0	T1	T2	T3
C	2.2 ± 0.4	3.5 ± 0.2	5.5 ± 0.2	8 ± 1
OX	2.2 ± 0.1	3.6 ± 0.4	5 ± 2	8.7 ± 0.4
SD	2.2 ± 0.4	3.8 ± 0.2	4.7 ± 0.9	8 ± 1
OF	2.8 ± 0.6	4.3 ± 0.3	4.3 ± 0.8	5.7 ± 0.6

Figure 3.1 Number of *S. erectum* individuals (line graph) in CWs systems along the experiment and average length (n = 3) of the third leave (bar plot) at T0 (day 52), T1 (day 80), T2 (day 108) and T3 (day 138). Pictures show the evolution of the microcosms with time. Arrows indicate different times of sampling. The table illustrates the dry weight of the third leave of *S. erectum* in CWs systems over time.

leaf, on average, at the beginning (T0) and the end of the experiment (T3), respectively (Figure 3.1). A similar plant growth rate was also observed in *Canna indica* and *Acorus calamus* in CWs treating industrial wastewater with metals, two ornamental aquatic plants with high tolerance to metals (Barya et al., 2022). Therefore, *S. erectum* could be an appropriate species choice to be used in CWs with LFD with metals, as they presented a healthy development throughout the experiment under northern Portuguese climate conditions, demonstrated good adaptability to LFD, and showed phytoremediation potential for metals accumulation. Doping LFD with antibiotics also did not significantly influenced plant growth. Despite no significant differences were observed, there seems to be a tendency for a higher number of individuals in the systems treated with antibiotics. Previous studies have shown that low antibiotic concentrations are beneficial for plant growth, whereas high antibiotic concentrations can induce toxicity (Migliore et al., 2000; H. Zhang et al., 2017).

Regarding *S. erectum* ability to accumulate metals, these elements were present in its tissues, including roots and leaves (Table 3.2). The concentrations of Fe and Cu in the roots of plants (per g of plant tissue) at T3 presented no significant differences with plants used to set up the experiments (Tsu). For Cr, Mn, Pb and Zn, their concentration in plant roots (per g of plant tissue) at T3 decreased significantly, as compared to the roots of plants used to set up the experiments (Tsu). Comparing the metal concentration in the leaves at the beginning (T0) and at the end of the experiment (T3), the concentration of Fe, Pb and Cr increased significantly, while the concentration of Cu decreased significantly, and levels of Mn and Zn had no significant changes over time. This suggests that plants collected in the stream (Tsu) were already accumulating trace metals probably present in stream waters where they were collected. *S. erectum* is a plant with a good metal accumulation capacity in aqueous alkaline conditions (Senze et al., 2023), and the natural environments, in this case, a region in the north of Portugal under different anthropogenic pressures, can carry metals that can be accumulated in the autochthonous macrophytes (Couto & Ribeiro, 2022). However, factors contributing to the decrease in metal levels were probably the desorption processes releasing metals previously captured, also observed in Zhou et al. (2019), and the growth of new individuals and biomass over time that increased the sorption surface and diluted the metal levels. Over time, some old leaves died and were replaced by young ones, which could contribute to reintroduce metals in the system. However, Knox et al. (2021) stated that in these cases, metals often deposited in a non-bioavailable species.

Table 3.2 Average concentration (± standard deviation, n = 3) of metals in *S. erectum* roots and shoots when CWs were set up (Tsu), at the beginning (T0) and at the end of the experiment (T3). C, OX, SD and OF indicate the different treating LFD.

Part of the plant	Time and treatment	Concentration of metal (µg metal/g dry weight)					
		Fe	Mn	Zn	Cu	Pb	Cr
Roots	Tsu	15375 ± 5677	99 ± 9	130 ± 24	9.4 ± 0.5	13 ± 3	11 ± 4
	T3 C	9132 ± 2358	40 ± 12	20 ± 2	7.5 ± 0.9	5 ± 2	2.8 ± 0.8
	T3 OX	11383 ± 4066	33 ± 12	19 ± 4	6.2 ± 0.1	5 ± 2	2.5 ± 0.5
	T3 SD	8952 ± 885	43 ± 9	26 ± 2	16 ± 3	7.9 ± 0.7	3.7 ± 0.1
	T3 OF	8222 ± 1204	48 ± 17	25 ± 4	17 ± 8	7 ± 2	3.4 ± 0.7
Leaves	Tsu	261 ± 23	113 ± 11	36 ± 9	4.5 ± 0.6	1.4 ± 0.8	0.51 ± 0.08
	T0 C	82 ± 14	96 ± 8	13 ± 2	2.7 ± 0.4	0.13 ± 0.03	0.13 ± 0.03
	T0 OX	118 ± 38	89 ± 17	11 ± 3	3.4 ± 0.8	0.16 ± 0.05	0.09 ± 0.03
	T0 SD	87 ± 5	97 ± 15	14 ± 4	3.2 ± 0.5	0.21 ± 0.03	0.08 ± 0.01
	T0 OF	83 ± 11	101 ± 12	12 ± 4	3.1 ± 0.6	0.099 ± 0.009	0.09 ± 0.01
	T3 C	154 ± 49	121 ± 15	9 ± 4	1.6 ± 0.1	0.4 ± 0.2	0.26 ± 0.09
	T3 OX	142 ± 21	85 ± 31	11 ± 3	1.49 ± 0.01	0.36 ± 0.05	0.30 ± 0.04
	T3 SD	152 ± 46	96 ± 14	15 ± 7	1.49 ± 0.02	0.492 ± 0.004	0.28 ± 0.08
	T3 OF	150 ± 30	117 ± 37	9.7 ± 0.8	1.9 ± 0.4	0.29 ± 0.02	0.24 ± 0.07

Previous works presented similar metal levels in roots and leaves (Almeida, Santos, Ferreira, Gomes, et al., 2017; Travaini-Lima et al., 2015). In Ghezali et al., (2022), metals were also removed from industrial wastewaters (from a refinery) with higher concentrations of metals in leaves and roots (except for Fe) in *Canna indica*. Moreover, in the present study, the bioconcentration factor of *S. erectum* in CWs was for Fe and Mn, above 28, and for Zn, Cu, Pb and Cr above 2.6 (Table 3.3). These results were higher bioconcentration factor values than the ones measured in *S. erectum* by Parzych, (2016), confirming that *S. erectum* is a good plant to be used in the phytoremediation of metals, especially for Fe and Mn. This species in the CWs configuration and treatment of the present study showed a higher bioaccumulation factor than other macrophytes

used in CWs removing metals from industrial wastewater, such as *Phragmites australis, Typha latifolia, Eichhornia crassipe*, or *Juncus articulates* (Khan et al., 2009). Therefore, Pavan et al. (2015) work on the screening of plant species' potential for the treatment of LFD is extended here, by showing the ability of *S. erectum* to grow in CWs treating LFD with metals and playing an important role in the removal of Fe, Mn, Zn, Cu, Cr and Pb from LFD.

Table 3.3 Average (± standard deviation, n = 3) bioaccumulation factor and translocation factor of Fe, Mn, Zn, Cu, Pb and Cr of *S. erectum* collected from the stream (Tsu), and *S. erectum* sampled at the end of the experiment (T3) in the different CWs systems.

Bioconcentration factor						
Time	**Fe**	**Mn**	**Zn**	**Cu**	**Pb**	**Cr**
T3 C	71 ± 26	34 ± 10	2.7 ± 0.2	4.4 ± 0.5	9 ± 3	7 ± 2
T3 OX	88 ± 39	28 ± 10	2.6 ± 0.5	3.6 ± 0.1	9 ± 3	7 ± 1
T3 SD	69 ± 7	37 ± 8	3.5 ± 0.3	9 ± 2	14 ± 1	9.8 ± 0.3
T3 OF	64 ± 9	41 ± 23	3.4 ± 0.6	10 ± 5	12 ± 4	9 ± 2
Translocation factor						
Time	**Fe**	**Mn**	**Zn**	**Cu**	**Pb**	**Cr**
Tsu	0.018 ± 0.006	1.1 ± 0.1	0.28 ± 0.03	0.49 ± 0.09	0.10 ± 0.04	0.05 ± 0.02
T3 C	0.017 ± 0.007	3 ± 2	0.5 ± 0.2	0.22 ± 0.01	0.10 ± 0.06	0.10 ± 0.05
T3 OX	0.014 ± 0.006	2.6 ± 0.2	0.59 ± 0.06	0.24 ± 0.01	0.08 ± 0.03	0.12 ± 0.03
T3 SD	0.017 ± 0.007	2.4 ± 0.9	0.6 ± 0.2	0.10 ± 0.02	0.06 ± 0.01	0.08 ± 0.02
T3 OF	0.019 ± 0.006	2.6 ± 0.8	0.40 ± 0.06	0.13 ± 0.09	0.05 ± 001	0.07 ± 0.04

Overall, Figure 3.2 shows a general picture of the distribution of metals across different compartments in CWs. Initially, in the moment of setting the microcosms up, Fe and Cu were predominantly concentrated in the LECA layer, being less than 10 % of abundance in plants' tissues. However, after the experimental period, the levels of these two metals increased in belowground parts of the plants (roots), and particularly, Cu also increased in the aboveground parts (leaves). The uptake of essential metals in plants is mainly driven by a non-selective active absorption process (Senze et al., 2023). In addition, a notable rise of Mn levels in aboveground parts was also observed over time, showing the highest increase among the measured metals. Only Mn had a translocation factor higher than 1 (Table 3.3), indicating high mobility from roots to leaves. The high root-to-

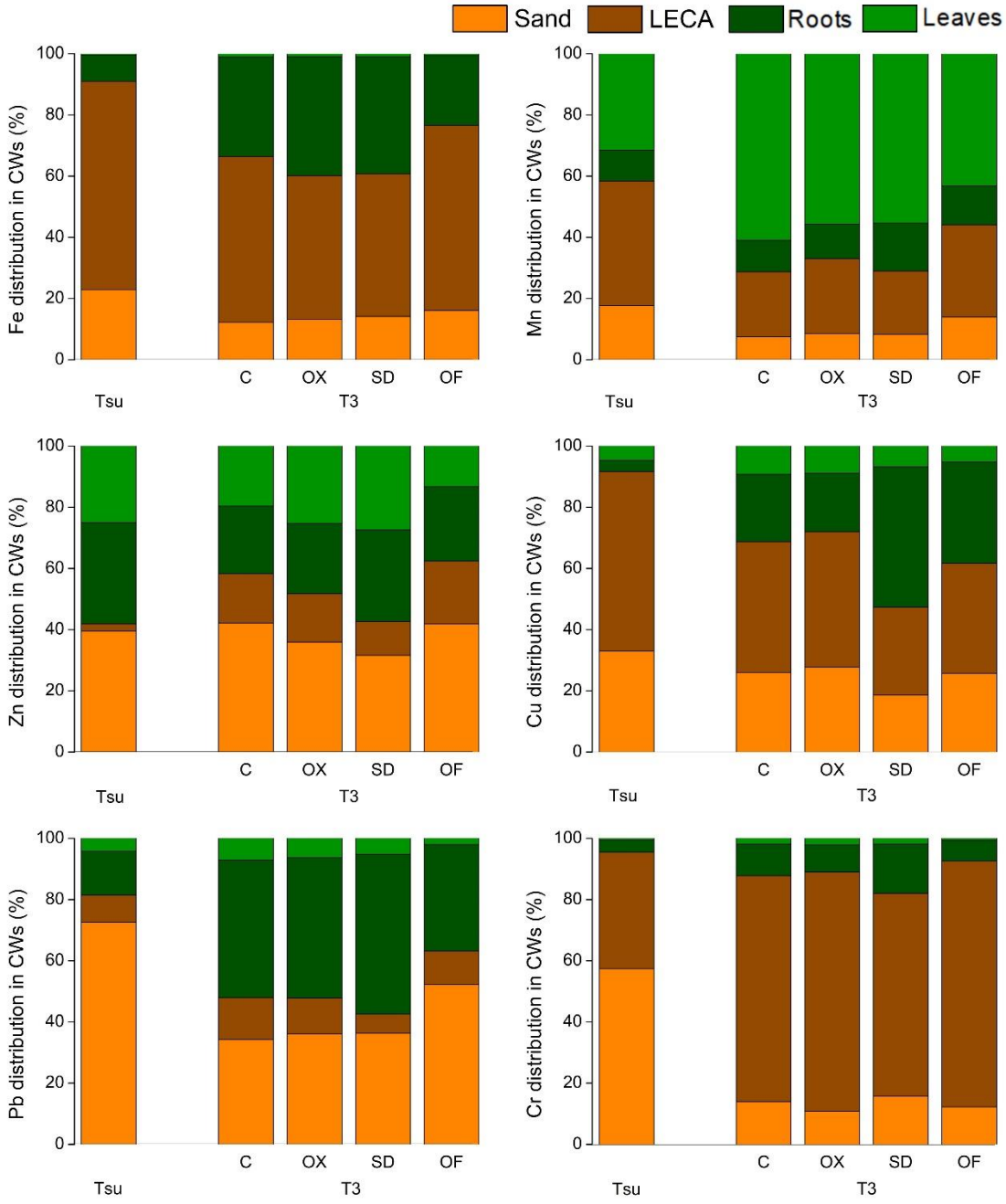


Figure 3.2 Distribution of Fe, Mn, Zn, Cu, Pb, and Cr in CW substrates (sand and LECA) and in plant tissues (roots and leaves) at the setting up of the experiments (Tsu) and after the three months of treating the LFD with or without antibiotics spiking (T3).

shoot mobility of Mn was seen in other aquatic plants, namely *Nelymbium speciosum, Nymphae stellata* and *Sagittaria sagittiflia* (Vardanyan & Ingole, 2006). The other metals measured in the present study had a lower translocation factor (below 1), and hence, were mainly accumulated in the roots, as reported previously (Vymazal & Březinová, 2015). Regarding Zn and Cr, an increase in their concentrations in LECA was noted after the LFD treatment in CWs (Figure 3.2). Walaszek et al. (2018) and Xiong et al. (2024)

reported that those two elements were involved in ion exchange on the superficial sediment, presenting a non-specific electrostatic adsorption on soil particles, especially in clays. Zn and Cr were easily mobilised in reducing conditions (Walaszek et al., 2018; Xiong et al., 2024). However, Vymazal et al. (2010) showed that the amount of Zn bound in the upper part of the plant was high. Here, although its translocation factor was below 1, over 20 % of total Zn mass, on average, at T3 was accumulated in the leaves. In the case of Pb, primarily in sand at Tsu, ended up being mostly accumulated in belowground parts of the plants after the treatment. Its uptake mechanisms have not been fully understood, but it has previously been reported that its accumulation is mostly in the roots as well (F. J. Zhao et al., 2022). As a nonessential element, Pb could have been taken up via Ca-permeable channels and accumulated strongly bound to cell wall components such as pectin in the apoplast, and partially as insoluble metal phosphate compounds (F. J. Zhao et al., 2022). Thus, each metal presented its particular removal pathway in vertical subsurface flow microcosms.

Metal ions can also form complexes with antibiotics, so, the co-occurrence of these organic and inorganic contaminants can affect metal uptake (Pulicharla et al., 2017; Sayen et al., 2019). Here, no significant differences were observed among treatments, LFD spiked or not spiked with one of the 3 selected antibiotics, at the end of the experiment neither in plants leaves nor roots metal levels, except for Cu. Hence, oxytetracycline, sulfadiazine and ofloxacin at the tested concentrations did not alter the removal driven by *S. erectum* of most metals, a fact supported by previous studies with other plant species and other antibiotics (Almeida, et al., 2017). However, Cu concentration in roots of C and OX systems was statistically lower than within SD and OF systems. Cupric ions have very high affinity in metalation, and in CWs, Cu could be mostly forming complexes with dissolved organic carbon and interacting with antibiotics with synergistic or antagonistic effects (Božić Cvijan et al., 2023; Foster et al., 2014; Sayen et al., 2019). In Ma et al. (2023), Cu bioaccumulation in plants was stimulated by sulfamethoxazole concentrations, a sulfonamide. However, in another CWs study, the presence of enrofloxacin, a fluoroquinolone, forming complexes with Cu, led to decreasing Cu plant uptake levels, even though the Cu-fluoroquinolone complexes were uptaken in roots (Sayen et al., 2019). Further efforts have to be made to elucidate the speciation, bioavailability and effects of metal-antibiotic complexes in phytoremediation processes.

4.2. Occurrence of ARGs in surface layer of CWs

The presence of contaminants, namely metals and antibiotics, in wastewaters/liquid digestate can exert selective pressure on CWs microorganisms. Consequently, ARGs may develop and be transferred within the population of the systems, turning CWs into hotspots for ARGs (Ohore et al., 2020). Numerous studies presented effective removals of ARGs from wastewater in CWs (Porras-Socias et al., 2024; Chen et al., 2019a; Du et al., 2020). The role of the substrate is crucial in the removal process of ARGs (Cui et al., 2023). To elucidate the fate of ARGs within the systems of this study, their absolute abundance in the top layer of sand (plant roots bed substrate) was measured (Figure 3.3). Over the time of the experiment, the bacterial charge in the top layer of CWs rose significantly ($p < 0.05$), from $9.6 \cdot 10^8$ at T0 to over $2.6 \cdot 10^9$ copies 16S/g sand at T3. The microbial growth in the superficial substrate was probably due to the constant addition of nutrients, organic matter, and bacterial charge from the influent in the systems over time, and the presence of oxygen, also reported in Zhang et al. (2017). The measured MGE, *intI1* gene, also increased from $3 \cdot 10^6$ to 4.3 - $7.24 \cdot 10^6$ copies *intI1*/g sand over the experiment. Although *tetW* abundance had no significant differences over time, *tetA* abundance increased from $3.6 \cdot 10^5$ at T0 to $1.0 \cdot 10^6$ copies *tetA*/g sand, on average, at T3. Similarly, a significant augment of *sul1* gene over the experiment was seen, rising from $3.7 \cdot 10^6$ to T0 to $5.7 \cdot 10^6$ copies *sul1*/g sand at T3, on average. However, a significant decrease in the relative abundance of *intI1* and ARGs was observed over time (Figure 3.4), suggesting that the increase of the absolute abundance of ARGs was due to the growth of the total population, and the transference of these genes was limited and were less prevalent after three months of experiment. Abundances of ARGs in the same order of magnitude were obtained in previous studies in the substrate layers (Chen et al., 2016). Finally, regarding *qnrS* gene encoding resistance to quinolones, both absolute and relative abundance increased over time reaching at the end of the experiment less than $8.5 \cdot 10^3$ copies *qnrS*/g sand in C, OX and SD systems (relative abundance of $0.5 \cdot 10^{-4}$, $1.2 \cdot 10^{-4}$, and $0.4 \cdot 10^{-4}$ %, respectively), and $2.5 \cdot 10^4$ copies *qnrS*/g sand in OF systems (relative abundance of $3.3 \cdot 10^{-4}$ %), also in the order of magnitude than Chen et al., (2016). The main removal processes of ARGs in CWs include adsorption to substrates and roots and microbial biodegradation (Ma et al., 2022). The capacity of sand in filtering microorganisms contributes to the removal of ARGs (X. Huang et al., 2017). LECA with its physico-chemical properties, especially the surface area of 250-300 m^2/m^3, provides a wide capacity for adsorption and microbial growth competing with bacteria carrying ARGs and biodegradation (Cui et al., 2023). In addition, planted CWs also contributed

to the reduction of ARGs dissemination increasing the adsorption surface and enhancing specific communities affecting the abundance of ARGs (Abou-Kandil et al., 2021).

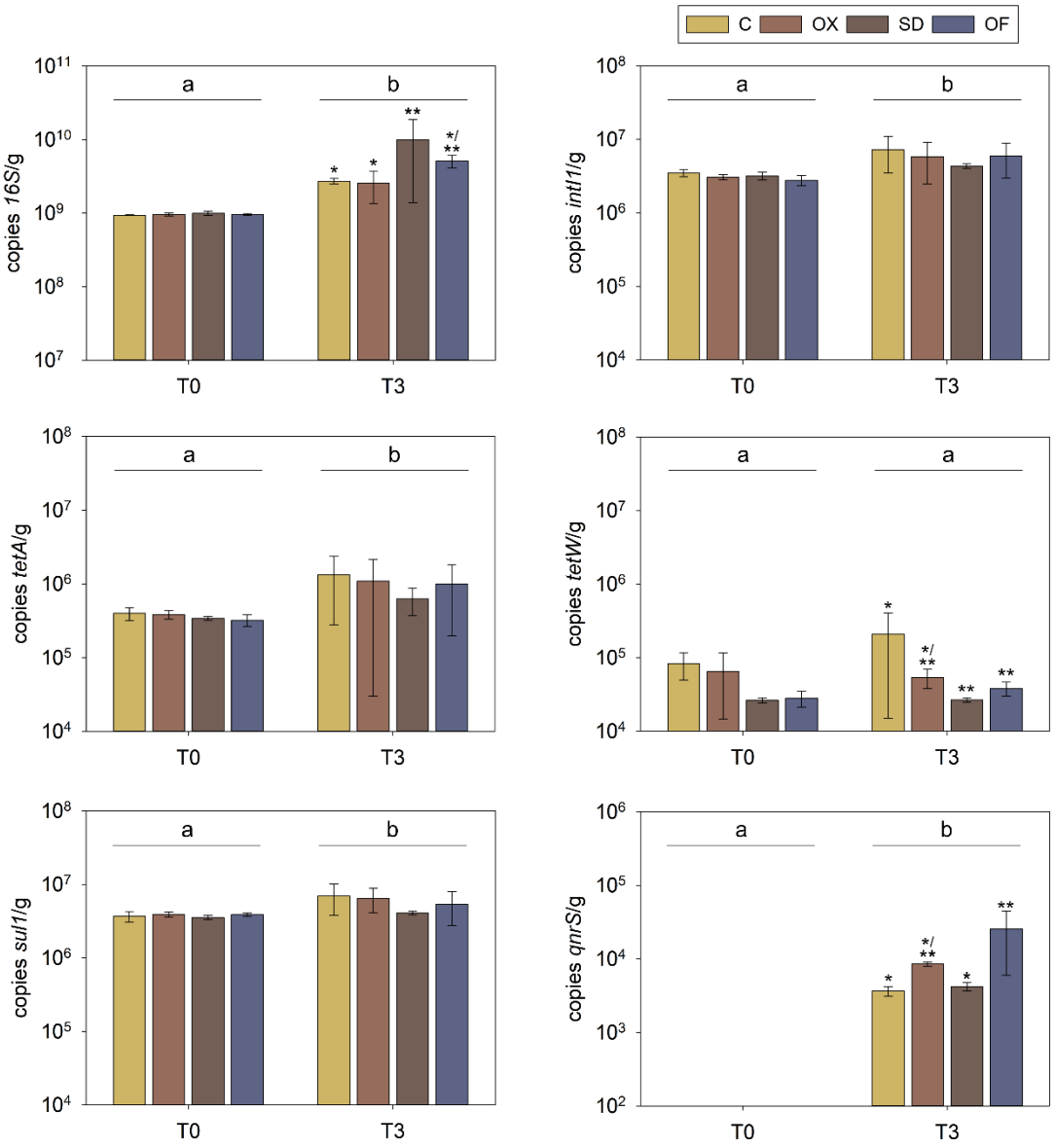


Figure 3.3 Absolute abundance of V3 region of *16S rRNA*, *intI1*, *tetA, tetW, sul1*, and *qnrS* genes in the top layer of sand, before (T0) and after (T3) the experiments in CWs. In the legend, C, OX, SD and OF indicate the different treating the LFD. The same letters show no significant differences between time ($p < 0.05$), and the same * illustrates no significant differences between treatments in a subset (when no * are presented, there are no significant differences between treatments).

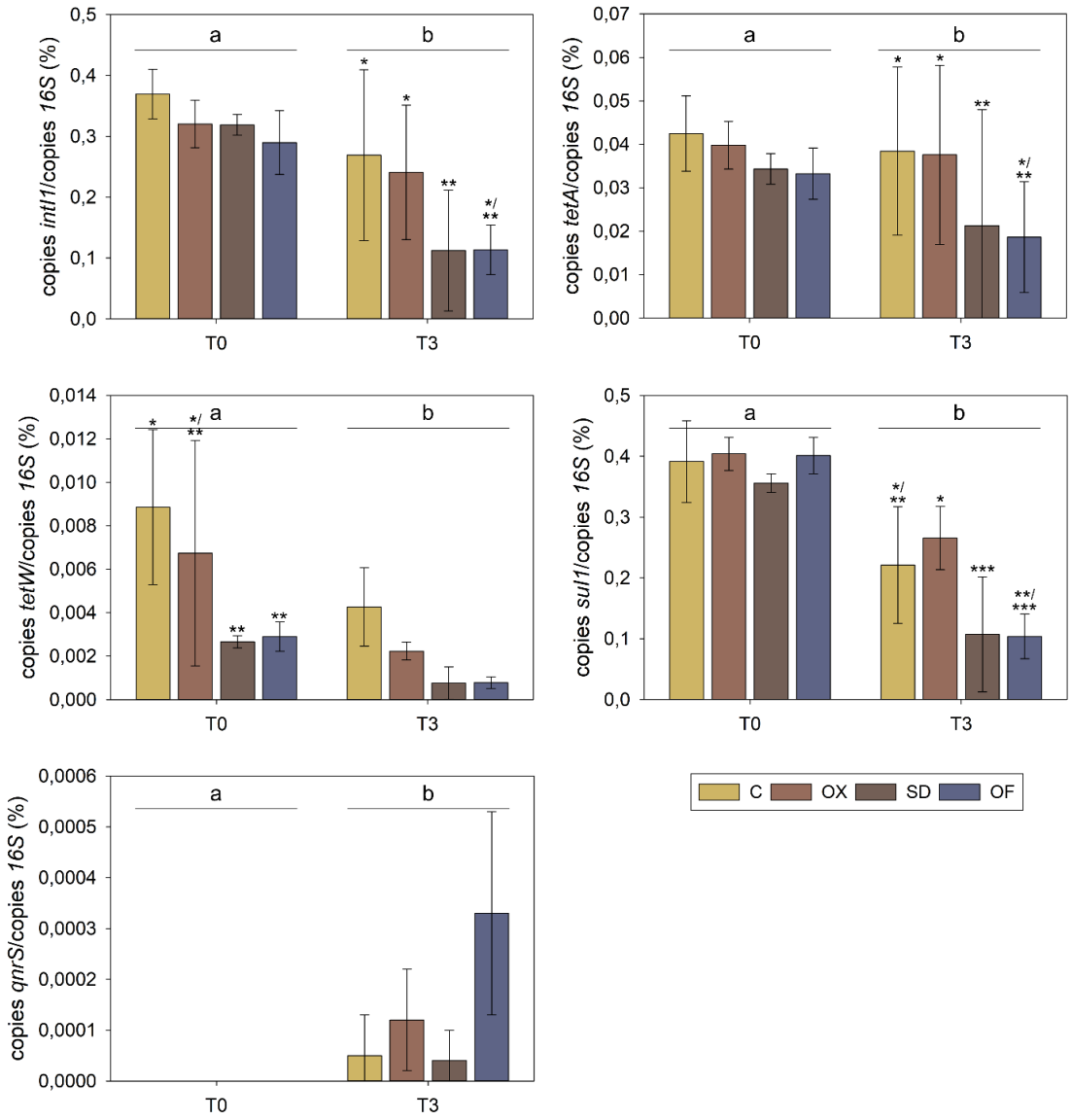


Figure 3.4 Relative abundance of *intI1*, *tetA, tetW, sul1*, and *qnrS* genes in the top layer of sand, before (T0) and after (T3) the experiments in CWs. In the legend, C, OX, SD and OF indicate the different treating the LFD. The same letters show no significant differences between time ($p < 0.05$) and the same * illustrates no significant differences between treatments in a subset (when no * are presented, there are no significant differences between treatments).

4.3. Prokaryotic community dynamics of CWs top layer over time

Silveira et al. (2022) reported that microbial communities of CWs from biofilms are significantly different than the ones from influent and effluent water samples which are more commonly studied. Biofilm samples are more representative of the system and

have higher density and activity than the ones from interstitial waters. The analysis of bacterial and archaeal communities in the superficial sand layer in CWs treating LFD with or without antibiotics addition over the experimental period (T0, T1, T2, and T3) included the analysis of the alpha and beta diversity, and the taxonomic profiling study.

First, the richness of samples, assessed with observed ASVs, exhibited an increasing trend with time (Figure 3.5A). At T0, each sample had between 469 and 792 different ASVs. This richness significantly increased at T1, with 680 – 863 observed ASVs, and continued to rise significantly at T2, reaching between 723 and 1057. However, there was a slight decrease at T3, with observed ASVs ranging between 652 and 843, resulting in no significant differences with datasets of T0 and T1. In general, the presence of antibiotics in LFD did not alter the richness of the community in the superficial substrate layer. Shannon diversity index results were always over 5.2, with no significant differences between treatments. This suggests that in the top substrate of CWs was colonised by numerous different species with a relatively even-distributed abundance. The lowest values were observed at T0 (5.21 - 5.76) and the highest at T1 (5.46 – 6.02). These results indicate that the rise in *16S rRNA* copies over time (Figure 3.3) involved not only a growth in the number of bacteria but also an increase in the diversity of the community. High diversity in the surface-sand community as compared with deeper layers was found in previous vertical subsurface flow of CWs works (Cheng, et al., 2022b; Guan et al., 2015). These results could be associated with the stable and favourable environment of the top substrate layer with an abundant presence of organic matter, nutrients, oxygen, and light, facilitating the colonisation of prokaryotic populations, and with the physical barrier that sand provides that could block particles and microorganisms, concentrating them in the surface (Ghadraoui et al., 2021).

