Instituto Tecnológico y de Estudios Superiores de Monterrey

Campus Monterrey

School of Engineering and Sciences

Characterization of the spatial variations in the structure and diversity of microbial communities within and between the stages of a wastewater treatment plant based on passive methods

A thesis presented by

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Submitted to the

School of Engineering and Sciences

in partial fulfillment of the requirements for the degree of

Master of Science

In

Biotechnology

Monterrey Nuevo León, November 24th, 2020

Instituto Tecnológico y de Estudios Superiores de Monterrey

Campus Monterrey

Dedication

Thanks to all my friends and family who supported me day by day during this long research journey. Thank you for showing your unconditional support and patience, for listen when I was stressed, helping me ease my problems and my anger, for reassuring me to continue on this path and rejoicing with me when finally, I achieve this goal.

Similarly, I would like to thank my boyfriend, Cesar, for being with me through thick and thin throughout this work, motivating me to give my best every day and supporting me. Likewise, I would like to thank my friends Alejandra, Jesus, Marialaura, Luis, Carlos, Karina, Walter, and Gary, for your patience, support, and always believing that I could achieve this goal.

I want to especially thank my thesis partner, Alberto, who was by my side day and night for these two years, showing me his unconditional care and cheering on me when stress seemed to overcome. He made this trip feel much shorter and more fun, besides allowing me to know him more and pushing me to give my best. I will always be eternally grateful to him.

Finally, I would like to thank my parents, Lourdes and Jesus, and my brother and sister in law (Jesus and Annie) to always believe in me and support me to fulfill this goal. Besides, I would like to thank my parents-in-law (Mirna and José) for believing in me and help me achieve this goal. They were always cheering me on and always believed in me. This work would not have been possible without these persons and their constant support.

Acknowledgments

Throughout the development of this work, I have received a great deal, support, and assistance.

I would first like to express my greatest gratitude to my supervisors which were my model. Firstly, Dr. Carolina Sénes for all the knowledge, patience, and faith she brings to me during this degree. Similarly, I would like to thank Dr. José de Anda for transmitting his extensive wastewater treatment expertise and the support and experience provided to make this work much more enjoyable. Not forgetting Dr. Sebastián Gradilla for the patience, dedication, and guidance provided throughout this work. Thank you for your valuable guidance throughout my studies. You provided me with the tools and the knowledge that I needed to complete this work.

I would like to thank M.Sc. Osiris Diaz for the support, patience, and training that she provided during this work and in the same way, I would like to thank Dr. Carlos Yebra for the experience provided.

I would like to acknowledge Tecnológico de Monterrey for the opportunity to study a master's degree and provide the facilities required to carry out the experimentation of this project. In the same way, I want to thank CIATEJ for providing the experimental system under analysis and analytical equipment. I want to thank Conacyt for the scholarship provided for the development of this project.

Characterization of the spatial variations in the structure and diversity of microbial communities within and between the stages of a wastewater treatment plant based on passive methods

by

Marycarmen Verduzco Garibay

Abstract

Wastewater is frequently discharged directly to water bodies without any treatment in developing countries. Despite the efforts to treat a higher percentage of sewage, wastewater treatment plants (WWTP) often end up abandoned due to the high maintenance and operational costs. Consequently, untreated wastewater often reaches water bodies and causes several pollution problems, such as eutrophication, affecting the communities' public health. To achieve the Sustainable Development Goal (SDGs) number six of the United Nations, which seeks to substantially improve water quality by 2030, lowering the release of pollutants and toxins into wastewater and safe reuse and recycling of treated water. It is crucial to switch from centralized to decentralized WWTP systems that require less maintenance and operation costs.

Microorganisms are essential in wastewater treatment since they are involved in nutrient and organic matter removal through several metabolic pathways. However, microbial communities are susceptible to environmental variation, design and operational features of WWTP. Moreover, few studies have been performed focusing on the microbial communities within the treatment stages of complete decentralized systems. Therefore, this work's general objective was to characterize the spatial variations of the bacterial communities occurring in a decentralized wastewater treatment plant. This work is composed of three chapters. Chapter one describes the problem statement and context, research question, solution overview, and main contributions. Chapter two describes the types of constructed wetlands (CW) and the pollutant removal processes that occur within them.

Additionally, this chapter discusses the effect of environmental parameters as well as operational and design factors on the structure, diversity, and activity of microbial communities. Chapter three presents the characterization of bacterial communities of a decentralized WWTP (the system under analysis) composed of a ST, an UAF, and a HFCW. Microbial characterization was carried out by high-throughput sequencing of 16S rRNA (V3-V4 region) to evaluate the spatial distribution of bacteria communities within a septic tank (ST), an up-flow anaerobic filter (UAF), and a horizontal flow constructed wetland (HFCW). Moreover, the effect of physicochemical parameters on the structure and diversity of bacterial communities was analyzed. Finally, chapter four describes future perspectives of this work and the importance of investigating the mechanisms to remove pathogenic microorganisms in the CW and the influence of iron in microbial communities' behavior during wastewater treatment.

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Chapter 1: Introduction

1.1 Motivation

Wastewater effluent is frequently discharged directly to water bodies without any treatment in developing countries [1]. Worldwide, more than 80% of the wastewater produced through human activities is released into the environment without treatment [2]. In 2017, according to the Mexican National Water Commission (Conagua by its Spanish acronym), 2,526 wastewater treatment plants (WWTP) were reported to be in operation throughout the country, which treated 135.6 m^3/s, and reached a national sanitation coverage of 63% [3]. Despite the efforts made by the states and municipalities to achieve a higher percentage of wastewater sanitation, most of the built conventional treatment plants end up out of operation and eventually abandoned due to the complexity of the systems and to the high maintenance and operation costs, allowing the untreated or partially treated to reach water bodies [4]. Therefore, the high investment made to build the existing centralized wastewater treatment plants has not substantially improved water quality [5].

Untreated wastewater contains a variety of pollutants that affect water quality and aquatic life as well as the public health of communities living in association with the water sources. The input of high loads of nutrients (nitrogen and phosphate), organic matter, and other contaminants into water bodies due to the municipal wastewater discharges and other non-point sources such as agricultural fields [6] frequently leads to a gradual decrease in the concentrations of dissolved oxygen which may cause the appearance of algal blooms [7,8], a process known as eutrophication. Besides, inadequate wastewater treatment leads to severe public health problems since direct exposure to wastewater can cause intestinal infection diseases such as cholera and diarrhea due to the pathogenic microorganisms in feces [9]. Mexico is among the top 20 countries regarding death by intestinal infectious diseases, and diarrhea is the fourth cause of infant mortality [10].

To achieve Sustainable Development Goals number six in Mexico, the development of decentralized systems with low initial investment and low operational and maintenance costs must be a priority to efficiently treat wastewater and prevent human diseases and protect the ecosystems' health. In the same way, it is essential to understand microorganisms' behavior in decentralized wastewater treatment since they are involved in pollutants removal. However, microbial communities are sensitive to environmental changes and design factors of treatment units that change the microbial composition and affect decentralized WWTP performance. Therefore, complete knowledge of microbial behavior within a decentralized WWTP may be possible to controlled and manipulated critical factors that may enhance treatment performance.

1.2 Problem Statement and context

Wastewater is defined as "water of varied composition from discharges of urban, domestic, industrial, commercial, agricultural, livestock, and in general, of any use" [1].In developing countries, it is common to discharge wastewater to water bodies such as lakes, rivers, and lagoons near human settlements, causing several environmental problems, such as eutrophication [11]. The discharge of wastewater exceeding the limits for water quality parameters dictated by the applicable regulation has significant implications for human and animal health [12].

Centralized systems are operated by public associations and are generally located far away from the generation site. In these systems, large volumes of wastewater are collected from large communities, thus require large pipes, major excavations, and manholes for access [13]. Furthermore, conventional centralized systems require high amounts of energy as well as higher maintenance and operation costs. Moreover, they are expensive to construct, frequently suffer deterioration due to low maintenance and require specialized personnel for operation [5,9,10,14]. Consequently, conventional wastewater treatment plants frequently start to reduce their operation capacity, suspend operation, and end-up being abandoned [13].

Decentralized wastewater systems collect, treat, reuse, and dispose of treated wastewater near the point of generation [15]. These systems generally have a simple design and are easy to operate and present low operation and maintenance costs. However, they require greater land areas but [14]. Decentralized systems commonly employ constructed wetlands (CW) since they combine physical, chemical, and biological processes to treat wastewater and at the same time present low costs and require low rates of energy consumption [16]. However, CW requires larger land areas and can suffer from clogging. CW have been recognized as an accepted low-cost technology beneficial to small communities and municipalities that cannot afford conventional treatment systems [17].

Microorganisms are essential for wastewater treatment since they are involved in nutrients and organic matter removal processes under aerobic and anaerobic conditions [18]. Microbial communities can be affected by several operational and design factors of CW, such as substrate media, hydraulic depth, and CW configuration [19], as well as environmental conditions [20]. However, there are few studies on the microbial communities within complete decentralized WWTP. More investigation is required to thoroughly understand the wastewater treatment process in decentralized systems and spatial variations of related microorganisms to improve wastewater treatment performance.