Second, the community composition dissimilarities between treatments and time are shown in Figure 3.5B. There were no significant differences between samples with presence or absence of antibiotics (p-value = 0.102). However, clusters representing temporal changes of the community reflected a progressive differentiation of the community through time, with a significant increase in dissimilarity between T0, T1, T2 and T3 ($p < 0.05$). Only at T3 there were significant differences between treatments in the prokaryotic community of the bed roots substrate. Many studies also showed differences over time, elucidating the temporal variation of microbial communities in CWs substrates (Li et al., 2018; Truu et al., 2019; Wang et al., 2016). In Weber & Legge (2011), it was only after 74 days of the mesocosm set up that the communities' structure

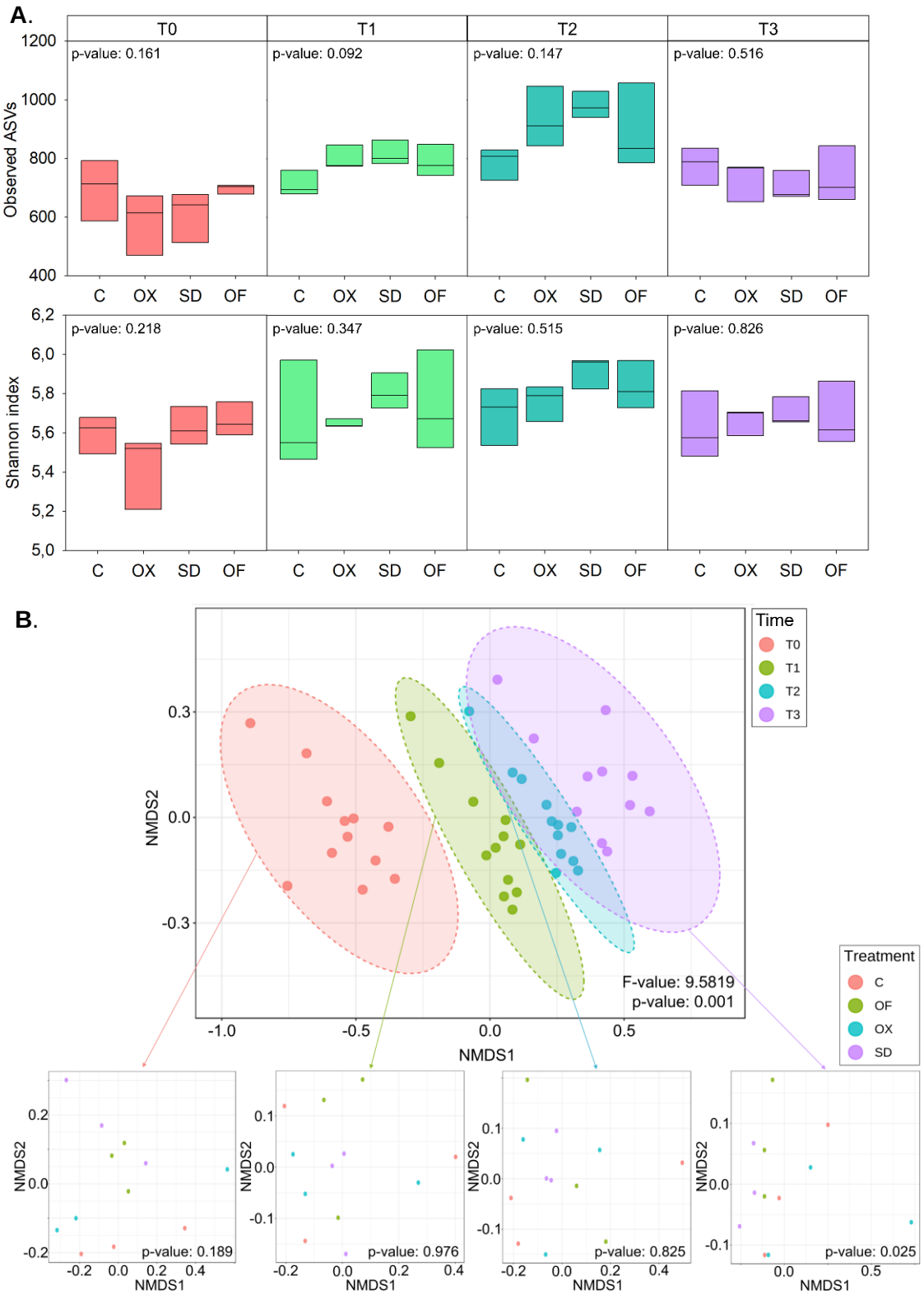


Figure 3.5 A. Boxplots of alpha diversity indexes (Observed ASVs and Shannon index) of the prokaryotic communities in the surface sand layer along the experimental period (T0, T1, T2, T3). B. Non-metric multidimensional scaling of prokaryotic communities of surface sand layer over time.

of the biofilms started shifting and being diverse between the tested conditions. During

the previous period, the communities were similar corresponding to the phase of establishment of the biofilms.

The most abundant taxa (over 1 and 2 %) in the top sand layer are presented Figure 3.6 at phylum and genus levels, respectively, belonging to the domain Bacteria. At T0, before the LFD with or without spiking started being added to CWs, the prokaryotic community was primarily dominated by *Proteobacteria*, constituting 35 to 45 % of the community, and followed by *Actinobacteriota, Planctomycetota, Firmicutes* and *Bacteroidota*, all with abundances between 6 and 17 %. Throughout the months treating LFD, the community maintained its composition with the previously mentioned predominant phyla, but with slightly different abundances. *Proteobacteria* showed a decreasing tendency over time, whereas *Acidobacteriota* and *Chloroflexi* exhibited a notable increase (from around 1 to 4 – 10 %). The phyla profile did not vary considerably between treatments. Only at the end of the experiments (T3), there were significant differences between treatments in the community composition, where in SD and OF systems, *Proteobacteria, Bacteroidota* and *Acidobacteriota* were more predominant as compared within the C and OX systems. On the contrary, *Actinobacteriota, Planctomycetota* and *Firmicutes* had a higher prevalence in C and OX systems than in the other ones. These abundant phyla were also observed in the top layer of previous studies of CWs treating wastewater, especially *Proteobacteria* as the most predominant phylum (Chen et al., 2019b; Guan et al., 2015; Liu et al., 2021; Wang et al., 2016). *Proteobacteria* is related to various metabolic functions including the biotransformation of organic matter and numerous organic compounds, the contribution to the nitrogen, sulphur and phosphorus cycles, and metal reduction (Mellado & Vera, 2021; Yarwood, 2018).

For a better comprehension on how the time and treatment affected the prokaryotic community of top layer CWs' substrate, the relative abundance was analysed at genus level. Previous studies highlighted the existence of a vertical gradient of microorganisms in CWs with different diversity and activity, and the main microbial-driven processes characterising the top layer area are degradation of organic matter, aerobic degradation of organic compounds and nitrification processes (Hassan et al., 2021; Weber & Legge, 2011).

Despite all systems were treated equally during the acclimation period, CWs showed variations between them at T0. Unclassified Isosphaeraceae, chemoorganotrophic aerobic bacteria with high glycolytic abilities found in wetlands and sewage sludge (Conejo-Saucedo et al., 2021; Dedysh & Ivanova, 2015), was one of the most abundant genera at T0 (4.0 %, on average), and its abundance dropped over time. Many functional

microorganisms were found to be related to the nitrogen cycle. On the one hand, genera associated with nitrifiers involved in ammonia oxidation, namely *Prosthecobacter* and

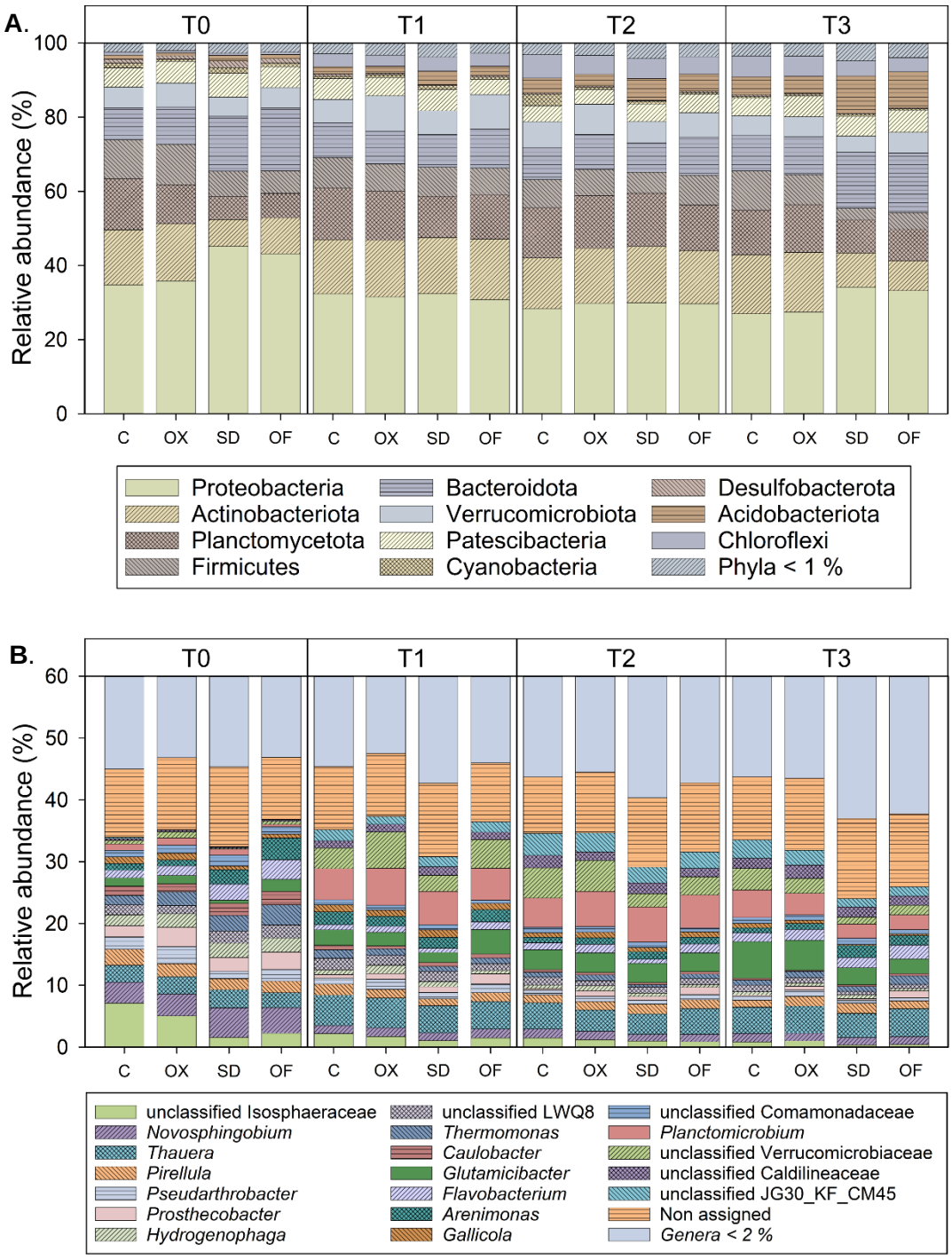


Figure 3.6 Taxonomic profile of prokaryotes in the superficial sand layer of CWs over time at the phylum level (A), and the genus level (B). Each bar represents the average relative abundance of the triplicated systems.

Pirellula (Xia et al., 2019, 2020), decreased over time. On the other hand, *Glutamicibacter* and non-assigned Verrucomicrobiaceae increased notably after T1, the former with heterotrophic nitrification and aerobic denitrification (HN-AD) bacteria, and some metal tolerant species, while the later with nitrite reduction processes and polysaccharide degradation in aquatic environments (Chen et al., 2023; Tong et al., 2023; Vishnupriya et al., 2024). Other genera related to denitrification processes were *Thauera*, *Thermomonas*, *Hydrogenophaga* and *Flavobacterium*, the two latter being associated with denitrifying anaerobic methane oxidation (Moazzem et al., 2023; Wang et al., 2022), were present along time with a total relative abundance of 6.3 – 9.4 %. Finally, *Planctomicrobium*, *Flavobacterium* and *Pirellula*, being associated with anaerobic ammonia oxidation (anammox) reactions (Wang et al., 2022; Zheng et al., 2021), presented a mild decrease over time. Therefore, in CWs, both aerobic and anaerobic bacteria were involved in N removal processes.

Regarding the genera associated with emerging contaminants removal processes, three of them, potentially associated with antibiotics removal, were among the most abundant ones. The unclassified JG30_KF_CM45, potentially related to the removal of organic contaminants and previously identified in CWs with positive correlations with diclofenac or benzotriazole (Ruppelt et al., 2020), presented an increase over the months of experiment, becoming one of the most abundant genera, especially after T2. However, *Novosphingobium* and *Pseudarthrobacter*, which were also related to organic contaminants degradation (Huang et al., 2019; Waigi et al., 2015), showed a decrease over time. Moreover, the decrease over time of *Pseudarthrobacter* and *Flavobacterium* genera could be associated to the decrease in the relative abundance of most ARGs, as they were previously found in contaminated sediments carrying various ARGs namely *tetW* or *qnrS* (Balan et al., 2018; Xu et al., 2023). Concerning metal biosorption-related genera, the *Hydrogenophaga* genus, previously associated with Cu^{2+} removal processes (Wang et al., 2022), was found in all systems with abundances around 2 % in T0 and stabilised to 1 % at T1, T2 and T3.

In the present study, it was at the end of the experiment, at T3, that a possible effect of sulfadiazine and ofloxacin was observed, decreasing *Planctomicrobium,* unclassified Verrucomicrobiaceae, unclassified JG30_KF_CM45 and *Glutamicibacter* relative, as compared with the control. This could lead to altered HN-AD and anammox processes. *Planctomicrobium*'*s* relative abundance was also altered by the antibiotics' presence in previous study of CWs treating antibiotics (Zheng et al., 2021).

4.4. Root microbiome of *S. erectum* at the end of experiments

Plant root microbiome play a crucial role in the performance of CWs, influencing the bioavailability of elements and stability of the systems regulating the biogeochemical cycles. The stress caused by pollutants can cause shifts on the root microbiome altering the secretions of the plant and the biofilms (Wang et al., 2023). In the present study, the alpha diversity indices showed that the rhizosphere richness or evenness was not altered by the antibiotics (p-value > 0.05), with 822 – 989 observed ASVs, on average, and a Shannon index value ranging from 5.63 to 6.27, on average (Figure 3.7). However, the episphere and endosphere prokaryotic communities' diversity was affected by the presence of antibiotics. Both the observed ASVs and Shannon index of the endosphere were significantly higher in SD and OF microcosms (960 – 990 and 6.20 – 6.25, on average, respectively), than in C and OX systems (averaging 700 – 764 and 5.15 – 5.21, respectively). In the episphere, variations caused by the antibiotics were less pronounced, where only the richness dropped in SD systems, reaching 567 observed

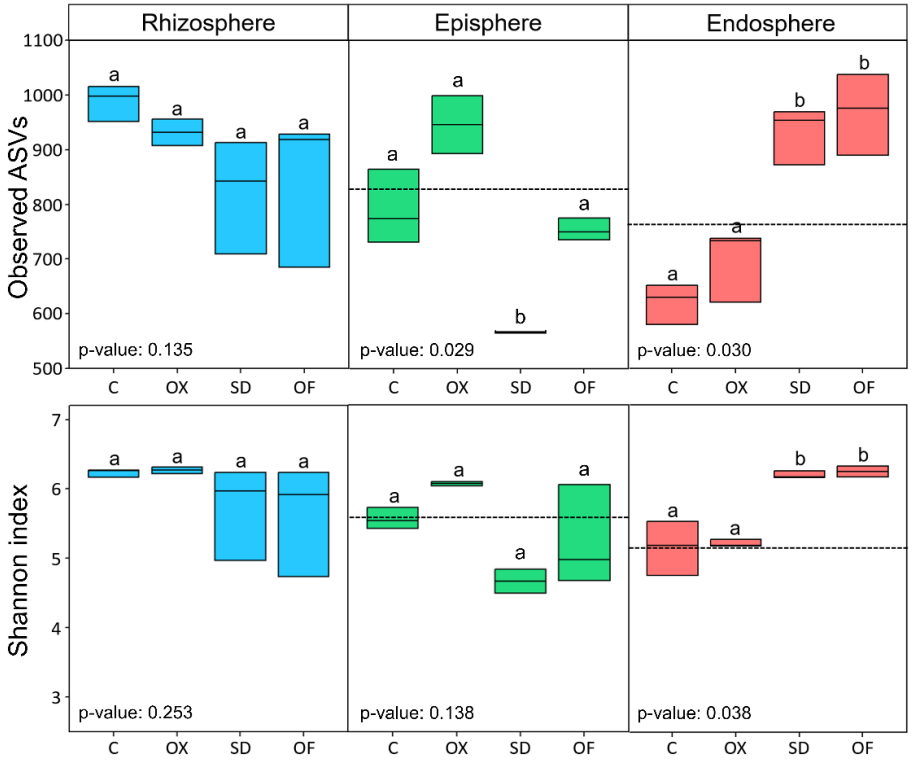


Figure 3.7 Boxplots of alpha diversity indexes (Observed ASVs and Shannon index) of the prokaryotic communities in root compartments (rhizosphere, episphere and endosphere) of *S. erectum* after treating LFD during the experimental period. The dashed black line indicates the alfa diversity indexes values of initial *S. erectum* before being transplanted in CWs (Tsu). No data was obtained for the initial rhizosphere.

ASVs, on average, presenting significant differences compared to C systems with 790 observed ASVs. Despite the differences in Shannon index values among CWs treatments, with lower values in SD and OF systems and higher in OX CWs, the variations in median values between treatment groups were not significant enough to exclude the possibility that the differences were due to sample variability and thus, no significant differences were observed. In Syranidou et al. (2018) there were no significant changes in the alpha diversity indexes of root endophytic microorganisms in low pollution influents, while a significant decrease in the diversity was found in high pollutant influent with Zn (400 mg/L), Ni (40 mg/L), Cd (2 mg/L), ciprofloxacin (100 µg/L) and sulfamethoxazole (500 µg/L). Thus, the concentration of pollutants has an important effect in the community.

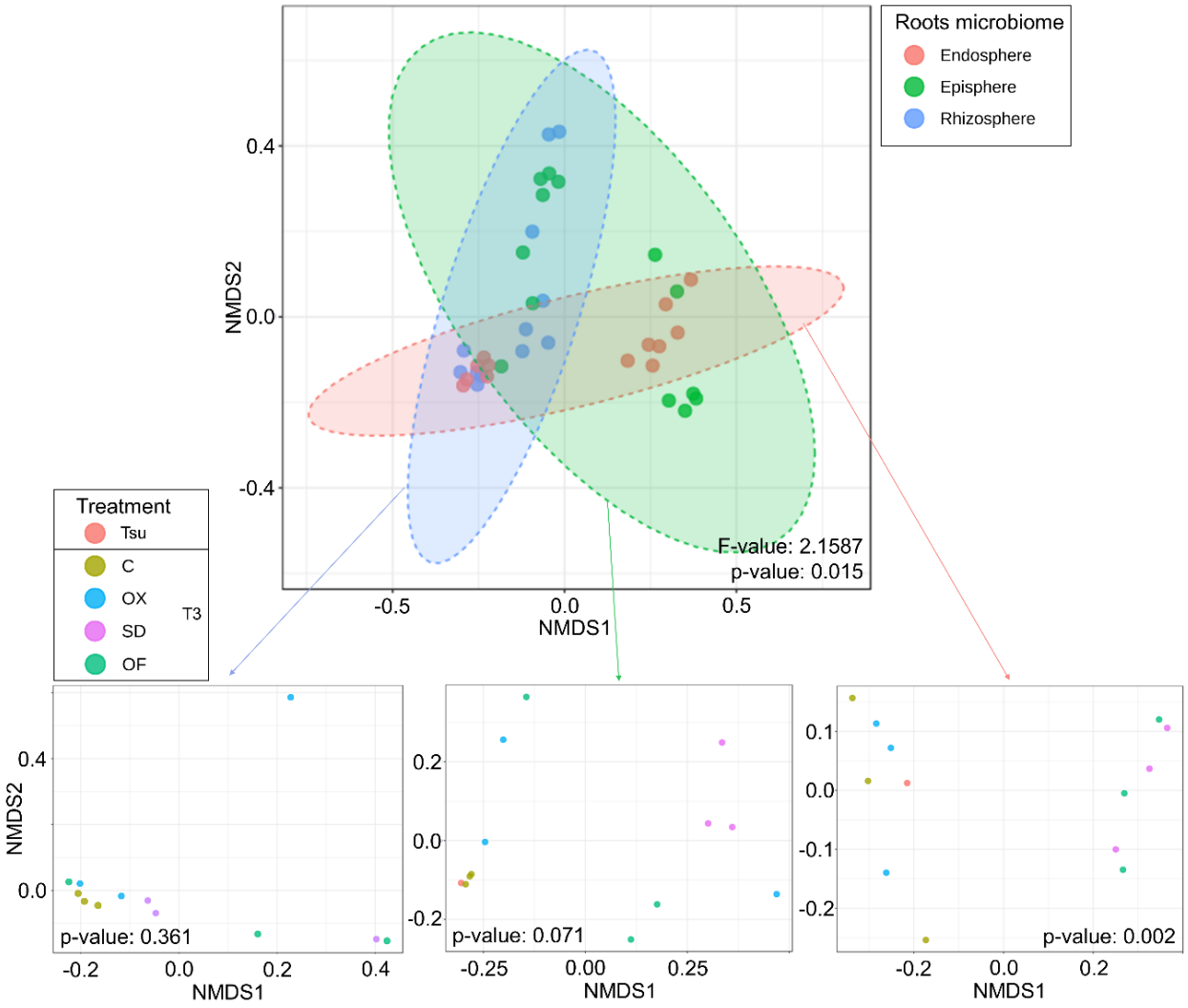


Figure 3.8 Non-metric multidimensional scaling of prokaryotic communities of all root compartments, with a focus on the dissimilarities between treatments in the rhizosphere, the episphere and endosphere (from left to right).

The beta diversity analysis of the roots' microbiome in the NMDS plot (Figure 3.8) resulted in significant differences in community structure between the roots rhizosphere endosphere and episphere compartments, despite some overlap in the NMDS clusters. Previous studies also observed differences between the rhizosphere and endosphere of other salt-marsh plants, such as *P. australis*, independently of the season, and part of the endosphere was influenced by the rhizosphere community (R. He et al., 2020). In the present study, no statistically significant differences were found between treatments in the rhizosphere or the episphere. The roots' episphere from the transplanted plants at Tsu, originally from the stream, showed noticeable similarities with the episphere from control systems at T3. In contrast, the endophytic communities presented significant differences between treatments (Figure 3.8). The NMDS plot of the endophytic communities clearly separates, in the left side, the samples representing the Tsu and the C and OX systems, and the right side of the plots, the samples representing the SD and OF systems. Some studies did not find clear differences in the endophytic community composition between plants in systems treating 2 mg/L of sulfamethoxazole plus diclorofenac and in control ones (Sauvêtre et al., 2020). However, in He et al. (2020), the presence of organic and inorganic contaminants produced changes in root community. Therefore, here, sulfadiazine and ofloxacin at 0.5 mg/L produced changes in the endosphere community of *S. erectum*.

Stress caused by pollutants influences the belowground microbiome, influencing the plant-microorganisms secretions and attracting specific populations enhancing the diversity (Wang et al., 2023). Figure 3.9 shows the taxonomic profile of *S. erectum* roots microbiome, and most predominant phyla were shared across the compartments but differed in terms of abundance in the community.

Specifically, in the rhizosphere, a general trend was observed in the four treatments with no alterations in the profile in CWs where antibiotics were added, also reported in a previous study where sulfamethoxazole and ofloxacin did not alter significantly the rhizosphere community structure of *P. australis* (Y. Lv et al., 2020). *Proteobacteria, Bacteoidota, Firmicutes*, and *Actinobacteriota* and *Planctomycetota* represented over the 73 % of the community, which is consistent with the most abundant phyla in *P. australis'* rhizosphere in previous studies (R. He et al., 2020; Man et al., 2020; Pietrangelo et al., 2018). However, in the episphere and in the endosphere the taxonomic profile varied with the addition of antibiotics.

In the plants' episphere from systems treating LFD with antibiotics, the *Proteobacteria* and *Actinobacteria* dominance decreased, while the *Firmicutes* presence increased.

Actinobacteria members can contribute to nitrogen fixation and plant-growth promotion, biodegradation of pollutants, decomposition of organic compounds and may also be pathogens (Lawson, 2017). Members of the *Firmicutes* phylum are also involved in plant growth promotion, biocontrol and bioremediation, even though there are also some opportunistic pathogens (Hashmi et al., 2020). In SD and OF microcosms, a significant growth of *Cyanobacteria*'s prevalence, related to carbon and nitrogen fixation, was observed being 12 – 22 % of the community. It has been previously reported that higher abundances of Cyanobacteria could potentially increase the removal of contaminants by producing oxygen from photosynthesis, providing an electron acceptor for pollutant degradation (Zhang et al., 2015). However, in systems treating LFD with sulfadiazine and ofloxacin, the relative abundance *Bacteroidota*, being versatile and diverse bacteria in funcions in nutrient cycling and ecosystem functioning, decreased. Moreover, regarding the differences between treatments in the endosphere community, variations in the taxonomic profile were observed in SD and OF systems, exhibiting resemblances between them. A decrease in the percentages of *Proteobacteria* and *Bacteroidota* was observed, consistent with the results of Sauvêtre et al. (2020) attributed to the effect of sulfamethoxazole and diclorofenac, but the strongest decline was observed in Cyanobacteria from 16 (in C and OX systems) to 1 % (in SD and OX systems). In contrast, the *Firmicutes* and *Actinobacteria* prevalence increased.

Furthermore, when comparing the taxonomic profile at both phym and genus levels of the episphere and endosphere of plants at T3 from C systems and transplanted plants from the stream (Tsu), their respective profiles closely resembled each other. This observation confirms the findings of the beta diversity results. The most predominant phyla of their episphere were *Proteobacteria* (49 – 52 %), *Bacteoidota* (16 %), *Actinobacteriota* (9 – 10 %) and *Firmicutes* (5 – 6%), differing from the ones from the endospheres being *Proteobacteria* (33 – 38 %), *Cyanobacteria* (15 – 17 %), *Bacteroidota* (11 – 14 %) and *Planctomycetota* (7 – 12 %). It was previously reported that the genetic lineage of the macrophyte has more significant influence on the structure of the bacterial community of the roots than the specific local environment (Bowen et al., 2017). Hence, *S. erectum* root epiphytic and endophytic microbiome remained stable across both natural environments and CWs control systems.

Focusing on a lower taxonomic level, differences (more pronounced in episphere and endosphere) were observed between treatments (Figure 3.9), as it was reported previously in plant-root microbiome in CWs treating wastewater with antibiotics (Man et al., 2020). In episphere, systems treating LFD with antibiotics presented variations in the

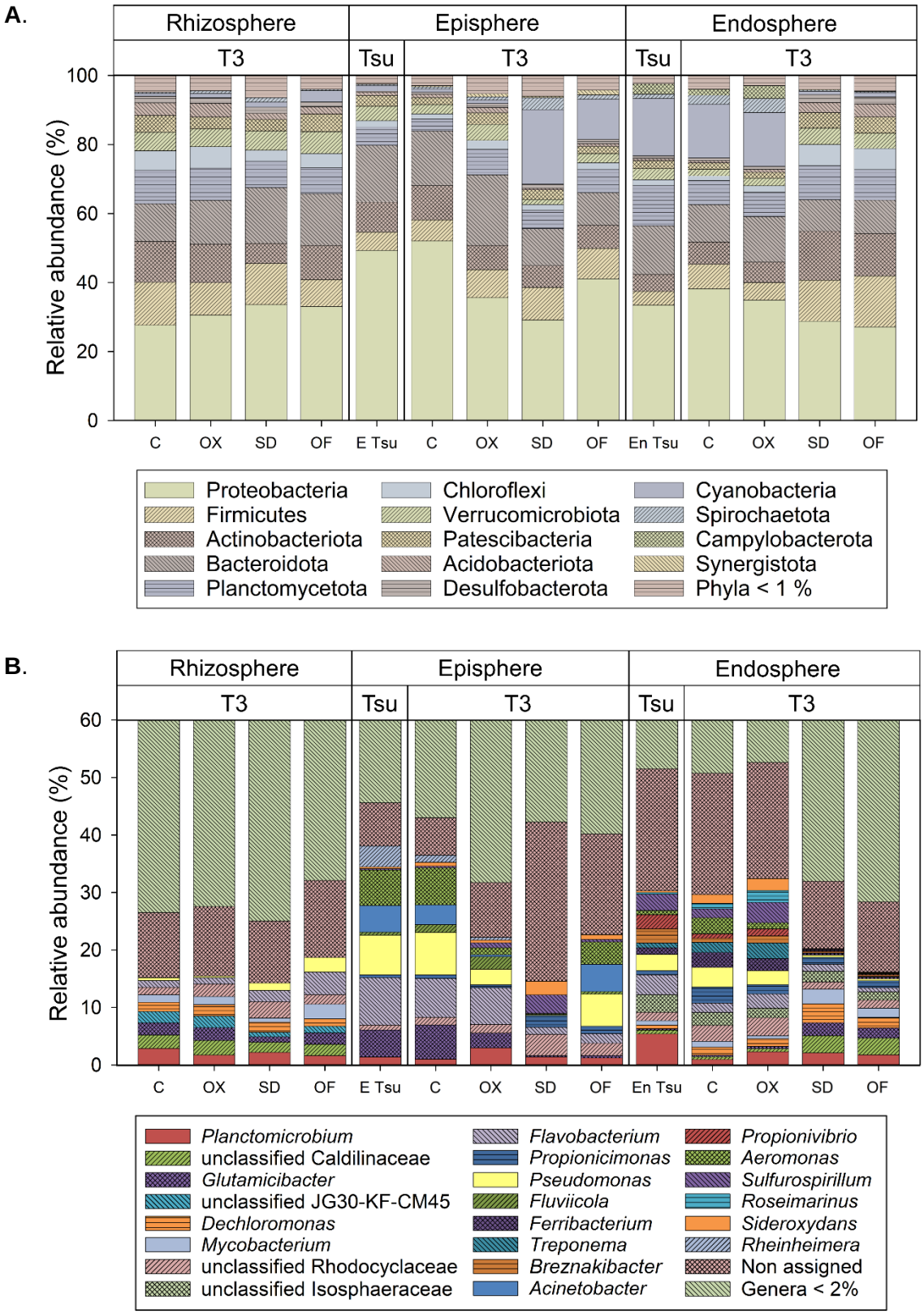


Figure 3.9 Taxonomic profile of rhizosphere, episphere and endosphere prokaryotes of *S. erectum* plants grown in CWs treating 4 LFD conditions. over time at the phylum level (A), and the genus level (B). C, OX, SD and OF bars represent the average relative abundance of the triplicated systems at the end of the experiments. The profile of one replica of the episphere and the endosphere of *S. erectum* collected in the stream is also presented here (Tsu).

composition, with OX systems showing a reduction in *Pseudomonas* and an increase in the less prevalent taxa and in *Planctomicrobium*., SD systems presenting an increase in Rhodocyclaceae (3.7 %), *Sulfurospirillum* (3.2 %), and *Sideroxydans* (2.4 %), and OF systems, reducing the relative abundance percentages of most genera. On the other hand, endosphere genera profile presented two main trends: from plants from C and OX systems (at T3) and from transplanted ones (at Tsu); and from plants form SD and OF systems. In SD and OF systems, the less prevalent genera, *Dechloromonas*, unclassified Caldilineaceae, and *Mycobacterium* increased, while unclassified Rhodocyclaceae, *Sulfurospirillum, Pseudomonas, Ferribacterium,* and *Treponema* dropped to less than 1 %.

Planctomicrobium, Glutamicibacter, Dechloromonas, Mycobacterium, Flavobacterium, Pseudomonas, Ferribacterium, and *Sulfurospirillum* are genera in the rhizosphere, episphere and endosphere of *S. erectum* related to plant growth-promoting rhizobacteria (PGPR). PGPR contribute to the sustainable development of plants by secreting specific compounds for plant health, facilitating the nutrient uptake from the rhizosphere, and contributing to phytopathology control (Hayat et al., 2010). Concerning the cycle of nitrogen, nitrifiers (*Nitrosomonas* sp. and *Nitrospira* sp. found in all compartments in < 1 %), denitrifiers (*Ferribacterium* sp., *Aeromonas* sp., *Flavobacterium* sp., and *Dechloromonas* sp.), and anammox bacteria (*Planctomicrobium* sp.), found in the root microbiome, are involved in nitrogen removal processes and previously found in CWs (Li et al., 2010; Tan et al., 2021; Zheng et al., 2021). Another alternative nitrogen removal process could be HN-AD, a process where nitrifications and denitrification steps are consecutive, under aerobic conditions, that could be performed by members of *Dechloromonas, Aeromonas, Ferribacterium, Acinetobacter, Fluviicola, Propionivibrio, Pseudomonas* and *Glutamicibacter* (Bai et al., 2021; Tan et al., 2021; Xi et al., 2022). HN-AD bacteria were found to grow fast in tidal flow CWs, using a wide range of carbon sources, converting NH_4^+, NO_2^- and NO_3^- in N_2 (Tan et al., 2021).

Moreover, some members of *Pseudomonas* and *Sideroxydans,* genera associated with *S. erectum* root microbiome*,* are PGPR producing siderophores which are organic compounds with a strong affinity to metal ions, important for the dissolution and chelation of these elements, making metal ions bioavailable and indirectly promoting plant growth (Swayambhu et al., 2021). In addition, siderophores produced by *Sideroxydans* could be conjugated to antibiotics that may bind to the outer membrane of pathogens, removing antibiotics and protecting plants from pathogens (Zheng et al., 2022). Another metal

removal process could be mediated by *Pseudomonas* or *Acinetobacter* species that can adsorb in the cell wall metals namely Cr (Martínez-Martínez et al., 2023).

Regarding carbon cycling, some species belonging to *Rheinheimera*, *Propionicimonas*, and *Propionivibrio* were related to the degradation of complex organic compounds, and fermentation of simple carbohydrates and fatty acids namely propionate (Li et al., 2022; Tao et al., 2023). Additionally, *Flavobacterium* and *Sulfurospirillum* are methanotrophs, crucial for greenhouse gas emission control in CWs, the latter can use sulphate or metals as electron acceptor for anaerobic methane oxidation (Mellado & Vera, 2021; Wang et al., 2022). Unclassified JG30_KF_CM45, also found in the top layer of sand, presented potential for the removal of organic contaminants (Ruppelt et al., 2020).

However, in the root microbiome some genera namely *Mycobacterium*, *Pseudomonas*, *Treponema*, and *Aeromonas* have species that could be pathogenic and *Rheinheimera*, *Mycobacterium*, *Pseudomonas* and *Acinetobacter* are known to harbour species with ARGs (Bartlett et al., 2022; Li et al., 2022; Norris et al., 2015).

To continue the validation of *S. erectum* as good candidate in CWs treating industrial effluents, it is crucial to conduct future research at a pilot-scale and full-scale CWs with a long-term exposure of LFD with antibiotics. A non-invasive sampling strategy should be developed to permit the collection of samples in the different wetland layers over time. To fully realise the potential of *S. erectum* ecosystem services in CWs, a metagenomic approach should be considered to provide a more detailed picture of how microbial function performs in this system. Ensuring that good removals are maintained with seasonal changes and controlling that neither ARGs nor antibiotics transformation products are disseminated, this CWs configuration can be integrated in biogas plants as a decentralised wastewater treatment strategy to support the expansion of the circular economy.

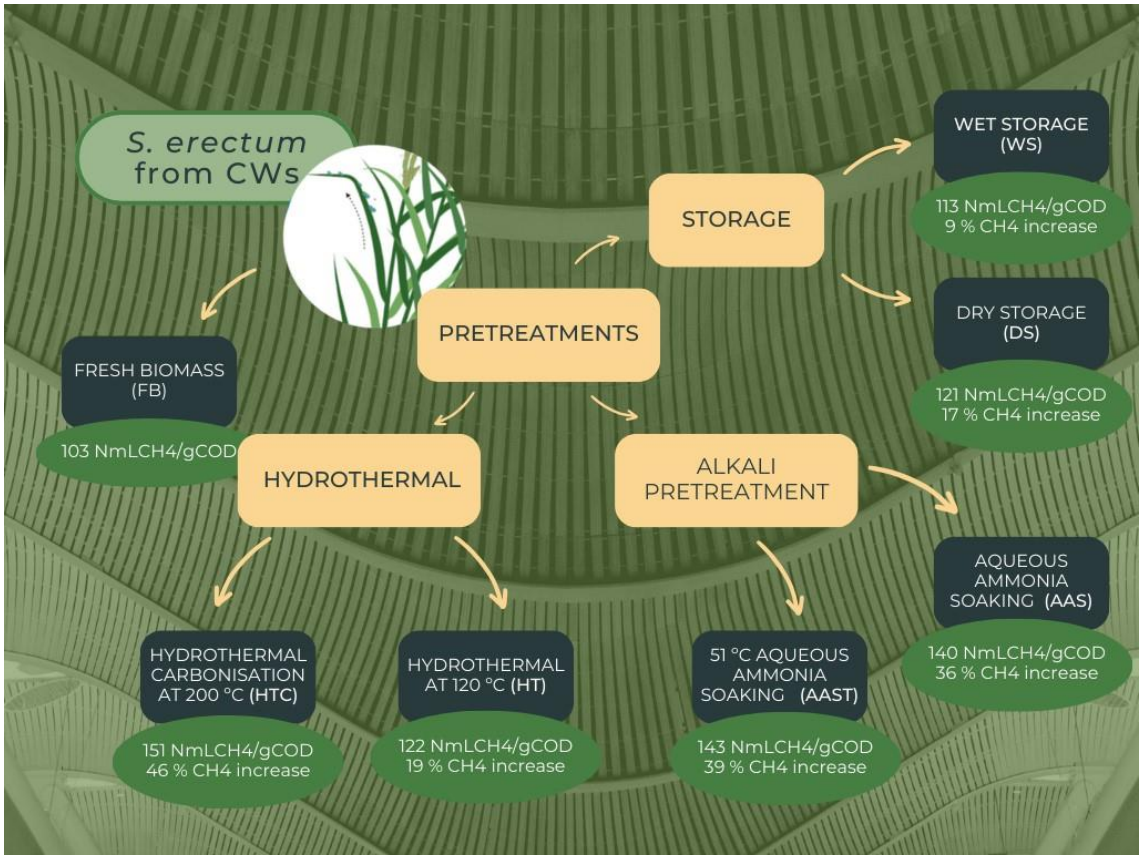
5. Conclusion

Vertical subsurface flow CWs successfully removed metals from LFD, whether spiked with selected antibiotics or not. *S. erectum* demonstrated good adaptation to the LFD, presenting a significantly growth in terms of both the number of individuals and amount of biomass over time. The *S. erectum* bioaccumulation factor for Fe and Mn was above 28 and for the other metals above 2.6, validating the phytoremediation ability of this plant species. At the end of the experiments, CWs showed notable accumulation of Fe, Cu and Pb in the roots, accumulation of Mn in the leaves, having a translocation factor over 1, and increase of Cr and Zn levels in LECA. Antibiotic spiking did not affect metal accumulation but influenced the microbial community structure. At T3, significant differences in the microbiome of the root bed substrate were observed in systems treated with sulfadiazine and ofloxacin compared to C and OX systems, despite their increase in richness and evenness over time with no significant differences between treatments during the two first experimental months. The ARGs prevalence among the microbial population decreased with time, except for *qnrS*. Moreover, regarding the microbiome in roots compartments, the spiking with antibiotics did not alter the composition of the rhizosphere but affected the structure of the episphere and endosphere, especially sulfadiazine and ofloxacin. The rich and even *S. erectum* roots' microbiome could potentiate its resilience in front of the stresses in CWs. Members of the most abundant genera were related to the geochemical cycle of carbon, the nitrogen cycle, the degradation of organic contaminants and metal biosorption. Finally, the episphere and endosphere of *S. erectum* roots maintained similar composition in both natural environments and CWs control systems. Understanding the microbial communities provides insights into ecological processes, evolutionary dynamics, and ecosystem resilience of CWs treating LFD with metals and other contaminants. This will contribute to improve the CWs management, ameliorating the removal processes in the future, and foster towards the integration of vertical subsurface flow CWs as an effective polishing process of AD, promoting environmental sustainability and SDG 6, SDG 7, SDG 9, and SDG 11.

Author Contributions:

PP-S: Conceptualisation, Methodology, Investigation, Validation, Formal analysis, Data curation, Writing - original draft, Visualisation; ; JPF: Methodology, Resources; MPT: Resources; ABM: Validation, Writing - review & editing, Supervision; GC: Conceptualisation, Project administration, Funding acquisition; BF: Writing - review & editing, Supervision; CRG: Resources, Writing - review & editing, Supervision; CMRA**:** Conceptualization, Methodology, Validation, Resources, Writing - review & editing, Supervision, Project administration, Funding acquisition; APM: Conceptualisation, Methodology, Validation, Resources, Writing - review & editing, Supervision, Project administration, Funding acquisition

Chapter 4. Effect of storage, aqueous ammonia soaking and hydrothermal pretreatments in anaerobic digestion performance of *Sparganium erectum* biomass



This chapter is based on the manuscript that is forthcoming in publication:

Pau Porras-Socias, Miriam Guivernau, Alexandre B. De Menezes, Carlos R. Gomes, C. Marisa R. Almeida, Ana Paula Mucha and Belén Fernández. “Effect of storage, aqueous ammonia soaking and hydrothermal pretreatments in anaerobic digestion performance of *Sparganium erectum* biomass”.

1. Abstract

Sparganium erectum is an emergent macrophyte commonly found among the harvested biomass collected in campaigns of environmental conservation, and with potential for application in constructed wetlands. New options for biomass valorisation are needed through anaerobic digestion. In this study, different pretreatment methods were assessed to enhance the methane yield of this plant biomass collected from constructed wetlands. The three tested pretreatments were storage (comprising dry storage through solar drying and wet storage via ensiling), aqueous ammonia soaking 15 % w/w (under two conditions: one at room temperature for 4 days, and the other at 51 °C for 27 hours), and hydrothermal pretreatments (one at 120 °C for 2 hours and the other at 200 °C for 1 hour). The effects of these pretreatments were evaluated through physicochemical and morphological analysis, biochemical methane potential tests, microbiome, kinetics and techno-economic analysis. Among all pretreatments, only the alkali and hydrothermal pretreatments presented differences in cellulose, hemicellulose and lignin content. While stored biomass showed no noticeable morphological changes, leaves pretreated with aqueous ammonia soaking displayed holes in the surface, and hydrothermally treated plants exhibited fissures in the cell wall. The centrate phase of the hydrothermal pretreatment at 200 °C for 1 hour yielded the highest methane production of 151 NmL/gCOD after 56 days, being the highest methane yield increase. At day 7, at the beginning of the methane exponential production phase for most treatments, prokaryotic abundance in reactors treating stored and hydrothermally pretreated biomass was higher than in untreated biomass. Although all pretreatments enhanced the methane yield, less than 50 % of COD was converted into methane, suggesting potential for further biodegradability improvement through more research on pretreatments or co-digestion options. Techno-economic assessment revealed significant benefits only with dry storage in terms of kWh/ton of substrate. Overall, this research underscores the significance of exploring pretreatment methodologies to recover energy from non-edible sources, highlighting the importance for sustainable energy production and environmental stewardship.

Keywords: Anaerobic digestion, pretreatment, methane yield, macrophyte, storage, aqueous ammonia soaking, hydrothermal pretreatment.

2. Introduction

The excessive natural growth of aquatic macrophytes poses a global problem disrupting human activities, and consequently, many resources are invested for their periodical removal. They are often viewed as water weeds and they are removed to prevent flooding, avoid clogging, facilitate irrigation, control plagues, and enhance boat traffic and recreational activities (Thiemer et al., 2021). These plants can also contribute for the removal of contaminants from natural waters (Fletcher et al., 2020).

Constructed wetlands are wastewater treatment technologies mimicking natural wetlands designed to efficiently treat various types of pollutants. With low operation and maintenance requirements, they can be integrated at different steps of the wastewater treatment process and exhibit robust performance in domestic, industrial, or agricultural effluents (Dotro et al., 2017). The presence of macrophytes in constructed wetlands enhances the removal of contaminants, improving the water quality (Brisson & Chazarenc, 2009). While removing pollutants, these systems are producing loads of plants biomass without competing with the human and husbandry food chain. However, these plants phytoextract pollutants, such as trace metals, and it is important to manage their biomass properly to prevent the pollutants from reintegrating again in water bodies (Kochi et al., 2020).

Presently, harvested macrophytes are primarily disposed of in landfills, incineration, or composting plants, independently from the source where they are collected. A highly promising cost-effective technology to manage the abundant, available, and non-edible aquatic biomass is anaerobic digestion (AD) (Cinar et al., 2022). Hence, this second-generation biorefinery offers two main advantages: the removal of pollutants from contaminated waters by plants, and the generation of bioenergy products from the plants biomass (Martínez-Gutiérrez, 2018). By valorising phytoremediation plants, circular economy is enhanced, contributing to the overall sustainability and environmental viability of the process. This impact is positive, measured in terms of global warming potential over a 100-year period, when the distance between the harvesting site and the biogas plant is up to 267 km (Vigil et al., 2022).

Aquatic macrophytes are primarily composed of moisture (>70 % wt) and organic matter (< 24 % wt), being polysaccharides and lignin the major organic components (Moeller et al., 2018; Rabemanolontsoa & Saka, 2013). Their cell walls are a matrix of complex fibers of cross-linked polysaccharides, namely cellulose, hemicellulose and pectin, lignin, and proteins, forming structures resistant to chemical and/or biological attacks, even at

high temperatures (Ahmed et al., 2019; Höfte & Voxeur, 2017). Consequently, the complex hierarchical structure of macrophytes, recalcitrant to microbial degradation in some cases, along with its high moisture content and the dependence on its seasonal availability, lead to a very limited use of this biomass as a feedstock for digesters (Martínez-Gutiérrez, 2018). To minimise the drawbacks and to enhance the biodegradability of aquatic plants, many pretreatments can be considered for breaking down the polymers before the AD process (Alam et al., 2021).

Pretreatments are classified into physical, chemical, physicochemical, and biological (or combined) processes and their effectiveness differs between different substrates (Abraham et al., 2020). Mechanical pretreatments, namely grinding or milling, reduce the particle size, thus incrementing the surface area of the plants and its accessibility to microbial degradation during AD (Abraham et al., 2020). The thermal drying of biomass has benefits for both waste management, by reducing its mass and volume, saving costs in storage, handling, and transport, and as an AD pretreatment, by improving biogas production. Greenhouse solar drying has been recently investigated as a cost-effective and sustainable pretreatment (Morey et al., 2023; Papastefanakis et al., 2023). Another storage technique is ensiling where the biomass is compacted to remove air and then sealed creating anaerobic conditions that promote lactic-acid production bacteria growth and pH drop. This fermentation process enhances lignocellulosic digestibility leading to an increase in the production of methane during AD (Sun et al., 2021). The aqueous ammonia soaking, being an alkali-based pretreatment, improves the removal of lignin and xylan without inducing significant changes in the cellulose allomorphic structure, enhancing the hydrolysis in AD (C. Zhao et al., 2020). The hydrothermal pretreatment is the process of heating water and biomass at a high temperature and pressure. This treatment solubilises hemicellulose and lignin at temperatures above 150 °C and can also alter cellulose crystallinity. But cellulose solubilisation into sugars, aldehydes and other molecules initiates above 230 °C (Ahmed et al., 2019).

Sparganium erectum is an emergent macrophyte that typically grows in shallow waters or very wet soils, found in various temperate regions of Europe, North Africa, Asia, North America, and Australia. This species is prevalent in the *Sparganium* genus, commonly known as bur-reeds (Gottsberger, 2020). Moreover, *S. erectum* is a plant with metal phytoextraction potential, making it suitable to be planted in constructed wetlands, even though its use is not common so far (Porras-Socias, 2024). A screening study in Germany of water weeds control showed that bur-reeds were the most abundant/frequent plants sampled and they presented a methane yield of 223 NmL

CH_4/g VS (Moeller et al., 2018). Therefore, *S. erectum* is potentially valuable for energy recovery through AD, given its biomethane potential, widespread presence in aquatic weed harvesting campaigns, and available for its utilisation as phytoremediation plant in constructed wetlands. However, to our knowledge, studies focusing on enhancing the biomethane production of this species are scarce. Thus, this chapter aims to evaluate the efficiency of different pre-treatments with available resources in biogas plants to enhance the valorisation potential of harvested *S. erectum*.

3. Materials and methods

3.1. *S. erectum* biomass

The substrate used in this study was the aboveground part of *S. erectum* harvested from laboratory-scale constructed wetlands (CWs). The constructed wetlands treated liquid fraction of the digestate from a biogas plant for 130 days, dealing with the organic fraction of municipal solid waste (Porras-Socias et al., 2024). *S. erectum* plants were collected on 26th November 2021, and subsequently, the biomass was stored at -20 °C in sealed plastic bags.

Only the aboveground of the plants was used in BMP experiments because in regular constructed wetlands maintenance, only this part is harvested. The belowground was not collected specifically to not alter all the substrate layers and the microbial community biofilms during the tearing of plants. Leaves (5 kg) were thawed and then cut with scissors into 1 cm pieces approximately (named FB or fresh chopped biomass). FB pieces were homogenised independently from the constructed wetland system of origin, to enable the collection of representative samples at our scale, mimicking a grinder or chipper machine.

3.2. Pretreatments

Chopped plants were subjected to three different pretreatments including storage, an alkali pretreatment with aqueous ammonia soaking and a hydrothermal pretreatment. Two conditions were tested by pretreatment (Figure 4.1). These pretreatments were selected considering their applicability and economic viability in a biogas plant. Every pretreatment was done in triplicate. We assumed that the biogas plant would have storage space, as well as an available quantity of ammonia aqueous solution, water, and energy. The effectiveness of each pretreatment was evaluated with the degradation percentage of fiber materials and with the morphological modifications of the leaf tissues.

3.2.1. Storage

The two storage conditions were dry and wet storage. On the one hand, the dry storage pretreatment (named DS pretreatment) was done by solar drying of the plants: 300 g of fresh chopped biomass was placed in three 8.4 x 10.6 x 3.7 cm^3 aluminium trays, distributed homogenously in a solar drying pilot plant described previously by Prenafeta-Boldú et al. (2021). The drying process, performed in triplicate, occurred for 14 days between 19th December 2022 and 2nd January 2023, while monitoring the temperature

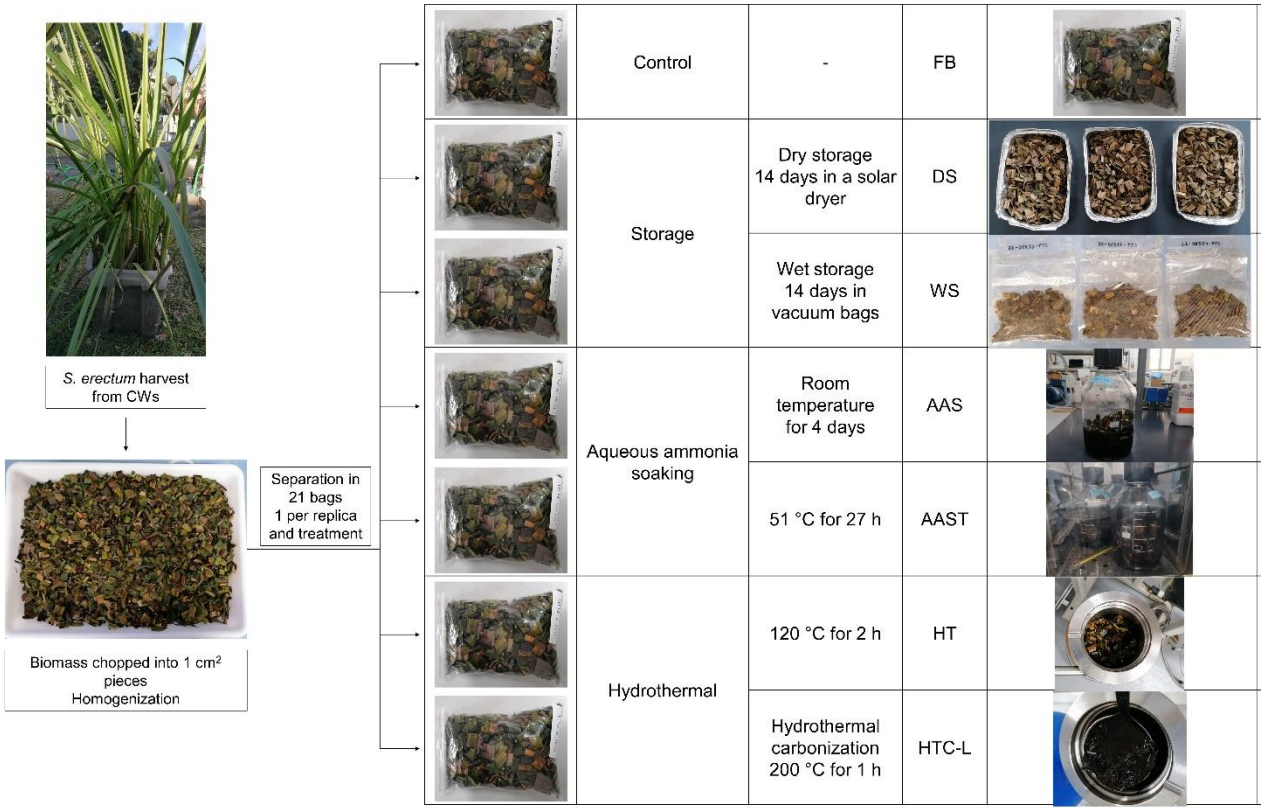


Figure 4.1 Summary of the methodology followed for the pretreatment of *S. erectum* biomass.

and the air velocity inside the solar dryer and the weight of the trays. The final moisture of biomass was 8.94 %. The dried biomass was stored in sealed plastic bags at -20 °C before sample analysis and BMP preparation.

On the other hand, the wet storage (named WS pretreatment) was conducted as a lab-scale ensiling. Vacuum bags were prepared with 100 g of fresh chopped *S. erectum* with a moisture content of 82.1 ± 0.3 %, in triplicates. The ensilage was set up at room temperature (25 ºC) in dark conditions for 14 days because the lactic fermentation of the biomass occurs during the first 14 days of ensilage, before the subsequent undesirable fermentations between days 14 and 120 (Hillion et al., 2018). At day 14, the ensiled biomass was stored at -20 °C in sealed plastic bags before sample analysis and BMP preparation.

3.2.2. Aqueous ammonia soaking

The aqueous ammonia soaking pretreatments of the biomass were conducted in triplicates at two different temperatures and times. For that, 100 g of bur-reed chopped pieces were added to Pyrex bottles (2 L) with 610 mL of an aqueous ammonia solution (15 %w/w) (NH_4OH solution 32 %, Sigma-Aldrich, Missouri, United States of America).

The liquid-solid ratio in each bottle was 1/1 (v/v), corresponding to 29.8 g of total solids (TS) of biomass per liter. Bottles were sealed to avoid NH_3 losses and were left intact protected from light. One set of bottles in triplicates was kept at room temperature (25 ºC) for 96 h (named AAS pretreatment), while another set was placed in an incubator at 51 °C for 27 h (named AAST pretreatment).

At the end of each pretreatment, to recover the treated biomass, first 610 mL of deionised water were added to each bottle to perform a vacuum evaporation of the solution. A rotary evaporator (Rotavapor™ R-100, Büchi, Switzerland) was used for this purpose and the evaporation was performed at 130 mbar at initial temperature of 20 °C (evaporator's water bath) and progressively raised up to 40 °C, 50 °C, 60 °C, and 80 °C with a total duration of 80 min following the methodology described in Lymperatou et al. (2017). After the vacuum evaporation, a liquid-solid separation of the substrate was performed by centrifugation at 13,000 g for 20 min at 4 °C (Eppendorf™ Centrifuge 5810 R, Germany). Both fractions (centrate named AAS-L and AAST-L; and cake named AAS-S and AAST-S) were stored separately at -20 °C before characterisation and BMP preparation.

3.2.3. Hydrothermal treatment

For the hydrothermal pretreatment, 200 g of chopped biomass and 1,220 mL of deionised water were added in a high-temperature and high-pressure reactor (model Autoclave Zipperclave Pressure Vessel, Iberfluids S.A., Spain), keeping a liquid-solid ratio 1/1 (v/v). Two different conditions were performed, each in triplicates: 2 h at 120 °C (named HT pretreatment) and 1 h at 200 °C (named HTC pretreatment, or hydrothermal carbonisation). Reactions were under equilibrium pressure (< 2 bar at 120 °C and 14 bar at 200 °C for HT and HTC, respectively). When the reaction stopped, the product was cooled to ambient temperature (25 ºC) and centrifuged at 13,000 g for 20 min at 4 °C. The centrate and the cake fractions (centrates named HT-L and HTC-L; cakes named HT-S and HTC-S) were stored separately at -20 °C before sample analysis and BMP preparation.

3.3. Biochemical methane potential (BMP) test and kinetics

BMP tests were conducted in 1.2 L flasks with a working volume of 500 mL. The inoculum was collected on 25th January 2023 in a mesophilic anaerobic digester treating sewage sludge (wastewater treatment plant La Llagosta, Barcelona, Spain) and stored for two weeks to consume the residual organic matter. The seven treatment groups of the experiment, all in triplicates, comprised the six pretreated biomasses (DS, WS, AAS,

AAST, HT and HTC-L) and the untreated biomass FB. At the moment of setting the BMP up, the AAS, AAST and HT samples were reconstructed maintaining the same percentage of centrate and cake phases, that were stored separately after centrifugation. In the case of HTC, only the centrate phase (HTC-L) was used for the BMP test, while the cake phase, namely the hydrochar, was kept for other revalorisation purposes. The substrate-to-inoculum ratio was adjusted to 1:1 based on volatile solids (VS) content, except for the three control flasks that contained no substrate (named C), being the initial inoculum and substrate concentration of 5 g VS/L and 5 g COD/L, respectively. 2.5 g of bicarbonate was added per flask to buffer the medium, maintaining an optimum pH between 8 and 8.5. The volume was adjusted to 500 mL with deionised water. The pH in the AAS and AAST bottles was neutralised with hydrochloric acid. All BMP experiments were set in triplicates. A total of 24 bottles were sealed with rubber stoppers and flushed with N_2 for three minutes to ensure an anaerobic environment.

The vials were placed under mesophilic conditions (37 °C) in an incubator chamber during a period of 56 days, from 8th February to 5th April 2023. All vials were stirred manually once per day and the biogas production was monitored along the experiment duration, three times a week through gas chromatography (CH_4, CO_2 and N_2 composition) Agilent CP-3800 unit equipped with a Hayesep packed column (Q 80/100 Mesh; 2 m × 1.8″ x 2.0 mm SS) and a thermal conductivity detector (Varian, USA).

From each flask, 1 mL of working volume was collected at days 7, 13, 19, 26, 35, 41, 48 and 56, was immediately centrifuged for 5 min at 20,000 g and 4 °C, and the pellets were stored at - 80 °C for microbial community analysis and the supernatants were kept at – 20 °C to measure the volatile fatty acids (VFA).

Total VFAs and the net cumulative methane volume produced were expressed in terms of COD equivalents, and the biodegradability of the substrates was measured using the COD conversion efficiency ratio by dividing the experimental methane yield (NmL CH4/g COD) by the theoretical yield (350 NmL CH4/g COD).