1.3 Research Question

The processes that occur within a decentralized wastewater treatment plant (WWTP) for eliminating organic matter and nutrients from wastewater are complex and involve multiple physical, chemical, and biological reactions. Additionally, the microorganisms involved in wastewater treatment are highly sensitive to physicochemical and environmental changes as well as wastewater composition and the design and operational features of each treatment stage of the WWTP. The performance and efficiency of these treatment systems can be affected as a result of variations of the structure and activity of microbial communities. It is important to understand how the microbial communities act within a decentralized WWTP to achieve an effective wastewater treatment. This knowledge may allow for the development of strategies to manipulate the microbial communities and their activity, thus improving the WWTP performance.

1.4 Solution overview

Molecular techniques and omics have allowed for a more extensive and systematic analysis of microbial communities' behavior and composition in complex ecosystems. Therefore, utilizing high-throughput sequencing of 16S rRNA (region V3-V4) may allow for a complete understanding of the variations in the structure and abundance of the bacterial communities involved in the nutrients and organic matter removal processes within a decentralized WWTP combining a septic tank (ST), an up-flow anaerobic filter (UAF) and a horizontal flow constructed wetland (HFCW).

Additionally, the knowledge of the spatial variations of these microorganisms as a response to physicochemical parameters is important for developing strategies to manipulate or control the crucial factors that enhance microbial activity and improve the WWTP performance.

1.5 Main contributions (of this work to state of the art)

Studies related to the characterization of microbial communities involved in wastewater treatment have been mostly reported for single treatment stages. Therefore, in this work, a focus was made on how the microbial communities change throughout a complete decentralized treatment system combining three treatment stages and their spatial variations within each unit. At the same time, we evaluated the effect of physicochemical parameters (pH, temperature, dissolved oxygen, and electrical conductivity) on microbial communities' structure, which is useful knowledge to develop strategies to enhance microbial removal processes and improve the performance of WWTP. On the other hand, to the best of our knowledge, this is one of the very few studies on the microbial communities of decentralized treatment plants located in tropical countries (such as Mexico), in which the climatic conditions play an essential role in the performance of these treatment systems.

1.6 Thesis organization

Chapter one includes the motivation, problem statement, context, solution overview, and main contributions of this work.

Chapter two presents CW's theoretical framework, including the classification of CW and the theory on the main removal processes such as nitrification, denitrification, anammox, comammox, organic matter degradation, and phosphate removal. Furthermore, this chapter discusses the effects of environmental, operational, and design factors on the structure, diversity, and activity of microbial communities, as useful information to improve CW performance. Factors such as temperature, pH, CW depth, substrate, availability of organic carbon, and plants' presence influence the CW's microenvironments, thus affecting the microbial communities' favorite different microbial metabolic pathways. The effect of these factors on microbial communities is explained in detail in this chapter. Additionally, the molecular techniques used to identify the microbial communities within the CW are presented.

Chapter three presents the characterization of bacterial communities by high-throughput sequencing of the V3-V4 region of the 16S rRNA gene from a decentralized WWTP conformed by a septic tank (ST), an up flow anaerobic filter (UAF) and horizontal flow constructed wetland (HFCW).

Finally, future perspectives and conclusions are presented in chapter four. The analytical techniques used to determine water quality are shown in detail in appendix A, and appendix B shows the rarefaction curve obtained from high-throughput sequencing and more information about statistic analysis.

Chapter 2: Structure, diversity, and activity of microbial communities in response to environmental, operation, and design factors in constructed wetlands

Submitted as a review titled **"Structure, diversity, and activity of microbial communities in response to environmental, operation, and design factors in constructed wetlands"** in the International Journal of Environmental Science and Technology: **https://doi.org/10.1007/s13762-021-03719-y**

Constructed wetlands are nature-like engineering systems implemented to treat wastewater through physical, chemical, and biological processes under controlled environments. In constructed wetlands, pollutant removal is primarily accomplished by microbial processes such as nitrification, denitrification, anammox, comammox, organic matter degradation, and phosphate removal. This review discusses the effects of environmental, operational, and design factors on the structure, diversity, and activity of microbial communities, providing useful information to improve constructed wetlands' performance. Factors such as temperature, pH, depth, substrate, availability of organic carbon, and presence of plants affect microenvironments, and thus the microbial communities within a constructed wetland, promoting different microbial metabolic pathways. Molecular techniques and omics technologies have allowed for a global understanding of microbial communities and their behavior in constructed wetlands. A greater understanding of the critical factors that can be manipulated or controlled to shift the dominance of different microbial groups and thereby enhance microbial activity and improve constructed wetlands' performance is still needed. Moreover, precise experiments manipulating critical factors and assessing microbial behavior as well as the performance of constructed wetlands may prove useful in developing strategies to optimize constructed wetlands' efficiency. Furthermore, future research should focus on the development of mathematical models able to predict the structure, diversity, and activity of microbial communities as a response of environmental, operational, and design factors in constructed wetlands. Indeed, these could become useful tools to optimize the functioning of constructed wetlands.

2.1. Introduction

The growth of the human population has led to an increase in municipal, industrial, and agricultural wastewater generation due to human activities [21]. Wastewater is the byproduct of diverse uses of water that occur within human communities, and it contains a variety of constituents that need to be treated before the water is re-used or released into the environment [22]. The constituents of wastewater can be classified in terms of physical, chemical, and biological components, with organic matter, nutrients (such as phosphate, P, and nitrogen, N), and suspended solids among the most common components of wastewater [23]. Moreover, in Mexico and other developing countries, it has become a challenge for municipalities to treat growing volumes of wastewater in order to avoid the contamination of water bodies and prevent public health risks [24,25].

Constructed wetlands (CW) are efficient nature-like technologies for wastewater treatment [26] that combine vegetation (emergent, floating, or submerged), substrates (rocks, soils, synthetic materials, among others), and microbial communities under controlled environments [27]. CW have been applied to treat wastewater from varied sources, such as landfill leachate, agricultural wastewater, municipal wastewater, domestic sewage, industrial wastewater, saline effluents, mine drainage, urban runoff, and water from polluted water bodies [28–41]. The main advantages of CW, compared with conventional wastewater treatment technologies such as activated sludge systems, are lower investment and operational costs as well as more simple operation and maintenance [13,42,43]. CW systems are used to remove suspended solids and reduce biological

and chemical oxygen demand, pathogens, and nutrients (N and phosphate) by various chemical, physical, and biological processes [44,45].

Microorganisms are essential for pollutant removal in CW because they are involved in primary transformation and degradation processes such as N, P, and sulfur removal and organic matter degradation [46,47]. Some of these communities are attached to the substrate's surface (filter media) and plant roots, forming a biofilm where the transformation and degradation of pollutants occur [48,49], while other microbial communities are found dispersed in water, forming flocs in some cases [50–52]. Microbial-mediated processes mainly depend on hydraulic conditions, wastewater properties, the quality and availability of nutrients, the filter (substrate) material, and the plant species in question [53]. Additionally, microbial communities are highly sensitive to environmental conditions [49].

Existing reviews of microbial diversity within CW have focused on the effect of environmental parameters on microbial communities [54] and their influence on the degradation capacity of the system [55]. In recent years, the expanded use of advanced molecular techniques such as qPCR and new generation sequencing has increased knowledge of CW's microbial ecology. This review further discusses the effects of environmental, operational, and design factors on the structure, diversity, and activity of microbial communities found in CW using different advanced molecular techniques. Special attention is given to the effect of these factors on different pollutant (N, phosphate, and organic matter) removal pathways and performance within each type of CW. More exhaustive knowledge regarding how the structure and the diversity of microbial communities are shaped in CW is useful for making future improvements to the operation and design of CW. In the first section of this study, the main features of different CW configurations are presented. Next, the microbial metabolic pathways for pollutant removal are discussed, with a focus on the differences that exist between different CW configurations, and on the related microorganisms that have been identified by molecular techniques in these systems. Finally, the effects of environmental, operational and design factors on the microbial communities within CW are discussed, and recommendations are provided in order to improve the performance and the stability of these systems.

2.2. Constructed wetlands

The combined effect of physical, chemical, and biological processes in CW allows the treatment of different types of wastewater effluents [56–58]. In CW, organic matter is mostly removed by both anaerobic and aerobic bacteria attached to plant roots and filter media. By contrast, N removal is mostly accomplished by ammonification, volatilization, plant uptake, microbial denitrification, nitrification, anammox, and comammox [59–61]. However, CW are more effective in removing nutrients (N and phosphate) than organic matter (carbohydrates, proteins, fatty acids) [62,63]. Commonly, CW have been used in combination with other technologies, such as aerobic or anaerobic bioreactors, which are often used in a previous treatment stage of CW to maximize their individual performance, such as to increase organic matter reduction [35,64].