The kinetics of the experimental methane production from the untreated and the pretreated biomasses were fitted to equation 4.1, the modified Stannard-Richards model (Zwietering et al., 1990). A non-linear least-square regression analysis was performed with the solver tool of Excel 2016.

$$BMP_t = A\left\{1 + \upsilon.\exp(1+\upsilon).exp\left[\frac{\mu_m}{A}(1+\upsilon)^{\left(1+\frac{1}{\upsilon}\right)}.(\lambda - t)\right]\right\}^{-1/\upsilon} \quad \text{(Eq. 4.1)}$$

Where, BMP_t is the net methane production (NmL CH4 /g COD), A is the maximum specific methane production potential (NmL CH4 /g COD), μ_m, is the maximum specific methane production rates (NmL CH4 /g COD · d), λ is the lag-phase time (d), t is the incubation time (d), and υ is the shape coefficient.

3.4. Analytical methods

3.4.1. Molecular analysis

DNA and RNA were extracted simultaneously from pellet samples collected at days 7, 13, 19 (exponential growth phase) of the BMP test, with the RNeasy PowerMicrobiome kit following the manufacturer's instructions (Qiagen Inc., Venlo, Netherlands), while residual DNA (of RNA aliquot) was removed with RNase-free DNase I 2 U/µL (Applied Biological Materials Inc., Canada). Then, 20 µL of mRNA was reverse transcribed to cDNA with PrimeScript™ RT reagent Kit (Perfect Real Time) (Takara Bio Inc., Kusatsu, Japan). cDNA concentration and purity were assessed through spectrophotometry (NanoDrop ND-2000 and Qubit 4 Fluorometer, Invitrogen, MA, USA).

V4 region of 16S rRNA gene was amplified with the primer pair 515FB (GTGYCAGCMGCCGCGGTAA) and 806RB (GGACTACNVGGGTWTCTAAT), following the protocols from the Earth Microbiome Project. Amplicons were subjected to sequencing on an Illumina MiSeq sequencer with V3 chemistry (Illumina, San Diego, CA, USA) at Genoinseq (Cantanhede, Portugal). Data was processed following the method described in Porras-Socias et al. (2024).

3.4.2. Morphological analysis

The morphology of *S. erectum* leaves was observed in lyophilised samples collected before and after the pretreatments, as well as after the BMP experiments, with a Scanning Electron Microscope (SEM) (ZEISS Merlin®, Carl Zeiss Microscopy GmbH, Jena, Germany). Samples were lyophilised in a CRYODOS-50 lyophiliser (Azbil Telstar, S.L.U., Terrassa, Spain). Prior to microscopy, samples were gold-coated to reach a ~200 Å thickness in a sputter coater K500X (Emitech, France).

3.4.3. Chemical analysis

Well-homogenised chopped samples of untreated and pretreated biomass were characterised with the content of total and volatile solids (TS, VS), pH, electrical conductivity (CE), total COD, total Kjeldahl nitrogen (TKN), total ammonium nitrogen (NH_4^+-N) (APHA et al., 2017). VFA, namely acetate, propionate, iso-butyrate, n-butyrate, iso-valerate and n-valerate acids concentrations were determined with a CP-3800 gas

chromatograph, (Varian, Palo Alto, CA, USA) fitted with TRB-FFAP (30 m · 0.32 mm · 0.25 µm) capillary column (Tecknokroma, Barcelona, Spain) and FID detection. The hemicellulose and lignin content were determined by gravimetric analysis with a NaOH treatment and a two-stage acid hydrolysis, respectively; while the cellulose content was calculated based on the acid detergent fiber (ADF) and the acid detergent lignin (ADL) methods (AOAC international, 2019). The content of total COD, hemicellulose and lignin were analysed from lyophilised powdered samples (CRYODOS-50 lyophiliser, Azbil Telstar, S.L.U., Terrassa, Spain). Only the cake fraction of AAS, AAST and HT was analysed for fibers characterisation, while the centrate fractions, including the HTC-L, were not analysed. The protein content was calculated with a conversion factor specific to vegetables following equation 4.2 (Mariotti et al., 2008).

$$\text{Proteins} = \text{TKN} - NH_4^+ \cdot 5.95 \quad \text{(Eq. 4.2)}$$

3.4.4. Data and statistical analysis

Each pretreatment was performed in triplicates, and each product was treated separately to set up three reactors, in parallel, testing the same condition. Technical analysis were also conducted in triplicates. The averages and standard deviations were calculated with Microsoft Excel 2019 and statistical analysis were conducted with Sigmaplot software v 14.0. The Shapiro-Wilk test was used to first test the normality of the dataset with a confidence interval of 95 %. If the distribution was normal, an ANOVA test was performed, followed by a Tukey test to calculate the significant differences between the pretreatments with p values < 0.05. If not, a Kruskal-Wallis one-way ANOVA on ranks was used.

3.5. Techno-economic assessment

The preliminary techno-economic assessment was performed to estimate the economic feasibility of the integration of harvest biomass from CWs treatment as biogas feedstock, after being submitted to the described pretreatments.

In this study, the assessment was performed based on the energy produced or consumed in kWh with 1 ton of substrate (untreated or pretreated) entering the reactor. For that, the equivalent tons of raw biomass were calculated, and the assumptions were made. Four possible scenarios were considered in this analysis depending on the hydraulic retention time (HRT) of the anaerobic mesophilic digester: 19, 25, 35, and 56 days (Elif Gulsen Akbay, 2024). The assumed methane calorific value was 10.97 kWh/Nm^3, and a thermal and electric yield of a combined heat and power unit of 45 % and 36 %, respectively (Misson et al., 2020). To assess the cost of the pretreatments,

the energy requirements of the chopper was 1.5 kWh/ton, of the vacuum evaporator was 150 kWh/ton and of the pasteurisation reactor is illustrated in equation 4.3.

$$Q = \rho \cdot V \cdot c \cdot (Tf - T0) \quad \text{(Eq. 4.3)}$$

with Q being the heat demand in kJ, ρ the density of the substrate in kg/m^3, V the volume of the reactor in m^3, c the specific heat capacity of the substrate in kJ/kg·°C, and T_f and T_0 the temperature at the end and beginning of the heating process, respectively (Lymperatou et al., 2022). The density and specific heat capacity of the substrates were considered that were the water ones.

4. Results and discussion

4.1. Untreated and pretreated *S. erectum* characterisation

The physicochemical characterisation of the aboveground tissues of *S. erectum* harvested in CWs is summarised in Table 4.1 Physicochemical parameters of the biomass of *S. erectum* before and after the pretreatments. Biomass presented a TS content of 17.83 %, a VS content of 86.27 % TS, comparable with the bur-reed literature values (Moeller et al., 2018), and an organic matter content of 254.37 g O_2/kg. The TKN and NH_4^+-N were 3.8 g N/kg and 12.2 % TKN, respectively, also coherent with TN content in bur-reed ranging from 2.2 to 5.3 g/kg of previous studies (Moeller et al., 2018).

The effect of the pretreatments on the physicochemical parameters of *S. erectum* is also presented in Table 4.1. Solar drying (DS) was the only pretreatment increasing the TS content of the biomass, reaching 91 %, while the VS content slightly decreased 86.0 % TS, on average (Figure 4.2). The moisture loss until reaching values around 10 % of moisture content and the maintenance of the VS was also observed in previous studies solar drying of other substrates namely food waste or aquatic biomass (Papastefanakis et al., 2023; Schmid et al., 2022). However, wet storage had a slightly diminishing effect on TS and VS content reducing them to 16.4 % and 84.1 % TS, respectively. The mild loss of organic dry matter was consistent with previous short-period ensiling studies (Herrmann et al., 2011). The ensiling process initiates with a bacterial fermentation step

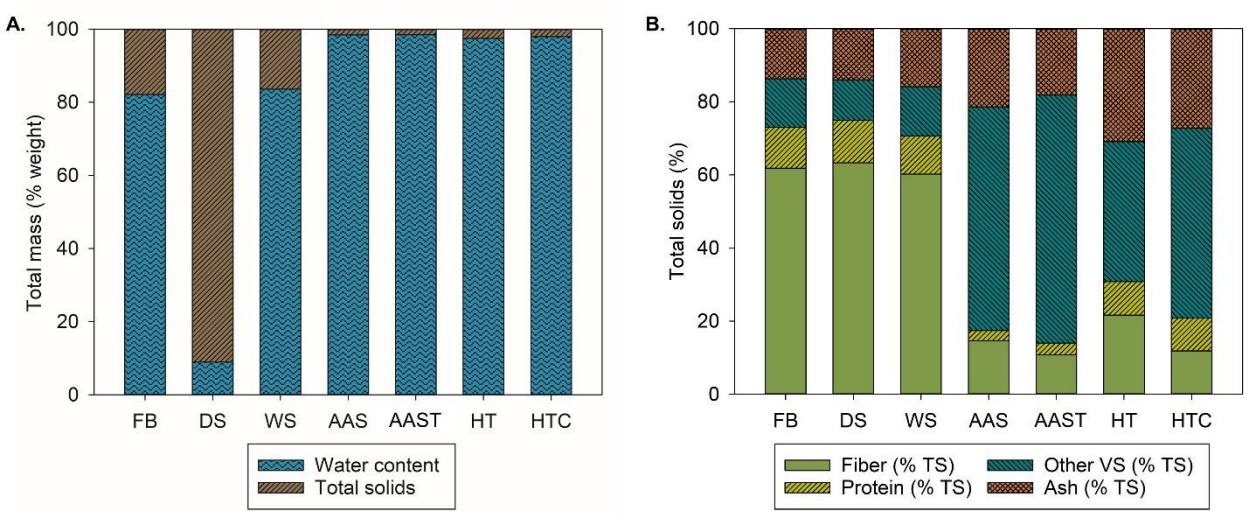


Figure 4.2 A. Average (n = 3) water and total solids (TS) content of untreated and pretreated *S. erectum* biomass samples. B. Average (n = 3) composition percentages of TS of untreated and pretreated *S. erectum* biomass samples, including fibers, proteins, other volatile solids (VS) and ashes.

producing lactic acid that leads to a pH drop to around 4.0 avoiding undesirable fermentations (Teixeira Franco et al., 2016), as observed in the present study.

The alkali and hydrothermal pretreatments significantly decreased both the TS to 1.56 - 2.53 %, as well as the VS of the reactor feedstock. This reduction resulted from the addition of aqueous reagents and water. No dewatering steps of the cake were included because each additional industrial process would increase the pretreatments cost. Consequently, the authors considered the biomass to be directly added to the AD reactor after the pretreatment. The VS content in the cake phase in AAS (4 days at room temperature) and in AAST (27 h at 51 °C) was 75 % TS and 79 % TS, respectively, suggesting the solubilisation of organic matter as the AAS treatments are known to make carbohydrates more accessible by breaking down and solubilising hemicellulose and lignin (Kim et al., 2016). Regarding hydrothermal pretreatments, the VS content of the cake was 69 and 55 % TS after HTC (1 h at 200 °C) and HT (2 h at 120 °C), respectively, which could also reflect the solubilisation of organic matter (especially sugars) from the plants to the liquid phase, as previously observed (Poomsawat & Poomsawat, 2021; B. Zhang et al., 2020). In Dutra et al. (2024) the HT pretreatment increased the lignocellulosic substrate's carbohydrates availability. The pH reduction to 3.77 of HTC-L was also observed in Poomsawat & Poomsawat (2021), being related to the production of organic acids during biomass hydrolysis. In both alkali and hydrothermal pretreatments, the one with higher temperature resulted in higher COD and VS content in the cake. Therefore, here, at the tested conditions, the temperature had a higher effect in organic matter solubilisation than the time of treatment. These results are consistent with the previous studies, even if for each substrate temperature, residence time and % VS have a different effect (Y. Li et al., 2015; Oliwit et al., 2020; Vakalis et al., 2022).

The TKN content of DS rose significantly, due to a concentration effect because of the loss of water content, while its NH_4^+-N percentage did not vary from FB. Despite a mild increase, the NH_4^+-N did not increase significantly during ensiling as compared to FB, thus, apparently, no protein degradation occurred (Sieborg et al., 2020). However, aqueous ammonia treatments increased significantly the TKN and NH_4^+-N of pretreated biomass to 13 – 14 g N/kg and 76 – 92 % TKN, respectively, due to the addition of NH_4OH solution during the pretreatment. This could also indicate an inefficient process of elimination and recovery of the added aqueous ammonia solution. Finally, hydrothermal pretreatments decreased the TKN content, due to the addition of water, and increased significantly the NH_4^+-N in % TKN, elucidating the potential breakdown of cell structures, proteins or other organic compounds releasing NH_4^+ (Ahmed et al., 2019).

Table 4.1 Physicochemical parameters of the biomass of *S. erectum* before and after the pretreatments (average values ± standard error, n = 3).

Treatment	Water content (% wt.)	VS (% TS)	Ash (% TS)	COD (g/kg)	pH	EC (mS/cm)	TKN (g N/kg)	NH_4^+-N (% TKN)
FB	82.1 ± 0.3 a	86.3 ± 0.8 a	13.7 ± 0.8 a	254 ± 4 a	5.80 ± 0.02 a	nd	3.8 ± 0.3 a	12.2 ± 0.7 ab
DS	9 ± 1 b	86.0 ± 0.7 ab	14.1 ± 0.7 ab	1185 ± 79 b	nd	nd	20 ± 2 b	12 ± 1 a
WS	83.6 ± 0.6 c	84.1 ± 1.6 ab	15.9 ± 1.6 ab	219 ± 20 a	4.26 ± 0.07 b	nd	3.3 ± 0.4 a	13.5 ± 0.9 ab
AAS*	98.39 ± 0.09 d	78.6 ± 1.3 c	21.4 ± 1.3 c	20.4 ± 0.5 c	11.4 ± 0.1 c	4.6 ± 0.2 ab	14 ± 3 c	76 ± 2 c
AAST*	98.44 ± 0.05 de	82 ± 1 bc	18 ± 1 bc	19.6 ± 0.4 c	11.35 ± 0.09 c	5.01 ± 0.02 a	13 ± 2 c	92 ± 5 d
HT*	97.5 ± 0.2 de	69 ± 3 d	31 ± 3 d	35 ± 3 c	5.4 ± 0.1 d	4.24 ± 0.07 b	0.51 ± 0.07 a	24 ± 4 e
HTC-L	99.27 ± 0.03 e	69 ± 1 d	31 ± 1 d	9.2 ± 0.6 c	3.77 ± 0.04 e	4.5 ± 0.3 b	0.202 ± 0.001 a	22 ± 7 be
HTC-S	92.1 ± 0.7	90 ± 2	10 ± 2	129 ± 2	nd	nd	1.09± 0.15	9 ± 2
AAS-L	99.60 ± 0.03	75 ± 2	25 ± 2	3.9 ± 0.2	11.4 ± 0.1	4.6 ± 0.2	16 ± 3	76 ± 3
AAS-S	94.3 ± 0.1	91.8 ± 0.5	8.2 ± 0.5	77 ± 7	nd	nd	8 ± 3	73 ± 11
AAST-L	99.43 ± 0.04	79 ± 1	21 ± 1	5.7 ± 0.2	11.35 ± 0.09	5.01 ± 0.02	14 ± 2	94 ± 5
AAST-S	94.0 ± 0.1	92.3 ± 0.9	7.7 ± 0.9	82 ± 2	nd	nd	9 ± 1	79 ± 3
HT-L	99.50 ± 0.04	55 ± 4	45 ± 4	4.1 ± 0.3	5.4 ± 0.1	4.24 ± 0.07	0.12 ± 0.03	15 ± 18
HT-S	94.2 ± 0.2	92 ± 1	8 ± 1	85 ± 4	nd	nd	1.2 ± 0.2	26 ± 3

Underlined treatments were the one used for BMP test.

** indicates the reconstruction of the coke (-S) and the centrate (-L).*

nd: not determined | The same letters indicate that there are no significant differences between groups with $p < 0.05$

Table 4.2 Effect of the pretreatments on the cellulose, hemicellulose and lignin content of *S. erectum* biomass and effect of AD on untreated (FB) and pretreated biomass (DS, WS, AAS-S, AAST-S and HT-S) (average values ± standard error, n = 3).

Treatment	Cellulose (% TS)	Hemicellulose (% TS)	Lignin (% TS)	Cellulose (% TS)	Hemicellulose (% TS)	Lignin (% TS)
	Before anaerobic digestion			After anaerobic digestion		
FB	35 ± 3 abc	20 ± 2 a	7 ± 1 a	11.74	5.50	8.99
DS	33 ± 2 ab	23 ± 2 a	7 ± 1 a	11.47	7.72	11.39
WS	30 ± 4 a	23 ± 2 a	7 ± 2 a	12.39	7.51	11.16
AAS-S	39 ± 2 bc	19.7 ± 0.7 a	5.7 ± 0.4 a	4.09	1.32	8.21
AAST-S	41 ± 2 c	12 ± 2 b	5 ± 1 a	3.66	0.70	6.02
HT-S	41 ± 2 c	18 ± 3 a	11.0 ± 0.3 b	10.27	3.24	9.62

The same letters indicate that there are no significant differences between groups with $p < 0.05$

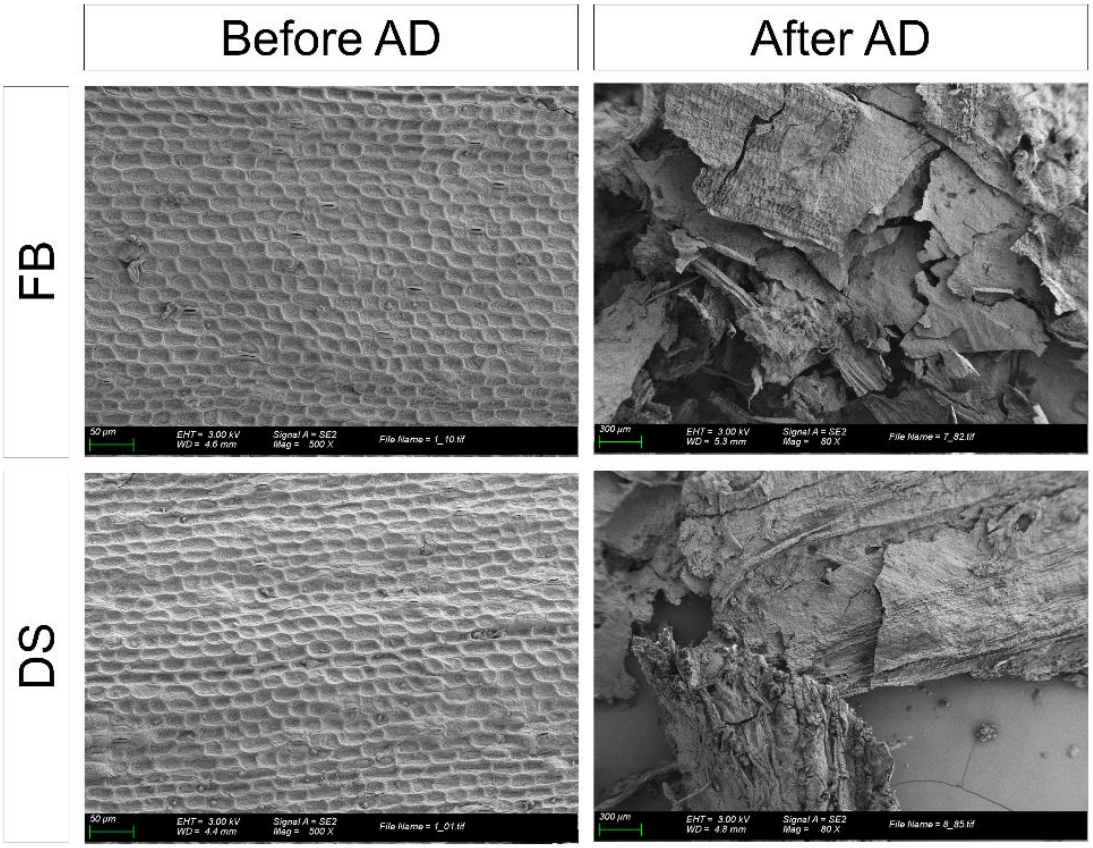
Focusing on the organic fraction of untreated *S. erectum*, cellulose, hemicellulose and lignin represented 35, 20 and 7 % TS, respectively (Table 4.2). The cellulose and hemicellulose content exhibited similar levels to other macrophytes namely *Phragmites australis*, *Typha latifolia* or *Carex elata* (Czubaszek et al., 2021). However, the lignin content of *S. erectum* was notably lower, akin to *Egeria densa* (Dutra et al., 2024). The variability in the lignocellulosic content can be due to the plant species, the geographical location, the environmental conditions, and the level of maturity, as lignification increases with the maturity (Barros et al., 2015; Czubaszek et al., 2021). After the pretreatments, both storage conditions resulted in a mild increase in the hemicellulose and lignin and reduction of cellulose content. The ammonia soaking provoked an increase in the cellulose content and a decrease in the lignin content, as previously observed (H. Yuan et al., 2020). Ammonia has a high lignin selectivity, cleaving its linkages with carbohydrates and making them more accessible (Kim et al., 2016). Regarding HT-S, both the cellulose and lignin contents rose to 41 and 11 % TS, respectively. Despite Dutra et al. (2024) showing a reduction of lignin content, other studies presented an increase in lignin with hydrothermal pretreatment due to its high recalcitrance at the tested conditions (Phuttaro et al., 2019). In addition, the pretreatment could have led to an undesirable loss of mass, concentrating the lignin. Therefore, with the results presented in Table 4.2, apparently, the pretreatment offering the best improvement of

anaerobic digestibility of *S. erectum*, would be AAST due to its high cellulose content, and lower hemicellulose and lignin content, diminishing the complexity of the leaf structure, facilitating enzymatic hydrolysis (Álvarez et al., 2016).

Observing the lignocellulosic cake's composition of AD products, there was a general trend of cellulose and hemicellulose percentage reduction, and an increase in lignin content when compared to the material before AD (Table 4.2), as previously reported (Luo et al., 2019). FB, DS and WS presented the highest levels of cellulose and hemicellulose, ranging 11.47 – 12.39 % TS and 5.5 - 7.72 % TS, respectively. Conversely, AAS and AAST exhibited the lowest concentrations, below 3.66 - 4.09 % TS and 0.60 - 1.32 % TS, respectively. Regarding the lignin content, DS and WS had the highest levels above 11 % TS and AAST the lowest being 6.02 % TS. With these results, it was expected to obtain greater conversion of organic matter to methane in AAST.

4.2. Structural and morphological changes of the pretreated and digested biomass

SEM is a useful tool to observe changes in the surface structure of the biomass (Pathan et al., 2010). The pretreatments aimed to improve the accessibility of the lytic enzymes, addressing the bottleneck in hydrolysis in AD reactions and boosting methane production. In the present study, Figure 4.3 illustrates the cell wall structure of *S. erectum*



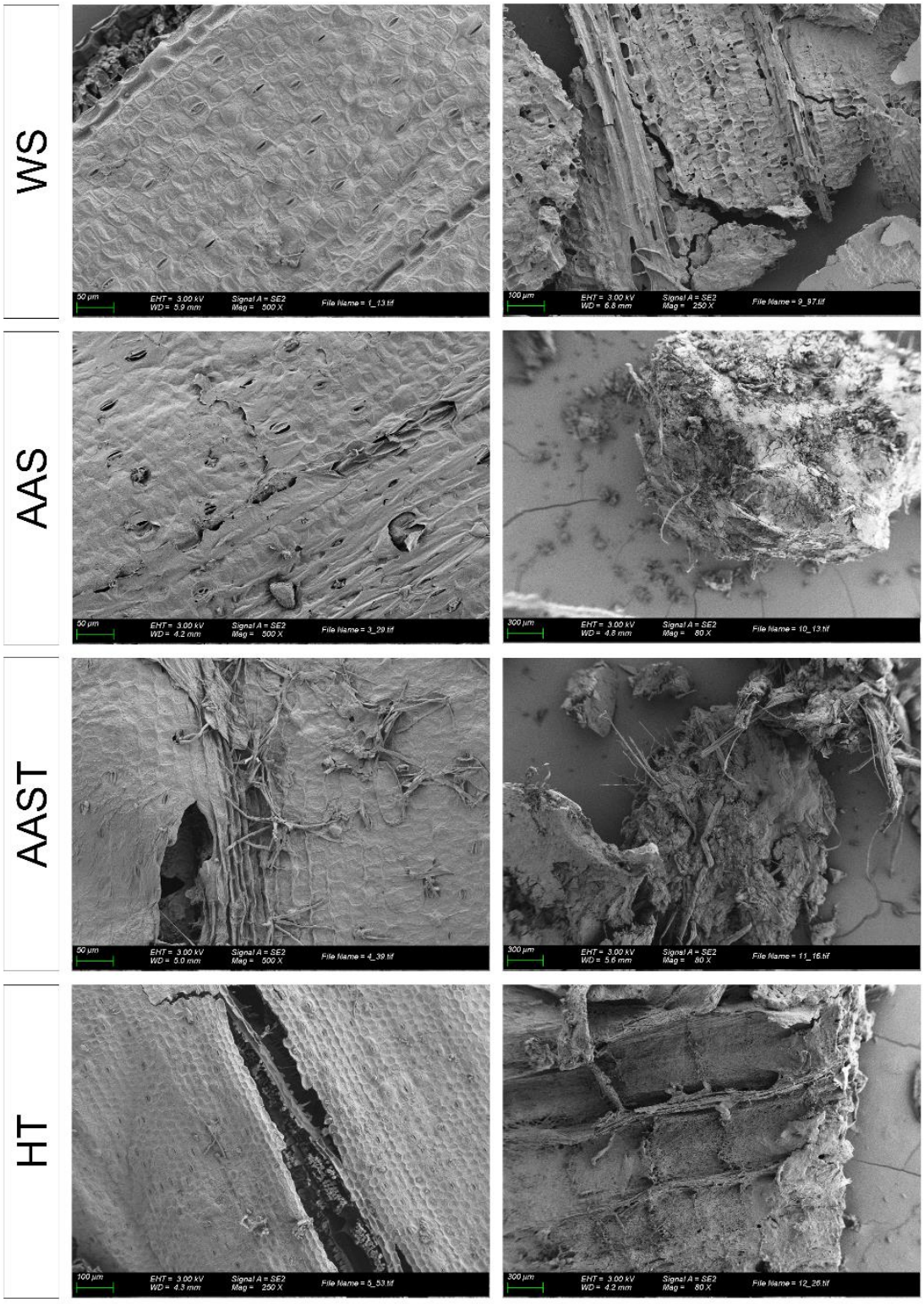


Figure 4.3 SEM micrographs of the plant tissues before and after the AD assays with untreated and pretreated *S. erectum* biomass.

leaves. The untreated biomass presented compacted epidermal cells and a smooth surface. The stored biomass (DS and WS) shared a similar morphology, featuring flattened cells with low relief and showing some pores in WS. Moreover, aqueous ammonia soaking induced changes in the morphology of *S. erectum*. On the one hand, AAS leaves exhibited numerous pores (smaller than 50 µm) in the cell wall. On the other hand, AAST biomass presented bigger pores (e.g. 150 µm of diameter) and it seems there is fiber detachment occurring. In HT pretreated biomass, a fissure, about 100 µm wide and over 600 µm long, opened up on the leaf epidermis, despite the remaining parts of the surface preserved integrity. Similar results were observed in previous works of alkali and/or HT pretreatments of *Miscanthus floridulus* (Fu et al., 2018), *E. densa* (Dutra et al., 2024) and rice straw (H. Yuan et al., 2020). Therefore, apparently, the pretreatment causing more damage to the epidermal morphology, allowing the anaerobes to increase the digestibility of the biomass were the AAST and the HT.

After AD, the surface structure of biomass was damaged in all reactors' cake products (Figure 4.3). Despite the degradation of the leaves tissues, FB, DS and WS products maintained epidermal or mesophyll cells and vascular tissues. However, in AAS and AAST, the initial morphology of the leaf surface was unrecognizable, forming a spherical conglomerate with fibers that hadn't been degraded. As for HT products, micrographs show the opening of the leaf, exposing the internal cavities undergoing degradation. Overall, the aqueous ammonia soaking pretreatments presented at the end of AD process more apparent biomass degradation, suggesting a higher potential for the transformation of organic matter into methane.

4.3. Effect of the pretreatment on the methane production

The modification of the structure and composition of biomass through pretreatments has an impact on its digestibility and on methane production (Kamusoko et al., 2019). Figure 4.4 shows the curves of the experimental net cumulative methane yield of BMP experiments. The untreated bur-reed presented firstly, during the lag phase lasting 13 days, a steady increase of the methane production, then, an exponential phase of approximately 10 days, culminating in a methane yield of 79.7 mL CH_4/g COD at day 25, and finally a gradual increase resulting in a final yield of 103.0 mL CH_4/g COD at day 56, which is a similar yield previously observed for untreated seaweed (Lymperatou et al., 2022). WS and DS showed a similar cumulative methane yield profile with slightly higher yields, reaching 112.6 and 121.0 mL CH_4/g COD on average, respectively, having the least effect on methane yield among all pretreatments (Table 4.3). Although several

studies presented an increase in biogas production when using dried biomass as a co-substrate with highly biodegradable or N-rich substrates (D'Silva et al., 2022; Papastefanakis et al., 2023), dried biomass appeared to hinder the bacterial growth and mobility due to reduced accessibility of moisture and nutrients (Moussaoui et al., 2024). In addition, low effects in methane yield were also observed previously in silage crops as compared with control ones (Herrmann et al., 2011), as its main function is the storage of the material without energy losses and the longer the silage periods, the higher the methane yield due to an increasing concentration of ethanol with time of storage (Villa et al., 2020).

In the case of biomass treated with alkali pretreatments (AAS and AAST), the lag phase extended to day 16, longer than observed in raw bur-reed. Probably, microbial population of AD reactor were initially inhibited by the high concentrations of ammonia of the substrate (Z. Yang et al., 2018). Ammonia could also exert selective pressure on sensitive microbes and promoted a microbial shift. Also, dissolved lignin produced during the alkali pretreatment could have also inhibited temporarily the methane production but

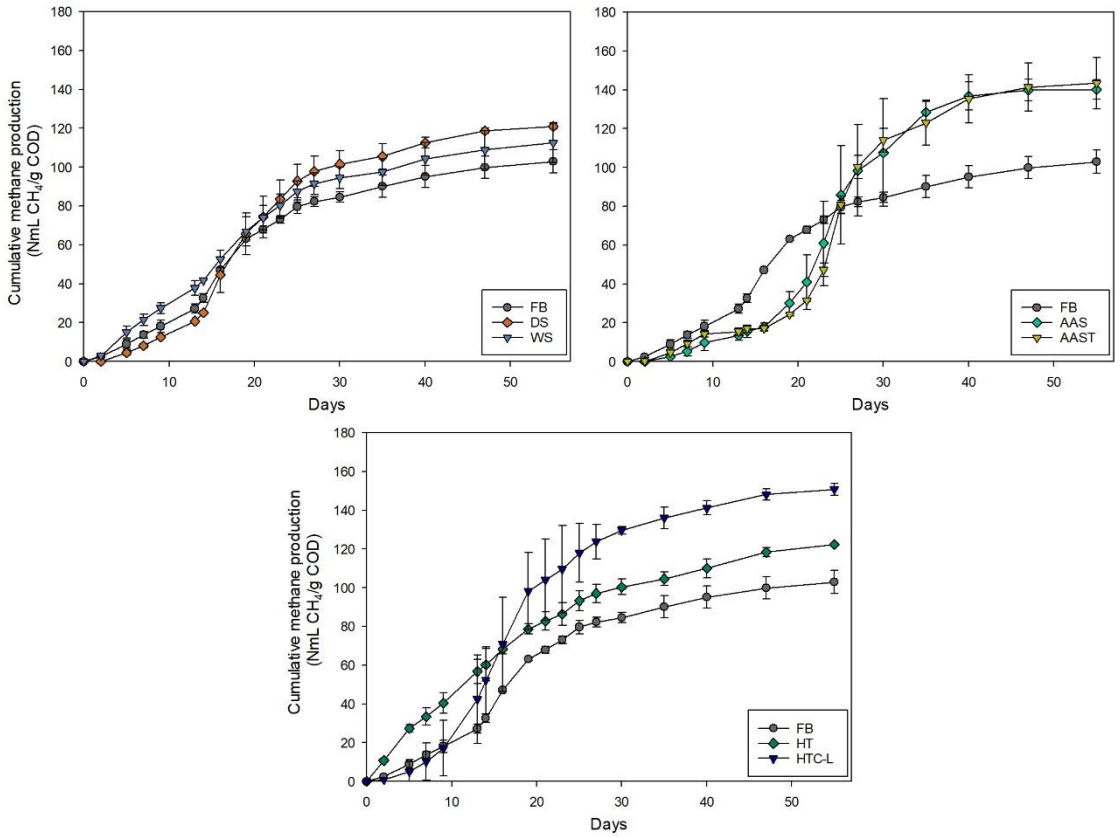


Figure 4.4 Net cumulative methane yield of BMP experiments of untreated and pretreated *S. erectum* biomass. Dots represent the experimental data, while the lines correspond to the fit to the Stannard-Richards model for each batch.

improved the AD efficiency in the long term (Koyama et al., 2017). Subsequently, the methane production exhibited an exponential growth, surpassing the methane yield of untreated biomass at day 25. At the end of the experiment, the cumulative methane yields were 140.1 mL CH_4/g COD for AAS and 143.4 mL CH_4/g COD for AAST (Table 4.3). No significant differences were observed in the methane production between the two AAS pretreatments. This represented an increase of 28 and 31 %, respectively, which is lesser than the 36 % obtained by Lymperatou et al. (2022).

Table 4.3 Cumulative biogas and methane yields of AD of untreated and pretreated *S. erectum* biomass after 56 days of digestion (average values ± standard error, n = 3).

Treatment	Biogas yield (N mL/g COD)	CH_4 yield (N mL/g COD)	CH_4 yield increase (%)	Biogas yield (N mL/g VS)	CH4 yield (N mL/g VS)	CH_4 yield increase (%)	CH_4 percentage in biogas (%)
FB	176 ± 14	103 ± 6		290 ± 22	170 ± 9		58 ± 1
DS	198 ± 9	121 ± 1	17 ± 1	310 ± 4	190 ± 8	12 ± 5	61 ± 4
WS	184 ± 18	113 ± 10	9 ± 9	292 ± 33	178 ± 19	5 ± 11	61.1 ± 0.4
AAS	217 ± 4	140 ± 5	36 ± 5	323 ± 13	208 ± 3	23 ± 2	64 ± 4
AAST	214 ± 33	143 ± 13	39 ± 13	309 ± 50	207 ± 20	22 ± 12	67 ± 4
HT	204 ± 3	122.4 ± 0.8	18.9 ± 0.8	328 ± 4	197 ± 2	16 ± 1	60 ± 1
HTC-L	239 ± 5	151 ± 3	46 ± 3	429 ± 3	271 ± 2	60 ± 1	63.25 ± 0.00

HT pretreated *S. erectum* presented the highest cumulative methane yield during the first 16 days. This yield gradually rose throughout the experiment reaching a final yield of 122.4 mL CH_4/g COD. This represents a 12 % increase in methane production, falling within the range reported in previous studies on HT pretreatments at similar temperatures (J. Du et al., 2019; Luo et al., 2019). In addition, the methane production of the centrate phase of HTC had a lag phase of 7 days and a consecutive exponential growth becoming the highest from day 16. The rise was expected since this reaction facilitates cell disruption, releasing available organic compounds such as sugars and lipids (Nguyen et al., 2023). The final yield was 150.9 mL CH_4/g COD, presenting an increase of 38 % in the methane yield. This increase was also reported with other aqueous phases of substrates such as microalgae and the organic fraction (37 %) treated through HTC (88 %) (Lucian et al., 2020; Marin-Batista et al., 2019).

The Stannard-Richards kinetic model successfully simulated the cumulative methane yield curves of BMP of both untreated and all pretreated biomass, as the model's simulated values closely matched the experimental ones (Figure 4.4). All the R^2 were higher than 99.05 % (Table 4.4). The parameters A and λ values, representing the maximum specific methane production potential and lag phase time, respectively, fitted

well with experimental results of each substrate. Notably, the model revealed that HT has no lag phase, while AAS and AAST showed the longest lag phase times, being 15 and 18 days, respectively, consistent with experimental results. Moreover, the kinetic model predicted that the most suitable pretreatment with the highest methane yield and with a relatively short lag phase time was HTC, as HTC-L biomass produced 146.43 mL CH_4/g COD with a lag phase of 7 days.

Table 4.4 Kinetic parameters of methane production in anaerobic reactors fed with untreated biomass (FB) or pretreated biomass (DS, WS, AAS, AAST, HT and HTC-L) of *S. erectum* fitted with the Stannard-Richards model.

Treatment	Experimental	Stannard-Richards model parameters				
	A exp	A	μm	λ	v	R^2
FB	103	100.29	4.33	5	0.235	0.9944
DS	121	117.00	6.14	9	0.298	0.9952
WS	113	112.12	4.19	3	0.058	0.9955
AAS	140	139.39	8.13	15	1.374	0.9979
AAST	143	137.57	9.75	18	2.624	0.9915
HT	122	120.00	4.31	0	0.001	0.9905
HTC-L	151	146.43	7.68	7	0.001	0.9970

To have a better understanding of biomass biodegradability and methane production efficiency, Figure 4.5 illustrates the production of VFA and methane in COD equivalents of the untreated and pretreated biomass, and their COD percentage transformed into methane. Untreated *S. erectum* showed the highest VFA production on day 7, reaching 3.9 % g COD and DS and WS treatments presented low VFA levels as well. Dutra et al. (2024) observed that the peak VFA production from macrophytes occurred in the initial 5 days, where easy-biodegradable compounds were hydrolysed and fermented involving hydrolytic and acidogenic microorganisms. Here, the lack of a VFA peak confirmed the absence of an exponential phase in methane production of FB, DS and WS, although the 2 % g COD as VFAs on day 13 led to the steepest increase in methane, suggesting a coordinated acetogenic-methanogenic activity. Despite a progressive COD conversion into methane was observed over time, reaching 29 %, 35 % and 32 % at day 56 for FB, DS and WS, respectively, these results remain relatively low, hinting the recalcitrance of *S. erectum*'s leaves. Even with the mild increase in COD conversion efficiency from dry

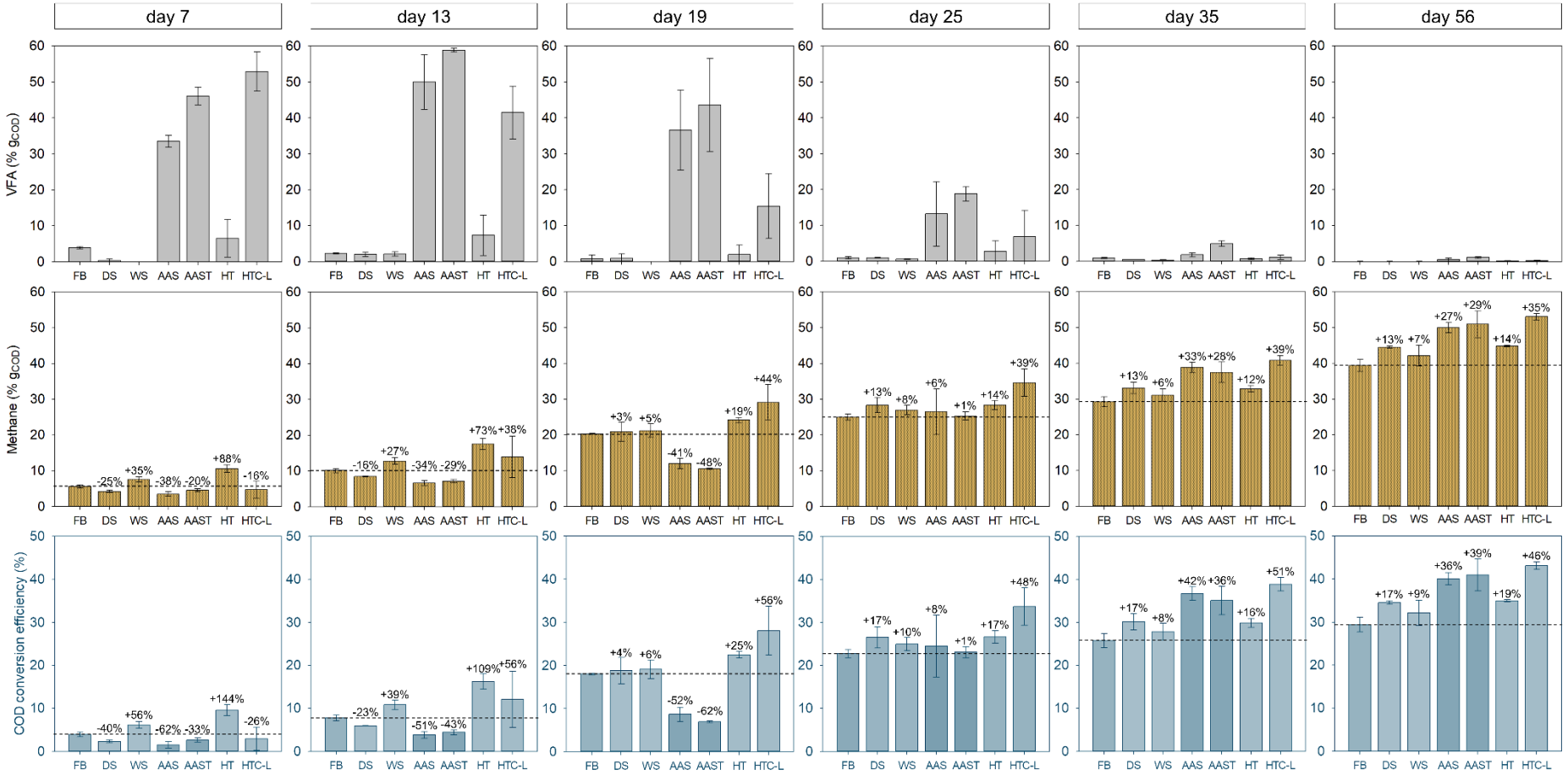


Figure 4.5 VFA and methane production, and initial COD percentage transformed into methane at days 7, 13, 19, 25, 35 and 55. The dashed black lines indicate the FB level and the percentages on the top of the bars present the increase of methane or COD conversion efficiency compared to FB.

and wet storage, these procedures did not overcome the biomass recalcitrance. The hydrothermal pretreatment at 120 °C, also presented low VFA levels. However, by days 7 and 13 there was a strong rise in the COD converted into methane, over 73 % as compared to the untreated biomass. Subsequently, after day 19, this value declined, maintaining a consistent range around 12 – 19 %. In previous studies, the VFA concentration level also reached the highest levels in the initial period, indicating that the pretreatment accelerated hydrolysis and acidogenesis (Luo et al., 2019). Henceforth, due to the recalcitrance of the biomass, hydrolytic and acidogenic activities slowed down. Thus, this pretreatment is suitable for AD processes with short retention times.

In AAS, AAST, and HTC-L, the pretreated biomass presented above 35 % g COD of VFAs at days 7, 13 and 19, which were the highest levels observed. On day 13, the methane yield in COD equivalents of HTC-L was 38 % higher than the untreated biomass and maintained the percentage across the experiment. Concerning biomass pretreated with AAS and AAST, the COD conversion efficiency did not reach untreated biomass levels until day 25, and from day 35 onward, it exceeded the untreated biomass levels by more than 36 %. When levels of VFA remain high for a period, as observed in the present study, acetogenesis and methanogenesis are being inhibited (Luo et al.,2019). Ammonia at high concentrations can inhibit the VFA degradation, and under mesophilic conditions inhibit s acetogenesis and methanogenesis (Bonk et al., 2018; Z. Yang et al., 2018). Overall, at the end of the experiment, the highest methane production was achieved by HTC-L, AAST and AAS, clearly confirming that these three pretreatments increased the biodegradability of *S. erectum* biomass, especially HTC if the retention time of the reactor is between 19 and 35 days.

4.4. Effect of the pretreatments on the microbial communities

The prokaryotic communities at three different stages (days 7, 13 and 19) of the exponential phase of methane production that were active during AD were analysed and compared. Considering both conditions of each pretreatment in the same data analysis group, alkali pretreated biomass presented the lowest prokaryotic richness, with 29 ± 20, 71 ± 9, and 76 ± 7 observed ASVs at day 7, 13 and 19, respectively, showing significant differences with FB (Mann-Whitney, $p < 0.05$), Ct ($p < 0.0001$), storages ($p < 0.001$), and hydrothermal pretreatments ($p < 0.0001$) (Table 4.5). The other pretreatments did not showed significant differences between them. Considering both evenness and richness, Shannon index was also the lowest in aqueous ammonia pretreatment, with averages between 2.11 ± 0.26 and 3.74 ± 0.17, presenting significant differences with stored

Table 4.5 Two alpha diversity indices, the observed ASVs and the Shannon index, estimated for the active prokaryotic communities of reactors at days 7, 13, and 19 digesting untreated biomass (FB), stored biomass (ST) including both WS and DS, aqueous ammonia (AA) treatments including AAS and AAST, hydrothermal pretreatments (HP) including HT and HTC-L, and the control with only the inoculum (CT) (average values ± standard error, n = 3).

α diversity index	Time	FB	ST	AA	HP	CT
Observed ASVs	Day 7	40 ± 13	59 ± 12	29 ± 20	100 ± 24	105 ± 8
	Day 13	99 ± 7	125 ± 7	71 ± 9	92 ± 20	99 ± 10
	Day 19	95 ± 15	107 ± 12	76 ± 7	96 ± 23	96 ± 9
Shannon	Day 7	3.1 ± 0.3	3.5 ± 0.2	2.1 ± 0.3	3.95 ± 0.09	3.9 ± 0.1
	Day 13	3.88 ± 0.07	4.15 ± 0.04	3.6 ± 0.2	3.87 ± 0.07	3.88 ± 0.08
	Day 19	4.0 ± 0.3	4.0 ± 0.1	3.7 ± 0.2	3.91 ± 0.04	3.8 ± 0.1

biomass ($p < 0.05$), hydrothermal pretreatments ($p < 0.0001$), and Ct ($p < 0.05$). In terms of beta diversity, only FB and stored biomass presented no significant differences (PERMANOVA, $p = 0.077$) (Figure 4.6). After the pairwise PERMANOVA analysis, significant differences were observed ($p < 0.001$) between all the other treatments suggesting that aqueous ammonia and hydrothermal pretreatments led to shifts in the reactors' prokaryotic population.

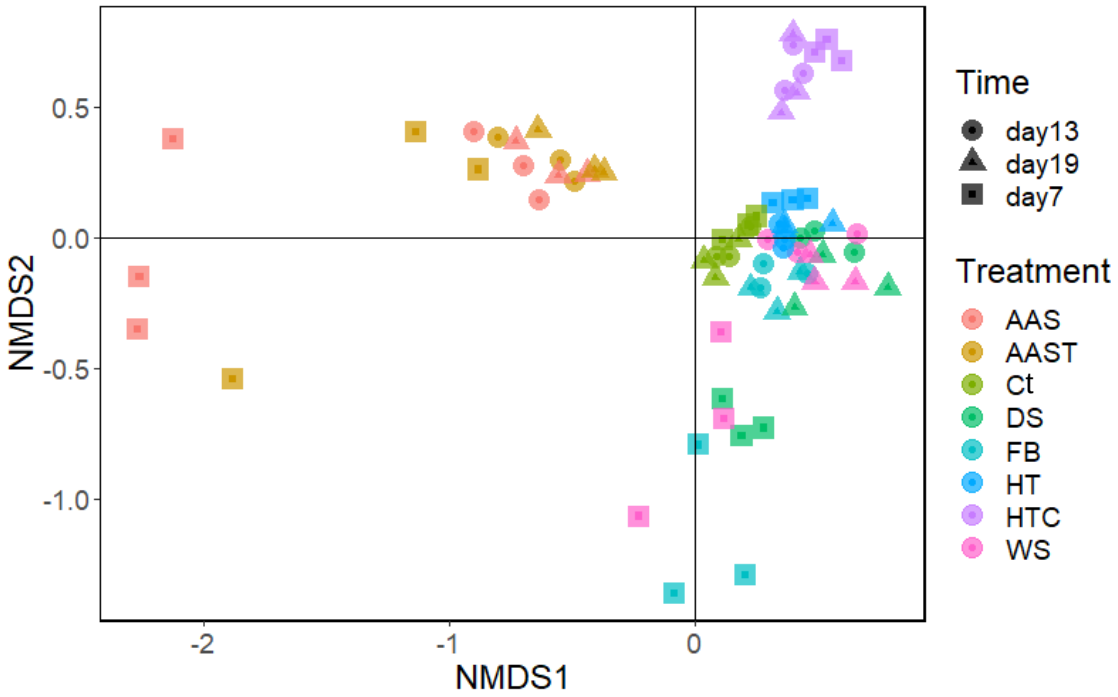


Figure 4.6 Dynamics of active prokaryotic community composition in reactors: non-metric multidimensional scaling analysis with Bray-Curtis dissimilarity index across time (days 7, 13, and 19), and substrate conditions (untreated or pretreated).

Figure 4.7 illustrates the taxonomic profiles of the reactors' microbiome at the phylum and genus levels. The most abundant phyla in FB at day 7 were *Acetothermia, Cloacimonadota* and *Halobacterota* (between 15.5 and 17.0 % of abundance), while with time, *Desulfobacterota* gained dominance until reaching 13.4 % at day 19. DS and WS presented similar profiles with FB. In addition, hydrothermal pretreatments did not present high variations in the taxonomic profile over time. In the case of HT, the dominant phyla were *Cloacimonadota, Actinobacteriota, Firmicutes* and *Acetothermia* (9.5 – 13.7 %). In general, the observed phyla in these treatments (FB, DS, WS and HT) presented high resemblance with the taxonomic profile of the Ct, suggesting that active microbial populations in the reactors were influenced by the initial inoculum used in the experiment, rather than being altered by the conditions of pretreated biomass. In fact, previous studies showed that the inoculum has a crucial role in shaping the reactor's microbiome and its functions (Han et al., 2016). Although *Firmicutes, Bacteroidota* and *Proteobacteria* are the typical dominant phyla in AD reactors, the observed phyla were also reported in previous studies (Harirchi et al., 2022; Lim et al., 2020; Mirmohamadsadeghi et al., 2021).

However, it is noteworthy that HTC-L, AAS and AAST presented a shift in the population as compared with Ct, confirming the significant differences observed in NMDS scaling. In HTC-L, *Actinobacteriota* gained prevalence (22.9 – 26.0 %) compared to the previously mentioned taxa and the *Actinobacteriota* relative abundance in Ct. This phylum has been positively correlated with high concentrations of proteins, fat, and sugars and has been described by its lignocellulose-degrading enzymes (Perman et al., 2022). *Actinobacteriota* species also showed syntrophic interactions with acetogens and methanogens producing propionate, lactate, acetate, and formate (B. Y. Li et al., 2022), and their capacity to adapt to low pH (Y. Liu et al., 2023), as HTC-L was a substrate characterised by a low pH and putatively a high sugar and organic acids availability (Nguyen et al., 2023). In AAS and AAST, *Firmicutes, Bacteroidota*, and *Spirochaetota* were dominating the community, presenting a notorious shift as compared with Ct. These phyla were also found in reactors with high levels of ammonia (Z. Yang et al., 2018). Ruiz-Sánchez et al. (2018) reported that *Firmicutes, Bacteroidota*, and *Chloroflexi* sp. were associated to syntrophic acetate oxidising bacteria, responsible for the metabolisation of the acetate excess to hydrogen and carbon dioxide due to the ammonia inhibition of acetotrophic methanogenic archaea.

At the genus level, numerous fermentative bacteria were active in the reactors, involved in many metabolic processes in hydrolysis, acidogenesis and acetogenesis. In general,

FB, DS, WS, HT and HTC-L showed a similar taxonomic profile than the control reactors only fed with the inoculum. In FB, DS and WS, Cloacimonadaceae W5, unclassified W27

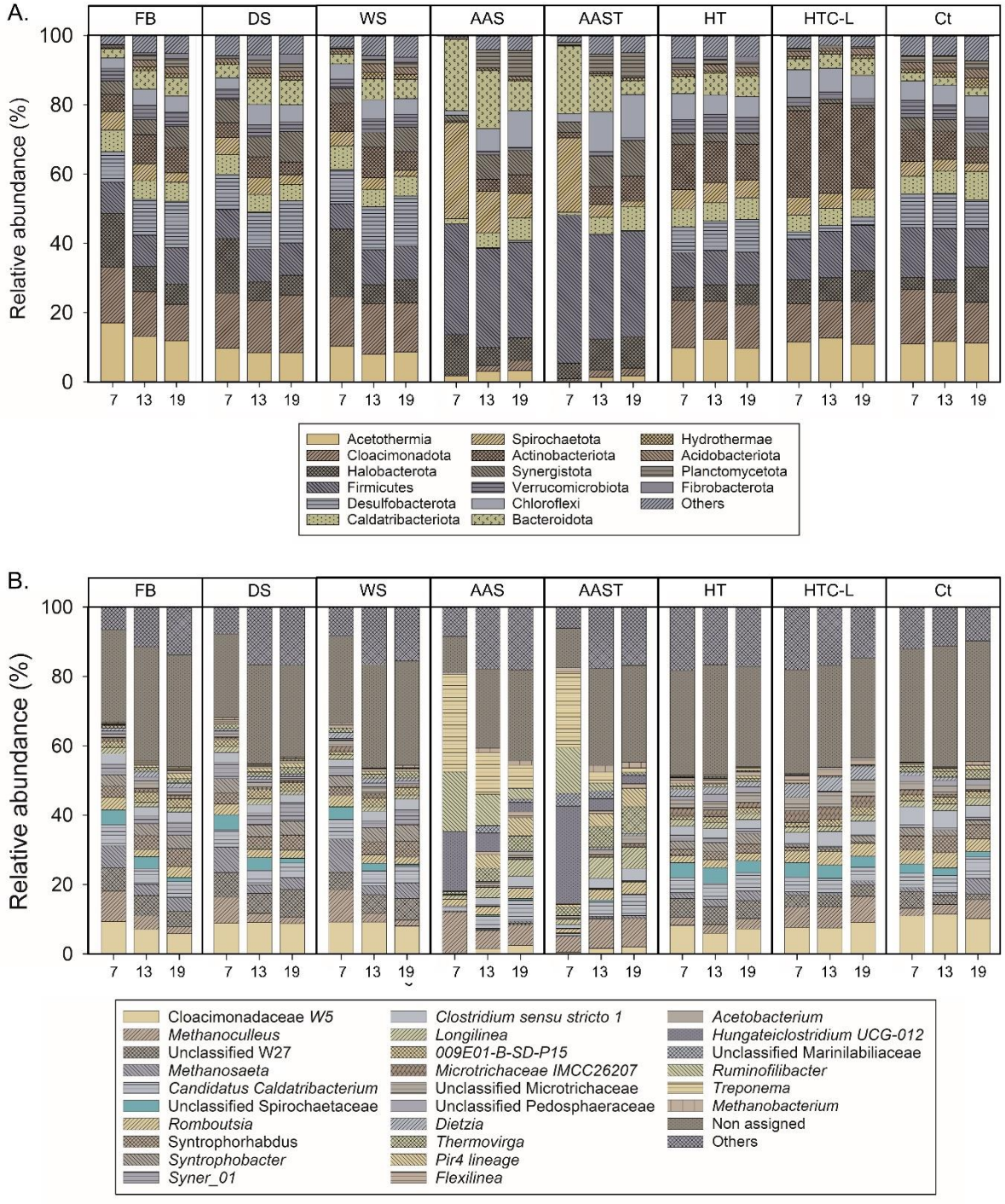


Figure 4.7 Taxonomic profile of most metabolically active prokaryotic communities in reactors at days 7, 13, and 19. Average (n = 3) relative abundance for each substrate conditions (untreated – FB; pretreated – DS, WS, AAS, AAST, HT, and HTC-L; and inoculum without substrate - Ct) are presented at phylum level (A) and genus level (B). Others correspond to phyla and genera with abundances below 2 %.

and *Candidatus* Caldatribacterium were the genera with abundances over 5 % at day 7 and, at day 19, *Syntrophorhabdus* presence increased, previously described as syntrophic bacteria degrading phenolic compounds in association with hydrogenotrophic methanogens (Usman et al., 2023). High prevalence of *W5* genus within Cloacimonadaceae family and W27 family within Cloacimonadales were previously reported in AD reactors (Perman et al., 2022). These members might be acidogens involved in syntrophic propionate oxidation (C. Li et al., 2022). *Candidatus* Caldatribacterium*,* on the other hand, were also identified in AD microbiomes associated with degradation processes of cellulosic metabolites, cellobiose, xylose, glucose, galactose, raffinose and xylan (Struckmann Poulsen et al., 2022). Other detected genera associated with hydrolysers were *Clostridium* sensu stricto 1, having cellulolytic activity and also playing a crucial role in maintaining the stability of the system (J. Ma et al., 2021; Struckmann Poulsen et al., 2022). Its relative abundance ranged between 2.1 and 3.1 % in FB, DS, and WS along time. Regarding hydrothermal pretreatments, the reactors' microbiota showed stability and robustness along time. HT and HTC-L were also dominated by Cloacimonadaceae W5, unclassified W27 and *Candidatus* Caldatribacterium and unclassified Spirochaetaceae. Microtrichaceae presented the highest abundances in HTC-L reactors, ranging from 3.7 to 4.6 %, and were related to the degradation of organic nitrogen and nitrification-anammox systems and to the hydrolysis of complex organic matter (R. Guo et al., 2022). This increase was probably due to the nature of HTC-L, presenting high levels of N, derived from protein degradation (Nguyen et al., 2023).

However, the taxonomic profile observed in AAS and AAST reactors differed from that of the Ct. On day 7, the most abundant genus were *Treponema* (28.0 and 21.8 %, respectively), previously reported to be crucial for the hydrolysis of lignocellulosic biomass, showing positive correlations with cellulase activity (Jensen et al., 2021), and *Hungateiclostridium UCG-012* (17.2 and 28.1 %, respectively), that have been related to hydrolysis and fermentation of carbohydrates and correlated with methane production inhibition (Cazaudehore et al., 2022). Both genera are characterised by ammonia assimilation (Tindall et al 2019, Waidele et al 2019). *Ruminofilibacter* are hydrolysers with xylan or hemicellulotytic activities that were also prevalent in these reactors (H. Wang et al., 2020). Thus, on day 7, in aqueous ammonia pretreated biomass hydrolysers and acidogens were the most prevalent genera in the community. However, on days 13 and 19, the less abundant taxa gained prevalence in the population, and on day 19, *Treponema, Candidatus* Caldatribacterium*, Pir4 lineage, Longilinea* and *Thermovirga*

resulted to be the most abundant genera. *Longilinea* uses fructose, sucrose, raffinose, or xylose to produce acetate, lactate, and H_2, and its growth is promoted in the presence of hydrogenotrophic methanogens (Yamada et al., 2007). *Thermovirga* are acetate-oxidizing bacteria with sulfate/Fe(III)-respiration (L. Wang et al., 2023). It was previously published that *Clostridium, Longilinea* and *Thermovirga* had a crucial role in the metabolic network within an AD reactor, with more frequent interactions with other members of the community and environmental parameters. *Longilinea* and *Thermovirga* showed inhibitions to increasing long-chain VFA concentrations (J. Ma et al., 2021).

Romboutsia was also detected in most reactors and might facilitate acidogenesis fermenting carbohydrates and single amino acids to generate principally formate and acetate. Some species are related to xylanase activity (Jensen et al., 2021). In addition, *Syner-01*, *Syntrophorhabdus* and *Syntrophobacter,* also present mostly in FB, DS, WS, HT and in minor abundances in HTC-L, AAS and AAST, are syntrophic VFA-oxidizing bacteria producing H_2, acetate and other VFAs, that live in syntrophy with methanogens (Hu et al., 2022; Lim et al., 2020). Another observed genus associated with acetogenic microorganisms was *Acetobacterium*, which consumes H_2 and CO_2 to form acetate (Lim et al., 2020).

Methanogenic archaeal community belonging to the Halobacterota phylum, exhibited relatively high abundance in FB, DS and WS reactors, ranging between 15 and 19 %, but falling below 7 % in the others reactors (AAS, AAST, HT and HTC-L), at day 7. At the genus level, *Methanoculleus* and *Methanosaeta* were found among the most abundant genera, while *Methanobacterium* and *Methanomethylovorans* were among the less prevalent ones. *Methanosaeta* are acetoclastic methanogens that can only use acetate and are typically associated with stable operational conditions with low ammonia concentrations. Conversely, high ammonia and acetate concentrations inhibit the growth of this genera (Hatti-Kaul et al., 2016). On the other hand, *Methanoculleus* are hydrogenotrophic methanogens that have been reported to be tolerant to high ammonia concentrations (Lim et al., 2020). On day 7, *Methanoculleus* and *Methanosaeta* presented relative abundances between 6.4 and 9.5 % in FB, DS and WS. In contrast, for AAS, AAST and HTC-L, only *Methanoculleus* emerged as one of the most dominant genera, with 11.7, 4.6 and 5.9 %, respectively, while *Methanosaeta* had minimal presence, ranging from 0.1 to 1.0 %. Similar results were found by Ruiz-Sánchez et al. (2018), where at low concentration of ammonia methanogenesis was carried out mostly by the obligate acetotrophic genus *Methanosaeta*, while at higher concentrations of ammonia, the process was carried out by hydrogenotrophic archaea, namely

Methanoculleus. On the other hand, by day 19, *Methanoculleus* decreased below 2 % in FB, DS, and WS, and reached abundances between 6.0 and 8.3 % in AAS, AAST and HTC-L. *Methanosaeta* maintained its levels below 2 % in AAS, AAST and HTC-L, and decreased in relative abundance in FB, DS, and WS to levels between 3.4 – 4.5 %. In HT, both *Methanoculleus* and *Methanosaeta* remained stable over time, between 1.4 and 2.9 %. Therefore, the structure of methanogenic community was not shaped by the type of substrates but by some operating parameters (Hatti-Kaul et al., 2016).

Thus, identified active microorganisms were related to syntrophic microorganisms showing good cooperation between VFA production and consumption, coupled with final methanogenesis. The pretreatments creating the highest shift in the prokaryotic community were the AAS, AAST and the HTC.

4.5. Techno-economic assessment of *S. erectum* pretreatments

The preliminary technical and economic assessment results are presented in Table 4.6 to compare the cost/benefit of each pretreatment with the untreated biomass. The analysis considered different scenarios depending on the HRT of the digestor. The only pretreatment that yielded better results compared to untreated biomass was solar drying, with a total outcome of 710.2 kWh on day 19 and 1314.3 kWh on day 56. This represents a 473 % increase on day 56 compared to untreated biomass.

Despite higher levels of methane produced, aqueous ammonia soaking and hydrothermal pretreatments had high energy requirements, leading to notorious costs that rendered the processes inefficient. These results deviate from previous studies where the outcome was consistently positive (Lymperatou et al., 2022). This discrepancy might be attributed to the high volumes of water introduced into the reactors, as less moisture, namely 100 g TS/kg of brewer's spent grain enhanced methane production (Peces et al., 2015). Future assessments could explore digesting only the cake fraction, potentially optimizing the outcome, even though it would involve incorporating a centrifugation step into the industrial process.

The presented study only provides a preliminary assessment of different pretreatments that could be feasible to be applied in biogas plants to enhance methane production. Roj-Rojewski et al. (2019) reported that the biomass quality from wetlands was generally unfavourable for the growth of methanogens due to its C:N ratio, which fell outside the optimal range of 20 to 30:1. However, the methane yield differed significantly depending on the season of the mowing with higher energy yields when the cutting period was

Table 4.6 Preliminary techno-economic assessment of pretreatments of *S. erectum* biomass for anaerobic digestion. The study considered the energy consumption to pretreat 1 ton of substrate entering the digesters and the energy produced based on experimental methane yields.

Substrate	Total outcome (kWh/t)				Energy consumption pretreatments (kWh/t)		
	day 19	day 25	day 35	day 56	Chopper	Heat	Vacuum evaporator
FB	140	177	201	229	1,5		
DS	710	1009	1146	1314	8,3		
WS	128	168	188	217	1,5		
AAS	-267	-257	-249	-247	0,2		271,97
AAST	-302	-293	-285	-282	0,2	34,34	271,97
HT	-91	-86	-83	-77	0,2	116,11	
HTC-L	-250	-248	-247	-246	0,3	257,31	

Substrate	Electric energy produced (kWh/t)				Thermal energy produced (kWh/t)			
	day 19	day 25	day 35	day 56	day 19	day 25	day 35	day 56
FB	63	79	90	103	78	99	112	128
DS	319	452	513	588	399	565	641	735
WS	58	76	84	97	72	94	105	122
AAS	2	7	10	11	3	9	13	14
AAST	2	6	9	11	2	8	12	14
HT	11	13	15	17	14	17	19	22
HTC-L	3	4	5	5	4	5	6	7

between July and August (Roj-Rojewski et al., 2019). Despite the variable availability of the macrophytes depending on the harvest campaigns, further research should be conducted to evaluate its potential in co-digestion with other substrates such as animal manure. This would optimise the C:N ratio for the entire process, particularly the hydrolysis and methanogenesis, resulting in higher biogas yields as compared with mono-digestion (Kaushal et al., 2022; Paranhos et al., 2020).

Nonetheless, this study showcases the energy recovery potential of *S. erectum* biomass along with the viability of DS options to both preserve the harvested biomass and enhance the methane yield. Further attention should be directed towards hydrothermal and aqueous ammonia soaking pretreatments to develop cost-effective technologies aiming to enhance methane production, considering the significant impact of pretreatments on the overall process cost.

5. Conclusion

This study evaluated three pretreatments namely storage, aqueous ammonia soaking and hydrothermal pretreatments, each under two different conditions, to enhance the methane yield of *S. erectum*, a plant harvested from constructed wetlands. The pretreatments showed different effects on the biomass morphology and physicochemical properties. While storage pretreatments resulted in mild alterations, aqueous ammonia soaking, and hydrothermal pretreatments led to more noticeable effects. All pretreated biomass improved the methane yield, but HTC pretreated *S. erectum,* at 200 °C for 60 min, presented the highest increase, reaching 46 %, with a value of 151 $NmLCH_4$/gCOD. Only the centrate phase was used as the substrate because the cake phase, known as hydrochar, holds potential interest for other valorisation purposes. Regarding the AD process at a microbiological level, the taxonomic profiles of FB, DS, WS, HT, and HTC-L (to a lesser extent) were primarily influenced by the inoculum, with numerous active fermentative and syntrophic bacteria genera observed during the exponential phase. However, aqueous ammonia soaking pretreated biomass led to a significant shift in the population with a decrease in the taxa abundance. Hydrogenotrophic methanogens were the prevalent methanogens in aqueous ammonia soaking and HTC pretreatments, while the other pretreatments also showed acetoclastic populations.

However, the techno-economic assessment illustrated that the implementation of aqueous ammonia soaking and hydrothermal pretreatments needs further optimisation to reduce expenses and achieve a balanced and feasible outcome. Remarkably, dry storage emerged as the most promising pretreatment option among those tested. Valorising the biomass from wetlands through AD not only enhances energy recovery but also fosters the principles of a circular economy, enriching synergy between resource utilisation and environmental conservation.

Author Contributions:

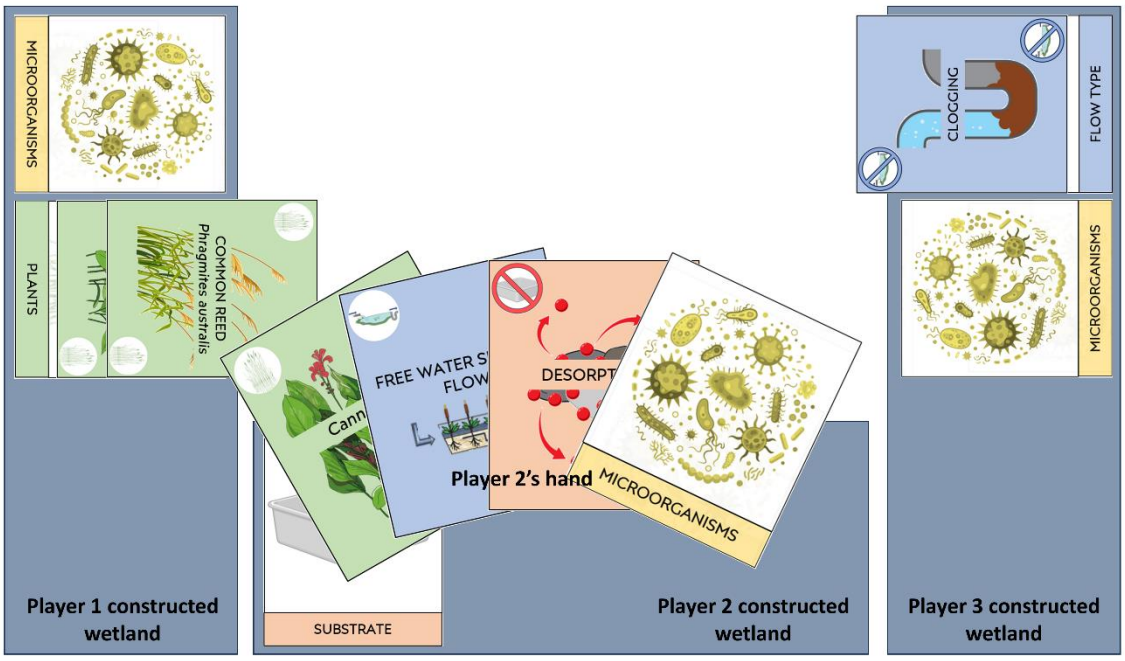
PP-S: Conceptualisation, Methodology, Investigation, Validation, Formal analysis, Data curation, Writing - original draft, Visualisation; MG: Methodology, Resources, Writing - review & editing; ABM: Writing - review & editing, Supervision; CRG: Writing - review & editing, Supervision; CMRA**:** Writing - review & editing, Supervision, Project administration, Funding acquisition; APM: Writing - review & editing, Supervision, Project administration, Funding acquisition; BF: Conceptualisation, Methodology, Validation, Resources, Writing - review & editing, Supervision.

Chapter 5. *Wetlands Wonders*: a constructivist-based game to improve motivation and learning outcomes in environmental engineering education.

This Chapter presents an educational activity focused on the engineering principles behind CWs, where participants learn to design and build their own eco-friendly wastewater treatment systems, showcasing their creativity and problem-solving skills. This is an original work planned to be submitted soon in *FEMS letters*.

Pau Porras-Socias, Alexandre B. De Menezes, Carlos R. Gomes, C. Marisa R. Almeida, Ana Paula Mucha and Belén Fernández. "*Wetlands Wonders*: a constructivist-based game to improve motivation and learning outcomes in environmental engineering education".

In the Appendix C is presented *Methanopoly*, another activity to disseminate the principles of AD that was created for *Science is Wonderful* fair with my colleagues Kris A. Silveira and Raffaello Mattiussi.



1. Abstract

Enjoyment plays a crucial role in student academic performance, highlighting the need to enhance motivation in STEM disciplines. In response, *Wetland Wonders*, an activity blending gamification with a constructivist approach, was designed for school students of the secondary grade. After an initial survey to activate prior knowledge and an introduction to nature-based solutions, students engaged in a contextualised challenge where they first collected materials through a card game and then collaborated in small groups to design an effective constructed wetland removing contaminants from a wastewater-type. Afterwards, teams presented their designs. A final survey was conducted to assess students' feedback. Results indicate students enjoy the activity and increased their understanding of constructed wetlands. *Wetland Wonders* activity also emphasises cooperative learning, the development of transversal skills, and metacognition to support meaningful learning outcomes.

Keywords: Gamification, constructed wetlands, STEM, meaningful learning, motivation, students.

2. Introduction

Nowadays, we are globally transitioning towards a new era known as the Fourth Industrial Revolution. The advancements in technology are blending the realms of the physical, digital, and biological, offering significant opportunities as well as potential risks. These developments are reshaping our lifestyles, jobs, and social interactions (Schwab, 2016). Governments worldwide are prioritising the enhancement of their citizens' STEM (science, technology, engineering, and mathematics) capabilities, as STEM education and research are progressively acknowledged as essential catalysts for national development, economic productivity, and societal well-being (Tytler, 2020). Students of the secondary education often find science disciplines very challenging, and many countries are encountering difficulties related to academic achievement (OECD, 2024), as well as the skills and attitudes of students, often exacerbated by traditional teacher-based instruction (Adesina & Gabriel, 2023). Moreover, the COVID-19 pandemic led to a decrease in the achievement of these students, especially younger ones and the ones from families with low socioeconomic status (Hammerstein et al., 2021). A study analysing the 2015 Programme for International Student Assessment (PISA) dataset discovered that the primary factor influencing performance in science class across all regions was the enjoyment of science learning (Lau & Ho, 2022). Consequently. there is a pressing need to boost students' motivation and focus in school classes and improve their enjoyment of science education.

On this premise, to engage students in the learning process and foster their interest in particular subjects, it is essential to employ innovative and interactive educational methods (V. R. Da Silva & Vieira, 2022). Interest can be defined as a "psychological state characterised by focussed attention, increased cognitive and affective functioning, and persistent effort", which is directly linked with motivation (Ainley et al., 2002). Motivation can be defined as the desire to "know, act, understand, believe, or acquire particular knowledge, skills, attitudes, or values". In education, it is related to the intellectual energy used to activate and maintain learning activities (Filgona et al., 2020). Gamification emerges a pedagogical tool that effectively motivates and engages students across academic disciplines, including environmental engineering (Gonçalves et al., 2019). This approach consists in the integration of game elements such as simple games, puzzles, quizzes, novel board, or computer games among others, into non-game environments (Robinson et al., 2018). Moreover, Johnson (2006) emphasised that all games, regardless of their educational purpose, should promote a scientific method of

exploring the environment, formulating hypotheses, testing them, and adjusting based on feedback.

Constructivism, the dominant paradigm of learning in science, is a pedagogy and philosophy based on people actively building their knowledge from their experiences and interactions with others, rather than taking information in a passive form (Cakir, 2008) . That is why it is important to activate students' prior knowledge and use relevant information to drive conceptual change, which will be unique for each student's mind (Cakir, 2008). The fundamental principles of constructivist teaching involve eliciting prior knowledge to build upon existing knowledge, creating cognitive dissonance through challenging problems, applying knowledge with feedback, and reflecting on learning to show the learnt outcomes (University at Buffalo, 2024). Additionally, in constructivist teaching, motivation also plays a key role in the construction of knowledge and mental models (Palmer, 2005).

In response to the need to improve motivation and enjoyment in science class, a constructivist-based activity named *Wetland Wonders* was designed to help school students of the secondary grade build mental models of biological processes applied by humans to achieve a specific goal, in the present case, to treat wastewater. For that, students must work in cooperative small groups, as cooperative learning has been shown to be highly effective in enhancing motivation, meaningful learning, and teamwork skills (Pujolàs, 2008). This game followed the already mentioned principles of constructivist teaching and was framed within a relevant context, as contextualisation provides more learning opportunities and demonstrates the practical relevance of science concepts (Tolbert et al., 2019). In addition, a scientific challenge was presented to promote reflection, critical thinking, communication, problem-solving, creativity, self-responsibility, motivation, and engagement among students (Taconis & Bekker, 2023). Finally, orientation guidelines (Figure B.1) and an evaluation rubric (Table B.1) were provided, to support the metacognition process (leaning to learn) and help students understand expectations, assess their work and enhance performance effectiveness (P. Shah et al., 2020).

3. Implementation

Students worked in groups of 4 or 5 over a 2-hour session. At the beginning of the activity, participants filled out a questionnaire regarding their prior knowledge, answering several questions on the topic (Figure B.2). Following this, an introduction to nature-based solutions was conducted, during which students identified various water-related environmental challenges using provided images and explored potential solutions. Then, constructed wetlands as a nature-based solutions were introduced, prompting each group to hypothesise about the purpose and functionality of these systems.

Subsequently, the main challenge of the activity was presented to the audience: *"the municipality is looking for a constructed wetland construction company to treat various wastewater types they needed to manage"*. Each group represented a company offering different constructed wetland configurations to effectively treat these wastewater types. The game was divided into two parts: first, gathering materials and different components; secondly, constructing the most suitable configuration of a constructed wetland adapted to the wastewater to treat.

3.1. Material Collection Game

To play this game, members of each group separated and paired up individually with one player from each company. This meant that in these new redistributions, each player interacted with one player from each original group. For example, if there were 6 companies of 4 members each, in this round, there would be 4 groups of 6 members, one from each company. Each group played independently in a card game to recollect components for their constructed wetlands.

The aim of the players was to be the first member to obtain all 4 components of constructed wetlands, signalling the end of the game. To set it up, participants shuffled the deck and distributed 4 cards to everyone. The deck was then placed face down on the table where all players could reach with the discard pile beside it, face up. When the deck ran out, the discard pile was flipped over without shuffling. There were three types of cards as presented in Figure 5.1: *wetland components cards* (substrate, plant, microorganism, and flow type), *type cards*, and *complication cards*. *Type* and *complication cards* were associated with one of the *wetlands component cards*. In addition, the card deck was complemented with *joker cards*.

During each turn, players were limited to taking only one action, and after completing their turn, the player drew cards until having 4 cards in their hand. Actions included

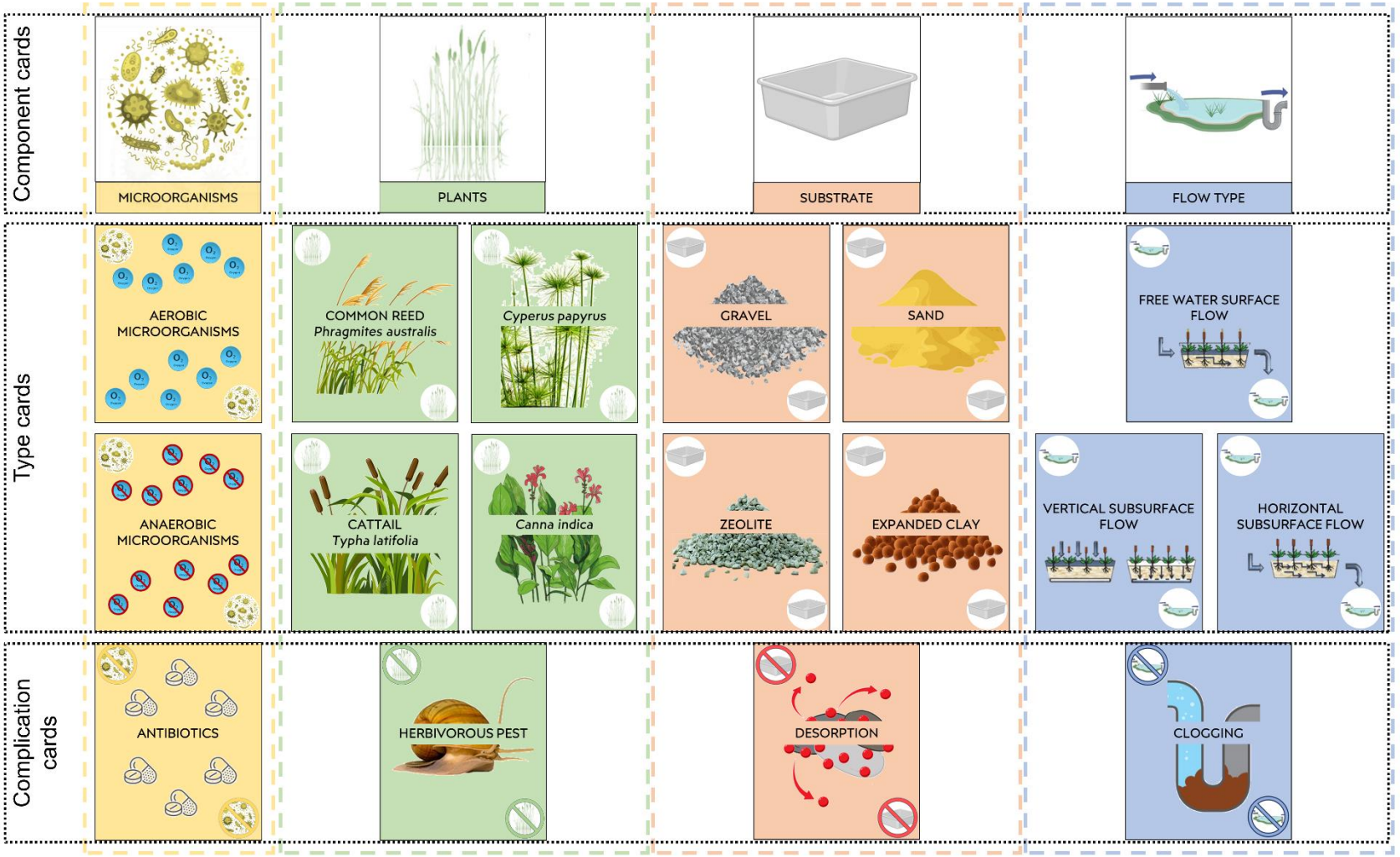


Figure 5.1 *Component*, *Type* and *Complication cards* used in the Material Collection Game

playing one card by placing it either in front of themselves or on an opponent's table or discarding as many cards as the player wanted. The space in front of each player represented their own constructed wetland, initially empty. Players had to strategically play *component cards* to fill their wetland, ensuring that no *component card* was repeated.

Type cards symbolise the constructed wetlands materials that players kept after the game to do the second part of the activity. These cards were placed on top of the *component cards* already in the constructed wetland on the table. Players could accumulate multiple *type cards* (one per turn) within one component, as long as each card added was of the same colour, and players were interested in accumulating as many as possible.

Complication cards were placed on top of the opponent's corresponding *component cards* to block the progress of other players in completing their constructed wetland before the player. If two *complication cards* were on a component, the component gets destroyed, and all three cards are sent to the discard pile. In addition, if a *complication card* was placed on top of a *type card*, both cards were immediately discarded. Conversely, a player could counter a *complication card* by placing a *type card* on top of it, resulting in both cards being discarded. Once a player had accumulated 3 *type cards* on a *component card*, opponents could not play a *complication card* on that component, creating a safeguard against future disruptions.

Finally, there were also *joker cards* that allowed players to:

- Swap a component (including all cards associated with it) with an opponent,
- Steal a component from an opponent and add it to the player's constructed wetland, if the player does not already have that component in their wetland.
- Transfer as many *complication cards* the player has in its one wetland to an opponent's wetland.
- Discard all the cards from all the opponents' hand, forcing them to spend their turn drawing cards for a new hand.

When a player successfully built their wetland with all 4 components, the game ended, and all players had to collect the type cards they had in their respective wetlands and return them to their colleagues of the company, the original groups.

3.2. Construction Process Challenge

Reunified groups were tasked with constructing an efficient wetland system to treat a specific type of wastewater with the *type cards* they had collected. For that, the reference documents provided included (i) a summary of what constructed wetlands are (Figure B.3), (ii) a table detailing contaminants present in each wastewater type (Table B.2), (iii) three additional tables outlining removal characteristics related to flow types (Table B.3), plant species (Table B.4), and substrate types (Table B.5), (iv) a competence-based self-assessment evaluation rubric (Table B.1), and (v) a letter soup with the wastewater type.

Initially, each group (company) had to decipher a letter soup to determine which wastewater type they would be addressing: pig slurry, marine aquaculture effluents, acid mine drainage, or sewage sludge, by solving a letters soup. Then, players had to identify the characteristics and contaminants associated with the assigned wastewater type. Also, each group had access to 3 *Special cards* where supplementary information was given (Table B.6). Groups could choose to share this valuable information with other groups or not. Considering the information provided in the tables and the available materials each group collected, they had to decide the best configuration to effectively treat all contaminants in their wastewater type. When the agreement was reached, they had to fill the 'Results and Discussion of the activity' part of the questionnaire (Figure S2).

3.3. Closure

Upon finalizing their configurations, each group was required to prepare a brief and concise presentation to the other groups sharing their model in a visual and creative form. During this presentation, players had to justify their chosen configuration and discuss the effectiveness of their treatment system, imagining they were pitching their product as a company would.

Following all the presentations, concluding remarks were made, and students were asked to fill out a questionnaire to provide feedback on the activity (Figure B.2). The survey included two parts: the 'Final evaluation of the activity' part, where students used the self-assessment evaluation rubric (Table B.1) to grade their competence levels from 0 (novice) to 3 (expert) for each skill; and the 'General opinion of the activity' part, which consisted of several affirmations for students to rate from 1 (strongly disagree) to 5 (strongly agree). Data from this questionnaire was analysed by calculating the average results of all 57 students of the self-assessment evaluation rubric items (values from 0 to 3), and also of the 'General opinion of the activity' rating from 1 to 5.

4. Discussion and conclusion

Wetland Wonders was delivered to school students of two 10th grade classes (last year of compulsory education in Spain, called 4th ESO), in the biology discipline to a total of 58 students. The self-assessment evaluation rubric results (Table B.1 Self-assessment evaluation rubric given to students at the end of the activity.) showed that for all evaluated items 52 students were in the “advanced” or “expert” level (Figure 5.1A).

After the activity, the feedback of students on the ‘General opinion of the activity’ (Figure B.2) was very positive, as the objective of enjoying while learning was achieved with almost all students voting “agree” or “strongly agree” in their enjoyment of the activity and their ability to explain what constructed wetlands are (Figure 5.2B). Many students answered that what they had enjoyed the most was the game and working in small cooperative groups.

A.

Item	Average ± standard deviation
Identification of the problem	2.4 ± 1.8
Teamwork and task organisation	2.6 ± 1.7
Understanding the concepts	2.3 ± 1.8
Quality of the CWs model	2.4 ± 2.0
Oral presentation	2.4 ± 1.9

B.

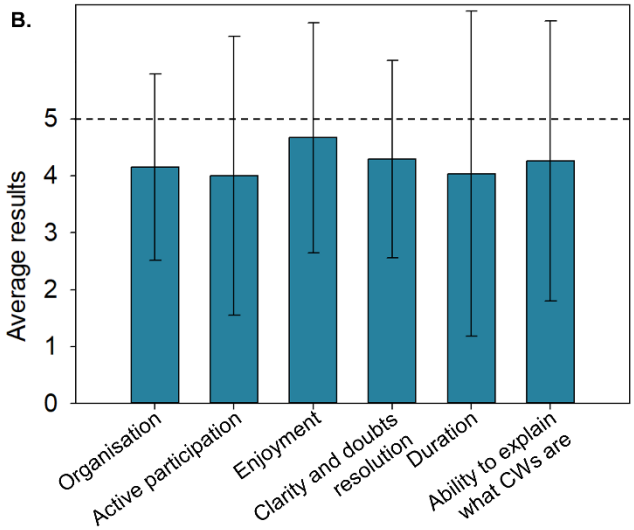


Figure 5.2 A. Table with the means and standard deviations of the self-assessment evaluation rubric results, ranging from level 0 (novice) to level 3 (expert) for n = 57 students. B. Bar plot summarising the average ratings of different items for the ‘General opinion of the activity’, with scores ranging from 1 (strongly disagree) to 5 (strongly agree). Error bars represent the standard deviation (n = 57).

Therefore, Wetland Wonders can be implemented in science classrooms to inspire student motivation and foster learning an applied biological processes. Despite being designed for 10th-grade students, the activity can be adapted and implemented in other

secondary education grades, as well as university courses. This pedagogical activity not only incorporates enjoyable gaming elements but also facilitates the development of essential skills known as transversal competences within the curriculum, namely critical and innovative thinking, inter and intra-personal skills, and information literacy (Care et al., 2019).

Although microbiology was already introduced in the school curriculum, most students held misconceptions about microbes viewing them solely as harmful and pathogenic. Through this activity, students also gained a deeper understanding of the fundamental role of microbial processes in wastewater treatment, recognising that microorganisms are influenced by environmental factors and can also interact with plants and substrates.

Thus, *Wetland Wonders* contributed to updating students' mental models to acknowledge the diverse functions of microorganisms in numerous daily processes. Combining constructivist teaching via gamification can be an optimal approach to improve students' motivation and performance in STEM disciplines, contributing also for the UN Sustainable Development Goals (SDGs), particularly SDG4 (Quality Education) and SDG5 (Gender equality), and in the current case to SDG6 (Clean Water and Sanitation).

Author Contributions:

PP-S: Conceptualisation, Methodology, Implementation, Validation, Formal analysis, Data curation, Writing - original draft, Visualisation; ABM: Writing - review & editing, Supervision; CRG: Writing - review & editing, Supervision; CMRA**:** Writing - review & editing, Supervision, Project administration, Funding acquisition; APM: Writing - review & editing, Supervision, Project administration, Funding acquisition; BF: Validation, Writing - review & editing, Supervision.

Chapter 6. Final remarks and future perspectives

1. Current challenges of liquid digestate valorisation

All the research presented in this thesis was carried out in the frame of the M2ex project which aims to exploit the metal-microbiome applications to expand the circular economy. More specifically, the work of this thesis is situated within the Work Package (WP) 6, which focuses on closing the loop to enable the bioeconomy, WP8, which addresses dissemination, exploitation, and public outreach, and WP7, a training network facilitated by secondments and collaborative connections with two universities, the University of Porto and the University of Galway, two research centres, the Interdisciplinary Centre of Marine and Environmental Research (CIIMAR) and the Institute of Agrifood Research and Technology (IRTA), also, a company specializing in MSW treatment named Tratolixo, and finally, Smart Waste Portugal, an association dedicated to promoting circular economy practices in Portugal.

Nowadays, AD biotechnologies are being promoted and expanding globally as a strategy for managing organic waste and producing bioenergy, addressing the challenges posed by the rising global energy demand and the increasing generation of waste. Recently, the perception of digestate has moved from a waste to recognising its value and potential benefits. Despite the fact that various innovative approaches have emerged for valorising the LFD, most methodologies are still in the developmental stage and come with significant maintenance and cost challenges (W. Wang et al., 2023). Additionally, regulations impose restrictions on the use of LFD as a fertiliser based on the substrate source of AD digesters (Sfetsas et al., 2022). Hence, it ceases to be a valuable resource and is seen as a potential source of pollution due to its high organic and nutrient content, metals, antibiotics, and other biological contaminants, such as pathogens or ARG. Consequently, proper management of LFD is essential to prevent adverse environmental pollution.

To reuse reclaimed water is essential for effective water resource management, guaranteeing a consistent water source, to face the challenging pressure on water bodies (Chen et al., 2021). CWs are low-cost biotechnological systems that remove pollutants from wastewater, enabling its subsequent agricultural, urban, industrial, or recreational reuse following a specific legislation (de Campos & Soto, 2024). For example, in the EU, the Water Reuse Regulation, applicable from 26 June 2023,

establishes minimum water quality standards for the safe reuse of treated urban wastewater in agricultural irrigation (European Commission, 2020, 2022). Previous studies have shown that CWs effectively reduced organic matter, nutrients, metals, antibiotics, pathogens, toxins, and endocrine-disrupting chemicals levels from urban streams, livestock wastewaters, and aquaculture effluents (Almeida et al., 2017b; Bavithra et al., 2020; Bôto et al., 2023; Dias et al., 2020; Gorito et al., 2018; Santos et al., 2019).

In this thesis, CWs experiments aimed to **study the systems from the perspective of the treatment of LFD with metals and antibiotics for water reuse purposes**. In the first experimental research, LFD from a reactor fed with mixed MSW, exceeding the irrigation guidelines standards set by WHO and APA for most the measured metals, and for total nitrogen and phosphorus (Ministério da Economia, 2015; World Health Organization, 2006), needed treatment to simultaneously reduce the concentration of these elements, organic contaminants, and biologic contaminants before irrigating soils. A new vertical subsurface flow CWs configuration was proposed, incorporating the study of a novel candidate species for wastewater treatment: *S. erectum*, thereby advancing research in the field of phytoremediation. In relation to this species, the role of the plant microbiome association was assessed. In addition, the second aim of the thesis was to **enhance the potential of valorisation of *S. erectum*, collected from CWs treating LFD**. For that, different pre-treatments were evaluated to improve the biogas production as compared with untreated biomass. Finally, the thesis delves into **the significance of public engagement outreach activities** related to AD and NBS, highlighting that their importance extends beyond just the scientific ecosystem.

Overall, the primary innovation of this thesis lied in the integration of CWs as part of the AD process. CWs served a dual purpose: first, as a polishing treatment of the LFD, and second, as a producer of additional feedstock; closing the loop of AD products' lifecycles and contributing to the transition towards a circular economy.

2. Constructed wetlands to reclaim liquid digestate

2.1. CWs configuration

The CWs configuration was a vertical subsurface flow with complete saturation and a daily recirculation for 14 days. The novel configuration (*S. erectum* planted in gravel, LECA, and sand bed substrate) showed high removals of organic matter (from 82 to 98 % COD), nutrients (over 98 % of ammonium and phosphate, 69 % of nitrate, and 90 %

of nitrite ions), metals (over 94 % of Zn, Cu, Pb, and Cr, over 92 % of Fe, and over 82 % of Mn), antibiotics (over 99 % of oxytetracycline, sulfadiazine, and ofloxacin), potential pathogens, MGEs, and some ARGs (*tetA, tetW* and *sul1*). Each metal presented a particular behaviour, with Fe, Cu, and Pb showing respective increases of 244, 627, and 180 %, in their relative concentrations in the plants' roots compared to their initial values. Additionally, on average, 57% of Mn accumulated in the upper part of plants by the end of the experiment, while Zn and Cr prevalence increased in LECA by 557 and 99 %, respectively.

2.2. *S. erectum*

S. erectum individuals adapted to the LFD, showing an average increase in population size of 407 %, and a growth in biomass weight of 110 %, on average, over time. Throughout the experimental period, plants primarily accumulated Mn in the leaves and Fe, Zn, Cu, Pb and Cr in the roots, showing promising potential for metal phytoremediation with bioconcentration factors of 35, 73, 3.1, 6.9, 11, and 8.2, respectively. To ensure optimal CWs management, a periodic harvest of plants is necessary. Its rapid growth and its considerable fresh biomass yield (22.1 kg/m^2) make it valuable for resource recovery. The metal concentration translocated to the upper parts of the plants was diluted due to the biomass growth and consequently, remained below the minimum inhibitory concentration for AD microbial communities.

2.3. Prokaryotic communities

Regarding the prokaryotic communities in these CWs systems, the bacterial charge of the effluent was reduced by more than 100 times compared to the influent, accompanied by an increase in species richness and diversity. CWs provided diverse microenvironments with different ecological niches, including oxygen gradients, substrate types, nutrient availability, and the presence of plant exudates, which supported a wider range of microbial communities. This diversity could potentially enhance pollutant removal, contribute to biogeochemical cycles, such as nutrient cycling or organic matter decomposition, promote plant growth, or compete with pathogens, thereby reducing their abundance, for instance leading to removal efficiencies over 85 % and 94 % of *Streptococcus* and *Clostridium*, respectively.

Over time, the number of bacteria increased over 5 times, and richness, and diversity within the root-bed substrate also rose, suggesting potential biofilm formation and growth. This phenomenon could contribute to the development of additional surface area, facilitating active sites for microbial activity. Notably, sulfadiazine and ofloxacin

affected the community structure only at the end of the experiment, after 3 months of dosing, while oxytetracycline showed no significant differences compared to the LFD control without antibiotics. Similar findings were observed in the microbial communities of the endosphere of *S. erectum*'s roots. Sulfadiazine and ofloxacin dosing resulted in changes in the community structure whereas oxytetracycline exhibited no significant differences in it. Although the episphere showed different taxonomic profiles in CWs treating LFD with antibiotics, no significant differences were detected in the rhizosphere and episphere between the presence and absence of antibiotics. Additionally, the taxonomic composition of the episphere and endosphere of *S. erectum* roots in natural environments resembled that of the episphere and endosphere, respectively, in CWs systems without antibiotic treatment.

2.4. MGEs and ARGs

Although there was a reduction in the absolute abundance of MGEs (87 % removal efficiency, on average) and ARGs in the effluents after CWs' treatment (over 98 % removals of *tetA, tetW, sul1*, and *qnrS*), there was a slight increasing trend over time in the relative abundance of *tetW* (from 1.15×10^4 to 1.55×10^5 copies/mL), and an increase of *qnrS* was observed in the effluent of OF systems (from 191 to 684 copies/mL). Antibiotics had no effect on the abundance of *intI1, tetA, tetW* and *sul1* in CWs effluents. In the root-bed substrate, the relative abundance of MGE and ARGs, except for *qnrS,* decreased over time. Hence, it appears that in general, CWs were not acting as reservoirs of ARGs, mitigating their dissemination. However, ofloxacin may have exerted selective pressure on ofloxacin-resistant bacteria and promoted the HGT of the *qnrS* gene, despite its minimal presence in the community.

3. Anaerobic digestion of harvested biomass to close the loop

S. erectum biomass from CWs proved to be a suitable substrate for AD. However, their cell walls are formed by a complex fibrous network of polysaccharides, primarily cellulose, hemicellulose, and pectin, along with lignin and proteins, creating resilient structures that are resistant to degradation. Three different approaches to enhance methane production were evaluated: storage, aqueous ammonia soaking and hydrothermal pretreatments. All pretreatments led to increases between 9 and 46 % in methane production, being hydrothermal carbonisation (200 °C for 1 h) the pretreatment with the highest increase, 151 $NmLCH_4/gCOD$, compared to untreated biomass. The pretreated biomass exhibited physicochemical and morphological changes. Plants pretreated with aqueous ammonia soaking presented the lowest concentrations of lignin

(~ 5.5 % TS) and the highest concentrations of cellulose (~40 % TS), like hydrothermally pretreated biomass (41 % TS). These pretreatments were also the ones associated with the most damage to the cell walls of the leaves.

Microbial communities in the reactor varied across the different pretreated and untreated plants. In general, aqueous ammonia soaking and hydrothermal carbonization induced significant shifts in the microbial composition, particularly in terms of taxa abundance and prevalence. Specifically, aqueous ammonia soaking resulted in the dominance of hydrolysers and acidogens in the early stages of methane production and acetoclastic methanogenesis was inhibited, whereas hydrothermal pretreatments exhibited stability and robustness in microbial community composition over time. Additionally, the methanogenic archaeal community showed varying abundance levels across different reactors.

However, when evaluating the techno-economic assessment, hydrothermal and alkali pretreatments required a lot of energy, that was not compensated by the increase in the methane production obtained, making the processes not feasible. Solar drying was the only pretreatment that enhanced the yield compared to untreated biomass, resulting in 1314 kWh/t of substrate, a 473 % increase in energy production on day 56. Nonetheless, hydrothermal carbonisation produced another byproduct that was not considered in the assessment, namely hydrochar, with valorisation potential applications as a soil amendment, adsorbent, or precursor to produce biochar-based materials.

4. Engaging future generations with the circular bioeconomy

Wetland Wonders provides a valuable educational tool for secondary school and university students, promoting engagement and understanding biological processes. Its adaptability across different education levels underscores its versatility and effectiveness in fostering essential skills such as critical thinking and information literacy. By addressing misconceptions about microbes and highlighting their role in wastewater treatment, this activity contributes to a more comprehensive understanding of environmental sciences. Furthermore, its alignment with SDG highlights its significance in promoting quality education and sustainable practices. Overall, *Wetland Wonders* exemplifies the potential of innovative pedagogical approaches to inspire learning and address global challenges.

The *Methanopoly* game illustrated the collaborative relationship among microorganisms that facilitate the AD process, enabling the production of methane from the degradation of organic wastes. The activity emphasised the crucial role of cooperation among the

microbes themselves, the students in class and the researchers within a project, in achieving the ultimate goal of renewable methane production from organic waste degradation. Students were actively involved in the learning activity, feeling very curious and enthusiastic. The activity addressed diverse learning styles of students to enhance understanding and retention of scientific concepts.

Gamification and the methodological approach of the activities resulted in an engaged and motivated audience involved in the activity and promoting meaningful learning outcomes. These activities promote circular economy principles disseminating sustainable practices prioritising resource efficiency, waste reduction and repurposing to minimise the environmental impact and maximise the economic value. More efforts are needed to bridge the gap between science and society.

5. Main conclusions

At the end of this work, we can outline the following take-home messages:

- Vertical subsurface flow CWs effectively removed pollutants from LFD.
- Fe, Mn, Zn, Cu, Pb, and Cr removals were over 82%. Mn was translocated into leaves, while Fe, Cu, and Pb were accumulated in the roots, and Zn and Cr in LECA.
- The microbial community of CWs effluent increased in diversity and declined in population and potential pathogens abundance, as compared to the influent.
- *S. erectum* conserved its specific microbiome.
- Although the treatment achieved high removal of oxytetracycline, sulfadiazine, and ofloxacin, antibiotics altered the episphere and endosphere community but not the rhizosphere.
- *intl1*, *tetA*, *tetW*, *sul1* and *qnrS* gene copy/mL decreased in the effluent, and ARGs relative abundance in the roots-bed substrate decreased over time.
- Storage, aqueous ammonia soaking, and hydrothermal pretreatments of *S. erectum* induced varied effects on the morphology and physicochemical properties.
- Hydrothermal carbonisation at 200°C for 60 min demonstrated the highest methane yield increase of 46%, reaching 151 $NmLCH_4$/gCOD.
- Aqueous ammonia soaking led to a decrease in taxa abundance and an hydrogenotrophic methanogenesis during AD process of pretreated biomass.

- Dry storage emerged as the most promising and feasible pretreatment option among those tested.

6. Future perspectives

This dissertation offered a preliminary approach at a laboratory scale that should be scaled up in future works. To scale up the systems, the main operation problem is clogging (Mitterer-Reichmann, 2012). A pretreatment for CWs should be assessed for LFD with high suspended solids load. Traditional methods for pre-treating wastewater to reduce the solids and organic matter are septic tanks, Imhoff tanks or primary decanters (Tchobanoglus et al., 2003). Integrating high-rate anaerobic systems such as anaerobic filters, or anaerobic fluidised bed reactors as a preliminary treatment for LFD in CWs could reduce the clogging of substrate beds and mitigate greenhouse gas emissions by collecting biogas (J. A. Álvarez et al., 2008).

Options to reduce HRT could be to dilute the influent with part of the effluent by recirculating it. Following the German design guidelines, the hydraulic loading rate should not exceed 20 g COD/m^2·d, so an approach could be to divide the surface area into various smaller systems to be charged independently, allowing an intermittent loading with a resting phase (DWA, 2017).

To evaluate if the simultaneous organic, inorganic, and biologic pollutants removal in the long term is maintained despite seasonality, further investigation would also be needed in the scaled-up systems. Previous works showed that organic matter, nutrients, and metals removals are maintained over the years of performance of CWs (Knox et al., 2021; Nilsson et al., 2020). However, no consensus on the effect of antibiotics in the long term exists (Ohore et al., 2022). Hence, further efforts are needed to understand the fate of antibiotics and transformation products in CWs and plants, and their interaction with other contaminants, to improve the design and configuration of the systems.

Finally, further research could be performed to improve the cost-effectiveness of the pretreatments of harvested biomass, to improve the biogas production, and enhance regional economies while implementing circular practices in bioresource management. Options such as dewatering steps or reducing temperature of the pretreatments could lead to achieve the revalorisation goal. A codigestion with organic fraction of MSW scenario could be also considered.

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Appendix A – Contaminants concentration in constructed wetlands' effluents

Table A.1 Final concentration of COD in effluents after the treatment in CWs along the six treatment cycles. C, OX, SD and OF represent the different experimental conditions (digestate with or without antibiotics).

Cycle	Concentration of COD in the effluent (mg/L)			
	C	**OX**	**SD**	**OF**
1st	963 ± 122	914 ± 124	1005 ± 47	873 ± 96
2nd	702 ± 58	842 ± 150	944 ± 290	650 ± 23
3rd	1496 ± 594	1129 ± 248	1283 ± 500	1367 ± 53
4th	1965 ± 215	1725 ± 34	2086 ± 449	1783 ± 217
5th	1610 ± 61	1647 ± 35	1806 ± 244	1531 ± 161
6th	630 ± 146	560 ± 64	625 ± 107	630 ± 37

Table A.2 Concentration of nutrients in the effluent of CWs treating liquid digestate spiked or not with antibiotics along 6 cycles.

Cycle	Nutrient	Concentration of nutrients in the effluent (mg N/L or mg P/L)			
		C	**OX**	**SD**	**OF**
1st	NH_4^+	4 ± 6	7 ± 12	1 ± 1	2 ± 1
	NO_3^-	1.1 ± 0.4	1.3 ± 0.5	0.8 ± 0.2	0.7 ± 0.4
	NO_2^-	0.1 ± 0.1	0.3 ± 0.3	0.06 ± 0.02	0.06 ± 0.03
	PO_4^{3-}	0.3 ± 0.2	0.24 ± 0.04	0.30 ± 0.06	0.25 ± 0.09
2nd	NH_4^+	0.9 ± 0.1	0.8 ± 0.2	1.4 ± 0.2	1.3 ± 0.1
	NO_3^-	1 ± 1	2 ± 1	1.1 ± 0.6	1.4 ± 0.5
	NO_2^-	0.05 ± 0.03	0.08 ± 0.08	0.04 ± 0.02	0.10 ± 0.07
	PO_4^{3-}	0.14 ± 0.02	0.15 ± 0.02	0.13 ± 0.03	0.13 ± 0.03
3rd	NH_4^+	0.7 ± 0.5	0.6 ± 0.6	0.6 ± 0.4	1.2 ± 0.5
	NO_3^-	3.5 ± 1	3.6 ± 3	3.1 ± 2	5.4 ± 3
	NO_2^-	0.12 ± 0.05	0.3 ± 0.3	0.18 ± 0.09	0.2 ± 0.2
	PO_4^{3-}	0.3 ± 0.1	0.17 ± 0.05	0.20 ± 0.07	0.22 ± 0.02
4th	NH_4^+	11 ± 16	4 ± 5	6 ± 3	16 ± 9
	NO_3^-	1 ± 1	2 ± 2	1 ± 1	5 ± 3
	NO_2^-	0.09 ± 0.06	0.06 ± 0.04	0.06 ± 0.03	0.11 ± 0.04
	PO_4^{3-}	0.3 ± 0.1	0.17 ± 0.09	0.20 ± 0.09	0.26 ± 0.06
5th	NH_4^+	12 ± 12	4 ± 4	8 ± 4	15 ± 6
	NO_3^-	1.1 ± 0.3	1.0 ± 0.1	3 ± 3	7 ± 3
	NO_2^-	0.06± 0.02	0.11 ± 0.03	0.06 ± 0.01	0.2 ± 0.2
	PO_4^{3-}	0.2 ± 0.1	0.28 ± 0.05	0.2 ± 0.1	0.19 ± 0.07
6th	NH_4^+	8 ± 3	6 ± 2	4 ± 1	9.77 ± 0.08
	NO_3^-	5 ± 2	5 ± 3	4.0 ± 0.6	6 ± 2
	NO_2^-	0.11 ± 0.03	0.14 ± 0.07	0.2 ± 0.2	0.5 ± 0.3
	PO_4^{3-}	0.18 ± 0.07	0.13 ± 0.02	0.21 ± 0.07	0.134 ± 0.005

Table A.3 Concentration of trace metals in the effluent of CWs treating liquid digestate during 6 treatment cycles. <LOD indicates that the concentration is below the limit of detection.

Cycle	Metal	Concentration of metal in the effluent (mg/L)			
		C	**OX**	**SD**	**OF**
1st	Fe	5 ± 2	5 ± 1	5 ± 2	5 ± 2
	Mn	0.2 ± 0.1	0.15 ± 0.09	0.14 ± 0.07	0.12 ± 0.02
	Zn	0.07 ± 0.05	0.03 ± 0.01	0.06 ± 0.03	0.04 ± 0.01
	Cu	< LOD	< LOD	< LOD	< LOD
	Pb	0.004 ± 0.003	0.0043 ± 0.0007	0.006 ± 0.004	0.003 ± 0.002
	Cr	0.0040 ± 0.0009	0.0035 ± 0.0003	0.0039 ± 0.0003	0.0034 ± 0.0003
2nd	Fe	2 ± 2	2 ± 1	3 ± 1	2.2 ± 0.4
	Mn	0.12 ± 0.03	0.11 ± 0.02	0.103 ± 0.006	0.11 ± 0.01
	Zn	< LOD	< LOD	< LOD	< LOD
	Cu	< LOD	< LOD	< LOD	< LOD
	Pb	0.004 ± 0.002	0.0030 ± 0.0005	0.0031 ± 0.0004	0.0028 ± 0.0004
	Cr	0.0031 ± 0.0005	0.0037 ± 0.0005	0.004 ± 0.001	0.0028 ± 0.0002
3rd	Fe	5 ± 2	3.4 ± 0.9	4 ± 2	6 ± 2
	Mn	0.14 ± 0.07	< LOD	< LOD	0.15 ± 0.06
	Zn	< LOD	< LOD	< LOD	< LOD
	Cu	< LOD	< LOD	< LOD	< LOD
	Pb	0.004 ± 0.002	0.0035 ± 0.0006	0.0041 ± 0.0005	0.0032 ± 0.0005
	Cr	0.006 ± 0.003	0.004 ± 0.001	0.004 ± 0.001	0.0056 ± 0.0007
4th	Fe	7 ± 2	8 ± 5	9 ± 5	4.6 ± 0.9
	Mn	0.15 ± 0.04	0.12 ± 0.02	0.11 ± 0.02	0.14 ± 0.05
	Zn	< LOD	< LOD	< LOD	< LOD
	Cu	< LOD	< LOD	< LOD	< LOD
	Pb	0.005 ± 0.001	0.006 ± 0.002	0.006 ± 0.004	0.004 ± 0.002
	Cr	0.0041 ± 0.0004	0.0043 ± 0.0008	0.0043 ± 0.0009	0.0043 ± 0.0001
5th	Fe	6 ± 2	5 ± 1	6 ± 2	3.4 ± 0.8
	Mn	0.17 ± 0.03	0.17 ± 0.02	0.15 ± 0.06	0.14 ± 0.03
	Zn	0.04 ± 0.03	0.06 ± 0.07	0.06 ± 0.06	< LOD
	Cu	0.07 ± 0.01	0.08 ± 0.01	0.09 ± 0.03	0.07 ± 0.01
	Pb	0.0077 ± 0.0008	0.012 ± 0.006	0.012 ± 0.009	0.0027 ± 0.0006
	Cr	0.007 ± 0.001	0.0061 ± 0.0006	0.008 ± 0.001	0.0061 ± 0.0005
6th	Fe	1.9 ± 0.8	1.5 ± 0.6	2 ± 1	1.4 ± 0.2
	Mn	0.12 ± 0.04	< LOD	0.11 ± 0.02	0.104 ± 0.006
	Zn	< LOD	< LOD	< LOD	< LOD
	Cu	< LOD	0.053 ± 0.005	0.056 ± 0.005	0.062 ± 0.006
	Pb	0.005 ± 0.004	0.003 ± 0.002	0.004 ± 0.001	0.0028 ± 0.0002
	Cr	0.009 ± 0.001	0.006 ± 0.001	0.0056 ± 0.0009	0.0058 ± 0.0006

Table A.4 Average relative abundance of *Clostridium* and *Streptococcus* genera in CWs treating in parallel the 4 different LFDs during the second, the fourth and the sixth 14-day treatment cycles.

Cycle	Genus	Relative abundance (%)				
		Influent	Effluent			
			C	OX	SD	OF
2nd	*Clostridium_sensu_stricto_1*	0.07 ± 0.01	0.03 ± 0.02	0.05 ± 0.04	0.03 ± 0.04	0.04 ± 0.03
	Clostridium_sensu_stricto_15	0.5 ± 0.2	0.02 ± 0.02	n. d.	0.01 ± 0.01	0.00 ± 0.01
	Clostridium_sensu_stricto_8	1.0 ± 0.2	0.01 ± 0.01	n. d.	0.01 ± 0.03	0.01 ± 0.01
	∑ *Clostridium*	1.5 ± 0.4	0.05 ± 0.03	0.05 ± 0.04	0.05 ± 0.05	0.05 ± 0.03
	Streptococcus	7.9 ± 0.2	0.7 ± 0.6	0.3 ± 0.2	0.5 ± 0.4	0.6 ± 0.3
4th	*Clostridium_sensu_stricto_1*	0.12 ± 0.02	0.01 ± 0.01	0.01 ± 0.01	0.02 ± 0.01	n. d.
	Clostridium_sensu_stricto_15	0.50 ± 0.06	n. d.	n. d.	0.002 + 0.004	0.002 ± 0.004
	Clostridium_sensu_stricto_8	0.090 ± 0.004	n. d.	n. d.	n. d.	n. d.
	∑ *Clostridium*	0.71 ± 0.05	0.01 ± 0.01	0.01 ± 0.01	0.02 ± 0.02	0.002 ± 0.004
	Streptococcus	8.3 ± 0.8	0.4 ± 0.1	0.4 ± 0.3	0.3 ± 0.1	0.3 ± 0.2
6th	*Clostridium_sensu_stricto_1*	0.158 ± 0.004	0.01 ± 0.01	0.009 ± 0.003	0.01 ± 0.01	0.01 ± 0.01
	Clostridium_sensu_stricto_15	0.6 ± 0.1	0.02 ± 0.03	0.05 ± 0.01	0.02 ± 0.01	0.02 ± 0.01
	Clostridium_sensu_stricto_8	0.5 ± 0.1	0.02 ± 0.01	0.05 ± 0.02	0.03 ± 0.02	0.06 ± 0.04
	∑ *Clostridium*	1.275 ± 0.002	0.05 ± 0.03	0.11 ± 0.02	0.06 ± 0.02	0.09 ± 0.04
	Streptococcus	9 ± 1	1.0 ± 0.3	1.3 ± 0.5	1.2 ± 0.4	1.4 ± 0.1

n.d.: not detected.

Appendix B - Materials of *Wetlands Wonders*

Orientation Guidelines

1. Identify the presented problem.
2. Extract information from the provided documents.
3. Identify the characteristics and contaminants present in the wastewater that need to be treated.
4. Evaluate which substrate, plant, configuration, and microorganisms can remove the contaminants from the wastewater, considering the provided supplementary information.
5. Represent the constructed wetland that removes the highest possible amount of contaminants from the wastewater with the provided arts and crafts materials.
6. Prepare a 4-minute speech selling your wetland proposal and highlighting its special features.

Figure B.1 Orientation guidelines of Construction Process Challenge helping students understand what they need to do.

Questionnaire of the activity:

Wetland Wonders

Centre:

Data: **Grade:**

Age:

Gender:

1. **Prior knowledge**

Identify three current challenges related to the water issue:

-

-

-

How can water be reused?

List the wastewater management systems you know:

What do you expect from today's activity?

2. **Results and discussion of the activity**

Name of your company:

Wastewater type:

Draw a sketch of the constructed wetland you will build

Write three characteristics that justify why your wetland is the most effective

-

-

-

Circle the most appropriate option: Here, effective means that the wetland...

... saves more energy. ... removes more contaminants. ... generates more water.

3. **Final evaluation of the activity**

Complete this self-assessment of the activity, considering the evaluation rubric.

	3	2	1	0
Identification of the presented problem	☐	☐	☐	☐
Teamwork and organization of tasks to be done	☐	☐	☐	☐
Understanding of concepts	☐	☐	☐	☐
Quality of the model representing the constructed wetlands	☐	☐	☐	☐
Oral presentation of your wetland	☐	☐	☐	☐

What did you like most about the activity?

Circle 1 item

The topic

The game

Working in small groups

Contact with people from outside the school

Learning new things

Others: _____

4. **General opinion of the activity**

Rate the following statements from 1 to 5:

1. Strongly disagree 3. Neither agree nor disagree 5. Strongly agree

	1	2	3	4	5
The activity was well organized.	☐	☐	☐	☐	☐
I have been able to actively participate.	☐	☐	☐	☐	☐
The facilitator was clear and resolved doubts.	☐	☐	☐	☐	☐
I enjoyed the activity.	☐	☐	☐	☐	☐
The duration of the activity was appropriate.	☐	☐	☐	☐	☐
I can explain what constructed wetlands are.	☐	☐	☐	☐	☐

Comments

Figure B.2 Questionnaire provided to the students (n = 57) that included the initial survey with prior knowledge, the self-assessment, and the final evaluation of the activity. The questionnaire was inspired by Carroll et al, (2020) and Fantastic DNA, Cell Explorer's activity.

Table B.1 Self-assessment evaluation rubric given to students at the end of the activity.

Items	Level 3 (expert)	Level 2 (advanced)	Level 1 (apprentice)	Level 0 (novice)
Identification of the presented problem	Offers a deep and comprehensive analysis of the challenge presented, identifying the steps to be taken to solve the problem.	Provides a satisfactory analysis with some shortcomings in terms of depth or detail.	Offers a basic analysis of the challenge with evident shortcomings in understanding the tasks to be carried out.	Lack of clear understanding of the presented problem.
Teamwork and task organization .	Active participation and cooperation among group members. Organization and problem-solving autonomously.	Satisfactory participation and cooperation. Autonomous organization, but occasionally the instructor had to intervene to guide and resolve doubts.	Limited participation and lack of cooperation among group members. The instructor had to organize the group and list the tasks to be done.	No participation or significant contribution from team members. There was no organization of tasks within the group.
Understanding of concepts	Clear demonstration of understanding of the principles related to wetlands during the game and discussions.	Accepted understanding, with some inaccuracies or lack of details	Basic understanding with many inaccuracies or evident shortcomings.	Lack of clear understanding of the concepts.
Quality of the wetland model	Detailed model, representing all elements of the wetlands and considering environmental characteristics.	Satisfactory model with some shortcomings or simplifications.	Basic model with evident shortcomings or little relation to reality.	Inadequate or absent model.
Oral presentation of the constructed wetland .	Clear and structured presentation highlighting the important aspects of the model.	Comprehensible presentation with some shortcomings in structure or emphasis.	Presentation with evident shortcomings in communicating key ideas.	Confusing or non-existent presentation.

Table B.2 Information about the contaminants present in different wastewater types.

Contaminants in wastewater	Pig slurry	Sewage sludge	Marine aquaculture effluents	Acid mine drainage
Suspended solids	+++++	++	+++	++
Organic matter	+++++	++	+++	
Total nitrogen	+++++	++	++++	
Ammoniacal nitrogen	+++++	++	++++	
Phosphorus	++++	++	++++	
Pathogens	++++		++	
Metals	+++			+++++
Antibiotics	+		+++	
Ibuprofen		++++		
Caffeine		++++		
Antidepressants and antiepileptics		++++		
Anti-inflammatories		++++		

+: the amount of '+' symbols indicates the concentration of the contaminant in the wastewater type, with no '+' corresponding to non-detectable levels and '+++++' corresponding to high concentration.

Table B.3 Information about the capability of flow types to remove contaminants.

Contaminants' removal	Free water surface flow	Horizontal subsurface flow	Vertical subsurface flow
Suspended solids	++	++	+++
Organic matter	+	++	+++
Total nitrogen	++	++	++
Ammoniacal nitrogen	+++	+	+++
Phosphorus	+	+	+
Pathogens	+	++	+++
Metals	+	+++	+++
Antibiotics	+	+	++
Ibuprofen	++	+	+++
Caffeine	++	++	+++
Antidepressants and antiepileptics	+	++	+
Anti-inflammatories	+	+	++

'+' symbols indicates the removal of the contaminant from the wastewater type. The amount of '+' corresponds to the levels of '+' in Table S2 that are removed in each flow type.

Table B.4 Information about the capability of plants to remove contaminants.

Contaminants' removal	***Phragmites australis***	***Typha latifolia***	***Canna indica***	***Cyperus papyrus***
Suspended solids				
Organic matter	+	+	+	+
Total nitrogen	+	+	+	+
Ammoniacal nitrogen	+	+	+	+
Phosphorus	+	+	+	+
Pathogens				
Metals	+	+		
Antibiotics	+	+		
Ibuprofen				
Caffeine				
Antidepressants and antiepileptics				
Anti-inflammatories				

'+' symbols indicates the removal of the contaminant from the wastewater type. The amount of '+' corresponds to the levels of '+' in Table S2 that are removed by each plant species.

Table B.5 Information about the capability of substrates to remove contaminants.

Contaminants' removal	Gravel	Sand	Expanded clay	Zeolite
Suspended solids	+	+		
Organic matter	+	+	+	
Total nitrogen			+	+
Ammoniacal nitrogen				+
Phosphorus			+	+
Pathogens		+	+	
Metals			+	+
Antibiotics			+	+
Ibuprofen			+	+
Caffeine			+	+
Antidepressants and antiepileptics			+	+
Anti-inflammatories			+	+

'+' symbols indicates the removal of the contaminant from the wastewater type. The amount of '+' corresponds to the levels of '+' in Table S2 that are removed by each substrate.

Table B.6 Information given in the *special card.*

You can build two constructed wetlands in series if you have at least one type of each component for each wetland.	You cannot have solely aerobic microorganisms in wetlands with horizontal subsurface flow.	You cannot have solely anaerobic microorganisms in wetlands with vertical subsurface flow.
You will gain two extra "+" signs in the elimination of the contaminant(s) you choose if you combine aerobic and anaerobic microorganisms.	You can plant two species of plants in the same wetland to combine the effects of each species.	You can have two layers with two different substrates to combine the effects of each substrate if you have wetlands with subsurface flow.
You can have a wetland without plants, but one "+" sign will be deducted from the elimination of each contaminant.	*Canna indica* and *Phragmites australis* can tolerate high salinity and maintain good levels of contaminant removal.	*Typha latifolia* and *Phragmites australis* are the plants that best tolerate high loads of organic matter.

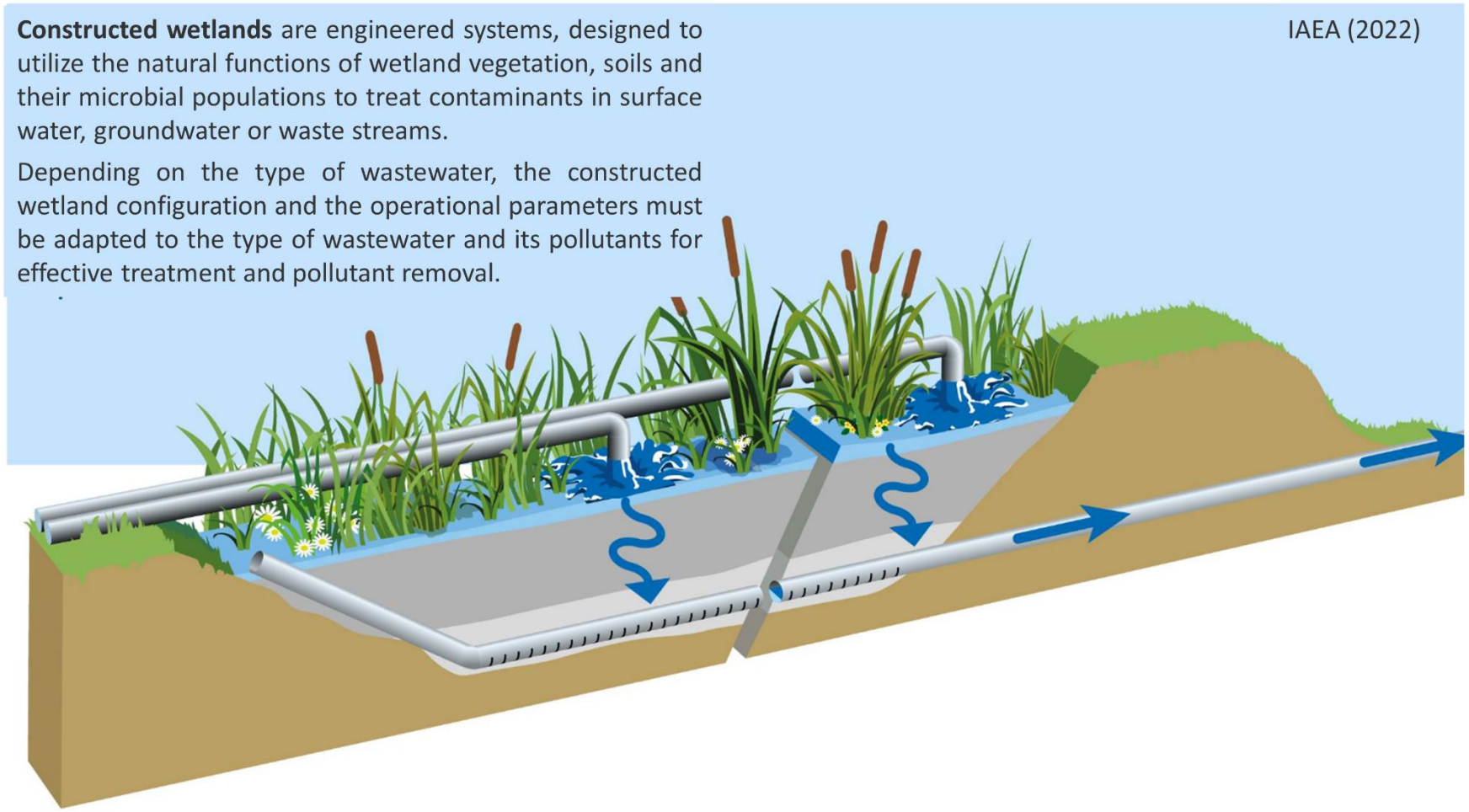


Figure B.3 Diagram of basic principles and definition of constructed wetlands.

Appendix C – *Methanopoly*, a giant cooperative game to learn the anaerobic digestion process

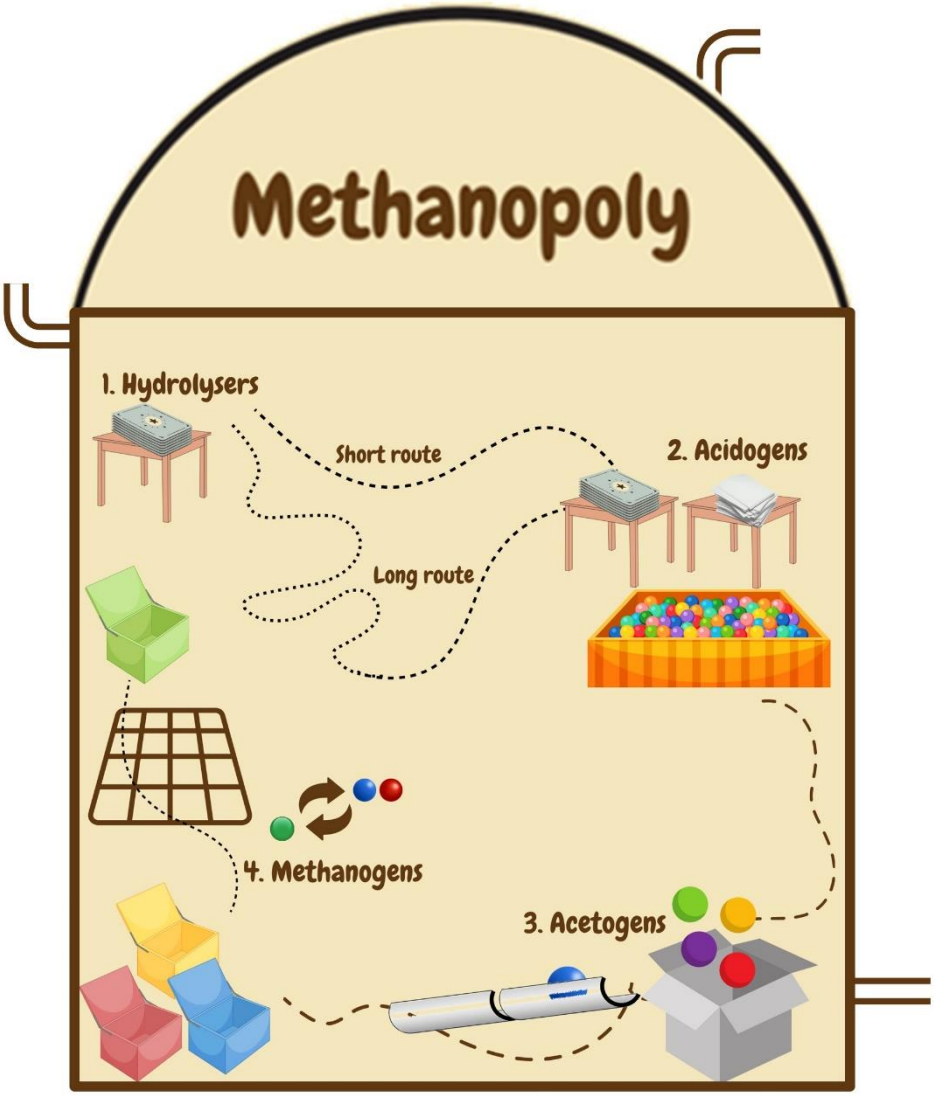


Figure C.1 General overview of *Methanopoly* game, with the relay of the anaerobic degradation process of organic waste.

This Appendix is part of a future publication targeting the *Journal of Microbiology & Biology Education* that has been written co-authored with Kris A. Silveira and Raffaello Mattiussi.

1. Introduction

The last decades have been characterised by a continuously changing world defined by rapid globalization, social dynamics, technological advancements, and climate fluctuations, which require adaptive strategies for societies. To face this instability and future challenges newer generations need to be properly educated towards these problematic and potential solutions (Serdyukov, 2017).

In such an evolving world, education must adapt as well and incorporate new methodologies for students to perform adequately (González-Pérez and Ramírez-Montoya, 2022). Gamification is defined as the use of any type of game element in a non-game environment (Kapp et al., 2012). It serves two primary objectives: first, aligning with learning objectives tied to the content, and second, fostering playful outcomes for students, including enjoyment, satisfaction, and creativity (Sailer et al., 2017).

Anaerobic digestion (AD) is a key technology towards valorisation of organic residues, ultimately achieving SD goals and aligning with EU Green deal directives. Understanding such technologies falls under the aegis of Green Education put forth by the European Education Areas (European Commission, 2024). Alongside the guidelines set out for American and European undergraduate courses in Biology incentivizing stakeholders to innovate in this aspect of learning (Allen et al., 2024; Sibirny et al., 2019).

Microbe-based biotechnologies for renewal energy production, whilst bearing promising solutions, still suffer from a lack of understanding. This can be explained both by existing misconceptions on microorganisms and by the complexity intrinsic to microbial communities functioning. Traditional curricula often undermine the essential role of microorganisms in the environment to focus too much on their role in human health, still too often restricting to their pathogenic roles (Lloyd & Berry, 2022)

Bringing to real life size a phenomenon from the invisible scale. Here, we simulate the presence of cooperating and interacting microbes within a complex anaerobic microbial environment (Bioreactor).

The activity demonstrates a deeper dive into microbial interactions sustaining AD whilst relying on meaningful learning through games and cooperation. The activity targets stakeholders from the AD ecosystem, complementing a lesson on renewable biotechnologies or as a demonstration of a microbial ecological interaction, that would like to disseminate their work to secondary or undergraduate students.

2. Procedure

This activity is designed for a 30-minute to 1-hour session for groups of 20 to 35 undergraduate students. Beginning with an introduction to organic waste management and anaerobic digestion process. The activity is adaptable to various background knowledge levels that students might have because the game facilitates a grasp of the general anaerobic digestion process.

Participants represent the microorganisms within an anaerobic digestor, and they aim to produce as much methane as possible. For that, they cooperate to facilitate the degradation of organic waste into methane. Participants are divided into 4 groups of microorganisms namely hydrolysers, acidogens, acetogens, and methanogens, that play independently conducting a specific task in the overall degradation process. In this giant cooperative game, molecules and organic waste are represented by coloured balls and tokens, respectively.

After a short trial for everyone to understand their own task, a 15-minute timer is activated. Throughout the game, some perturbations occur, requiring participants to adapt to continue their tasks. When the time is over, participants must count the number of green balls produced. If several groups play, players can compare their performance with other groups based on the amount of methane produced. Then, the instructor stimulates a discussion to understand what happened and explain the main concepts.

The activity requires a spacious, obstacle-free area to simulate an anaerobic reactor. Four designated areas within this space correspond to the place where each group conducts their “degradation” task. The input, comprising all organic waste to be degraded, is placed next to the hydrolyser area, while the methane box serves as the output, where methanogens deposit the products of their reactions, as shown in **Figure C.1**. Participants had cue cards with the instructions, as a reminder (**Figure C.2**).

2.1. Game 1: hydrolysers

Crossing an obstacle course to breakdown & transfer degradable wastes (organic waste tokens, **Figure C.3**). Team members work together to tear the organic waste tokens and move through an obstacle course ensuring to walk only on laminated sheets. Based on the difficulty of degradability, take the easier course or complex course respectively, see long or short route in **Figure C.1**. Ultimately, exchanging an equivalent token & handing it over to the next team.

2.2. Game 2: acidogens

Participants will receive the waste tokens card and flip them (**Figure C.3**). They will read the flip side which contains a series of instructions. Their goal will be to pick out coloured balls out of a container and fill a bag with them, following instructions given on the back of the instructions cards (**Figure C.4**). Once one bag is prepared, they will transport it to the next group. The transportation method might also be subject to specific instructions contained on the waste tokens. At the end of their path they find three boxes, each one aimed to receive a specific colour of ball. The bag must be emptied, and balls dropped into the appropriate boxes.

2.3. Game 3: acetogens

Team members must transport balls and sort them into the three final colour boxes. Each participant has a half-pipe tubes, that will be the tool they need to transport the balls. First, one team member must pick one coloured ball from the initial box and place it on the pipe. The other participants must place the pipe continuously with the first team member and roll it into the next player's pipe. When the ball rolls into their own pipe, participants are not allowed to move; once the ball has passed, players can move and place their pipe at the end of the pipeline. Thereby, making a continuous chain, participants will be able to distribute each coloured ball into respective boxes.

2.4. Game 4: methanogens

Convert to Green: Team members must follow a specific Conversion Card (**Figure C.5**), pick the exact number of coloured balls required and collect the corresponding number of green balls from an instructor.

Uncover and pass a 4X4 methanogen grid: Team members must now take turns through trial and error & support from your instructor to find the hidden path on the grid (**Figure C.6**), you must remember the correct steps and only use these steps to pass the grid. After that drop the green ball in the Methane box. Voila! The team just created Methane.

2.5. Perturbations

At minute 5, the instructor communicates that there is a temperature decrease. All players will carry out the task in silence. A penalty of stopping all activity for 5 seconds will be applied to all players if violated.

At minute 10, the organic waste carried antibiotics that affect microorganisms in the reactor. Each group must choose 3 players to sacrifice them. They leave the group and go to another group.

3. Conclusion

The described activity's aims at depicting the microbial cooperation sustaining the AD process which permits methane production from organic waste degradation. Care was put into designing an activity which materialises key points of the real biological phenomena through a sequence of involving mini games. Each mini game is thought to be as nature-relatable as possible by integrating but simplifying concepts such as syntrophy, stoichiometry and reaction rates. The necessity of all these combined activities to reach a final common goal stresses the essential aspect of cooperation for both the players and for the actual microorganisms sustaining the real-life process.

The different groups of players represent the broad functional subdivision of microorganisms commonly used to describe the AD process (Hydrolysers, Acidogens, Acetogens and Methanogens). Each of these groups performs a degradation or recombination step in the transformation of organic matter into methane.

Each session of the game was concluded with a collective discussion based around a set of questions. The goal was to tick some important learning points (sequential aspect of AD, community-wise functioning of microorganisms, functional specificity of each trophic group...) using/asking about players' first-person experience.

This activity was also implemented during the Science is Wonderful 2023 fair where classes of age groups spanning from 5 – 16 years old experienced it.

How to play 'Hydrolysers' mini game

1. Decide with which substrate (organic waste card) you want to feed the digester.

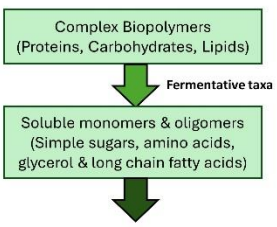
2. All members must transport the substrate through the hydrolysis pathway (obstacle course) until you reach the acidogenic area.
There are two routes, the **shorter**, for **easy** biodegradable waste and the **longer**, for waste that is more **difficult** to degrade.

3. Begin the route by always placing a laminated sheet for the first player to step on it and start the movement. You cannot step on the floor; you always must step on laminated sheets. Only one player can step on a sheet at a time. Your team can only transport one organic waste card at a time.

4. Continue the route bringing the organic waste card with you. The last member in the route must pass the last sheet to the member in front of him/her. The first player places the sheet in front of him/her and takes a step forward. All teammates must follow him/her in a trail while progressing through the route by always staying on a laminated sheet.

5. Give the hydrolysed organic waste card to the acidogens players when you finish your pathway. 6. Return to the starting point through the free path (you can walk on the floor) and restart again.

A Hydrolysers job



Hydrolyser facts

- Hydrolysis is a rate limiting step within Anaerobic digestion.
- Pre-treating the waste makes this process easier & faster
- For eg. Heating, Acid or base treatment.

How to play 'Acidogen' mini game

1. Collect the organic waste card from the Hydrolysers and identify which waste it is; then take a matching card and flip it to discover the number of coloured stickers shown.

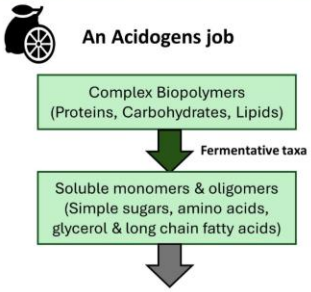
2. Transfer the combination of coloured balls indicated on the organic waste card
from the box to a bag. The bag corresponds to a mix of soluble monomer & oligomer molecules.

3. Now, pick a white card. This card will indicate the way in which all the acidogens players have to cross the acidogens pathway.

4. Transport the molecules bag through the acidogen pathway.

5. Empty the content of the bag content into the molecule box for the acetogens. The whole team returns to repeat the process with a new organic waste card.

An Acidogens job



Acidogen facts

- One of the fastest & flexible process within Anaerobic digestion.
- Rapid increase in volatile fatty acids lead to digester failure.
- Stronger acidogens are key to a resilient process.

How to play 'Acetogen' mini game

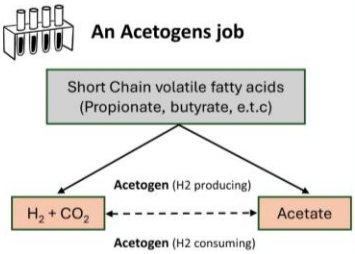
1. Any one team member must pick one colored ball from the molecule box to place on the pipe then cannot move from their position now.

2. The other team members must place the pipe continuously with the first team member and roll it into next player's pipe. Once the ball has passed their pipe, players are allowed to move and place their pipe at the end of the pipeline.

3. Thereby, making a continuous chain and eventually distributing each colored ball into respective boxes.

4. Repeat the process to sort all the colored balls into the respective boxes.

An Acetogens job



Acetogen facts

- Acetogenesis is a H_2 sensitive process
- Interesting syntrophic relationships arise between classes of microbes at this level
- Other VFA compounds need to be converted to acetate

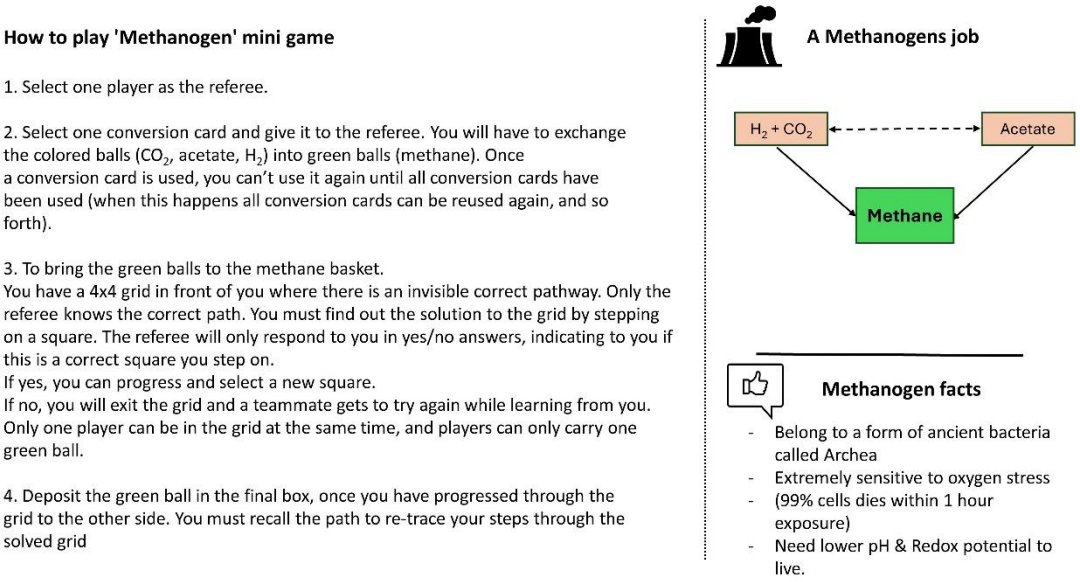


Figure C.2 Cue cards given to participants summarising the key points of their tasks and reminders of the process of the microorganisms they represent.

Figure C.3 Organic waste tokens. There were two piles: at the reverse of one pile there were indications of the easy/complex biodegradability of the materials, and at the reverse of the other pile there were coloured stickers indicating the corresponding combination of balls.

Only 4 feet and 4 hands in total can touch the ground to cross.	You have to cross in a single line, sorted in alphabetical order of your grandmother's (mother of your mother) last name.
You all have to cross together wearing something black (each one of you).	You all have to cross without touching with your feet on the ground.
Without speaking, you all have to cross in a line, from the one that has the smallest feat to until the one that has the largest one.	You have to transport the bag with the balls and none of you can touch with your hands the balls nor the bag, All players have to participate.
Instead of the bag, you have to use 8 different objects to transport all the balls.	You have to transport the balls until the end, only by blowing on them (making them roll on the floor). If you touch them, you have to restart.
You all have to cross together wearing something in your head (each one of you).	Only 3 feet and 6 hands in total can touch the ground to cross.
 You have to cross like this sorted in alphabetical order of one of your hobbies	 You have to cross like this sorted in order of the day of the month of your birthday

Figure C.4 Instruction cards for acidogens to perform acidogenesis and continue the degradation process. These were the indications of how participants had to collect and transport the balls (molecules).

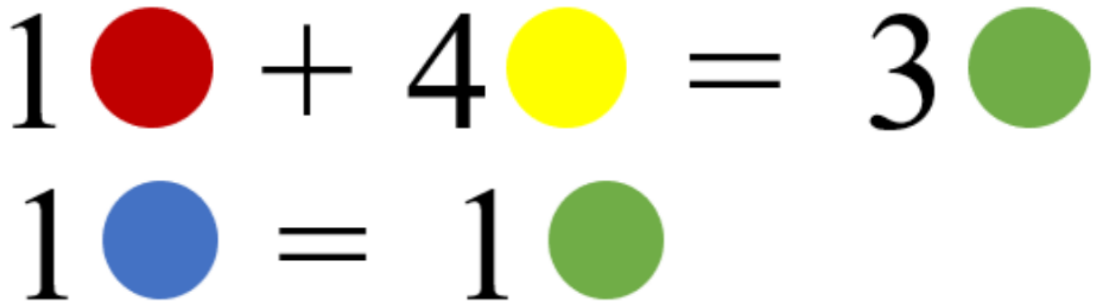
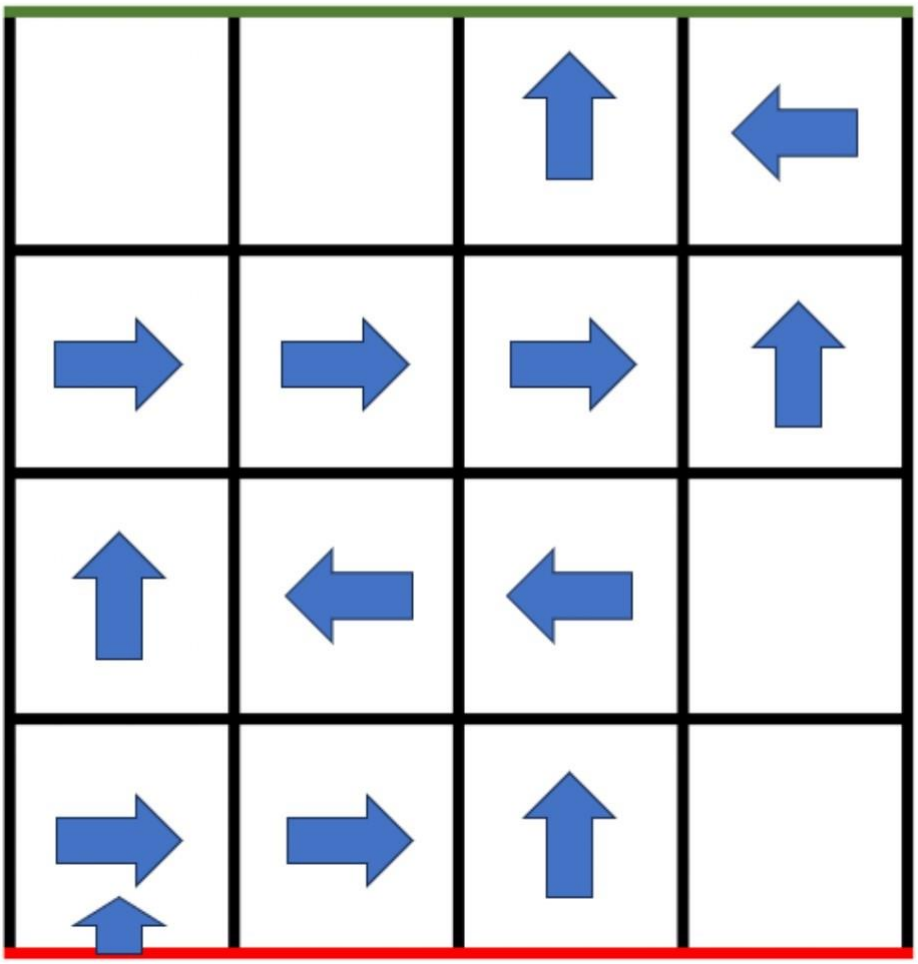


Figure C.5 Stoichiometric changes of red (CO_2), yellow (H_4), and blue balls (acetate) into green balls (CH_4).

The methanogenesis pathway follows the <u>blue arrows</u>.

You can only say <u>YES</u> or <u>NO</u> to where your partner has to step next.



The methanogenesis pathway follows the blue arrows

You can only say YES or NO to where your partner has to step next.

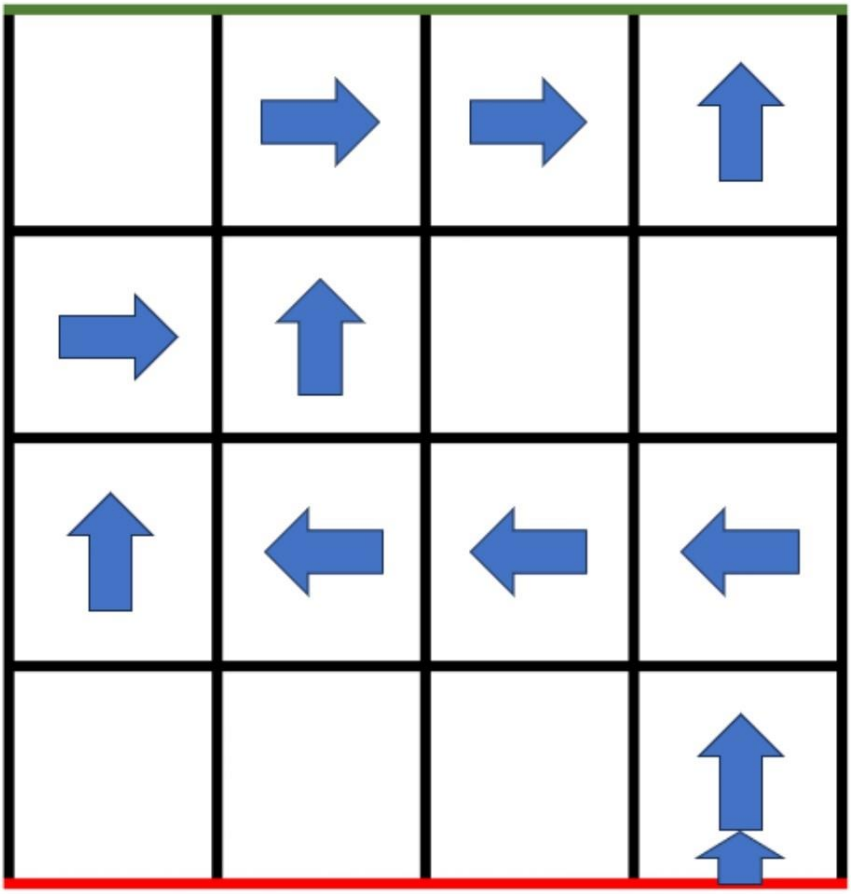


Figure C.6 Examples of two methanogens grid. Blue arrows show the "invisible" methanogenesis pathway where methanogens have to transport the green balls until the end.