CW may be classified according to the type of macrophytes used in the system, which may be free-floating, rooted emergent, or submerged [65]. In addition, CW may be classified according to the hydrology and the flow regimen of the wetland, e.g., free water surface (SF) or subsurface systems (SSF) [66]. Subsurface systems are further classified, considering their flow direction, into vertical flow (VF), horizontal flow (HF), or hybrid (Hy), which may integrate surface flow constructed wetlands (SFCW), vertical subsurface flow constructed wetlands (VFCW) and horizontal subsurface flow constructed wetlands (HFCW) in different arrangements [55,67]. Each CW type is described with detail in the following section.

2.1.1. Surface flow constructed wetlands

Surface flow or free-water surface flow constructed wetlands (SFCW; Fig. 1a) consist of an exposed water system containing floating or emergent rooted vegetation with a high diversity of microorganisms [68]. Exposed wastewater may contain pathogens that can be a health risk to humans and wildlife and a suitable environment for mosquito reproduction [23]. Despite these disadvantages, SFCW have been successfully applied to treat agricultural wastewater and rural wastewater and flooding control by retaining stormwater [69–71].

2.1.2. Subsurface flow constructed wetland

Subsurface flow constructed wetlands (SSFCW) are characterized by water passing through a granular matrix, referred to as the substrate, and a varying depth depending on the plant root characteristics [72]. Wastewater is in contact with the rhizomes and the granular matrix, providing a surface for microbes. SSFCW are further classified based on their hydraulic configuration: vertical flow or horizontal flow [73]. In SSFCW, a higher organic removal performance is attributed to a large surface area in contact with wastewater [74]. Biological and physicochemical routes accomplish the removal of nitrogen compounds (organic and inorganic) from wastewater. Biological and physicochemical routes accomplish the removal of N compounds (organic and inorganic) from wastewater. Biological routes include ammonification, denitrification, nitrification, plant uptake, anammox, and biomass assimilation, while physicochemical roles include ammonia volatilization and adsorption [74].

2.1.2.1. Horizontal flow constructed wetland

Horizontal subsurface flow constructed wetlands (HFCW; Fig. 1b) consist of a bed filled with granular materials with different levels of porosity (such as rocks, sediments, soils and synthetic materials), planted with macrophytes [75]. Plants are among the most critical components in all types of CW. The macrophytes most commonly employed in HFCW are *Phragmites*, *Typha*, *Scirpus*, and *Iris* [74]. However, in many countries, local and ornamental plants are employed in these systems, such as *Zantedeschia aethiopica*, *Strelitzia reginae*, and *Agapanthus africanus* [13]. The wastewater flows horizontally by gravity through a granular medium and has direct contact with the substrate (and the biofilm), the roots, and the rhizomes [23]. The materials that constitute HFCW create a suitable environment for microorganisms to grow and remove pollutants [76]. In this type of system, organic matter is degraded by aerobic and anaerobic microbial processes; however, anoxic/anaerobic processes prevail due to the continuous saturation of the filtration bed [64]. As a consequence, denitrification rates in HFCW are high, whereas nitrification is limited [64,77]. Clogging is the major operational problem of HFCW and is caused by the retention of inorganic and organic particles, biofilm formation, and plant root growth [78].

2.1.2.2. Vertical flow constructed wetland

A vertical subsurface flow constructed wetlands (VFCW; Fig. 1c) system consists of a bed planted with macrophytes, filled with graded gravel and topped with sand (a larger size fraction in the bottom and smaller in the top layer [65,79]. Like HFCW, VFCW are usually planted with reeds such as *Phragmites australis* [79,80] and *Typha angustifolia* [81]. An upward or downward vertical flow characterizes VFCW. Downflow is the most common operational mode, in which wastewater is introduced in large volumes onto the surface of the system to flood the surface layers, creating saturated conditions, and the air that is trapped is forced to move downward. The water is then drained vertically by gravity through the porous medium, while the air in the atmosphere enters the system, replacing the drained water volume. Intermittent flooding and draining improve oxygen transfer from the atmosphere, enhancing aerobic conditions [30,82]. In contrast to in HFCW, in VFCW successful ammonia removal occurs, but very limited denitrification is

accomplished [82]. Significant advantages of VFCW are high hydraulic loading rates, greater oxygen transport capacities, and low land area requirements [81,83]. VFCW systems are suitable for chemical oxygen demand removal and nitrification [84,85]. VFCW have been used to treat high-strength wastewater municipal wastewater [79] as well as for other specific purposes, such as sludge dewatering [86]. Table 1 mentions the advantages and disadvantages of each CW type.

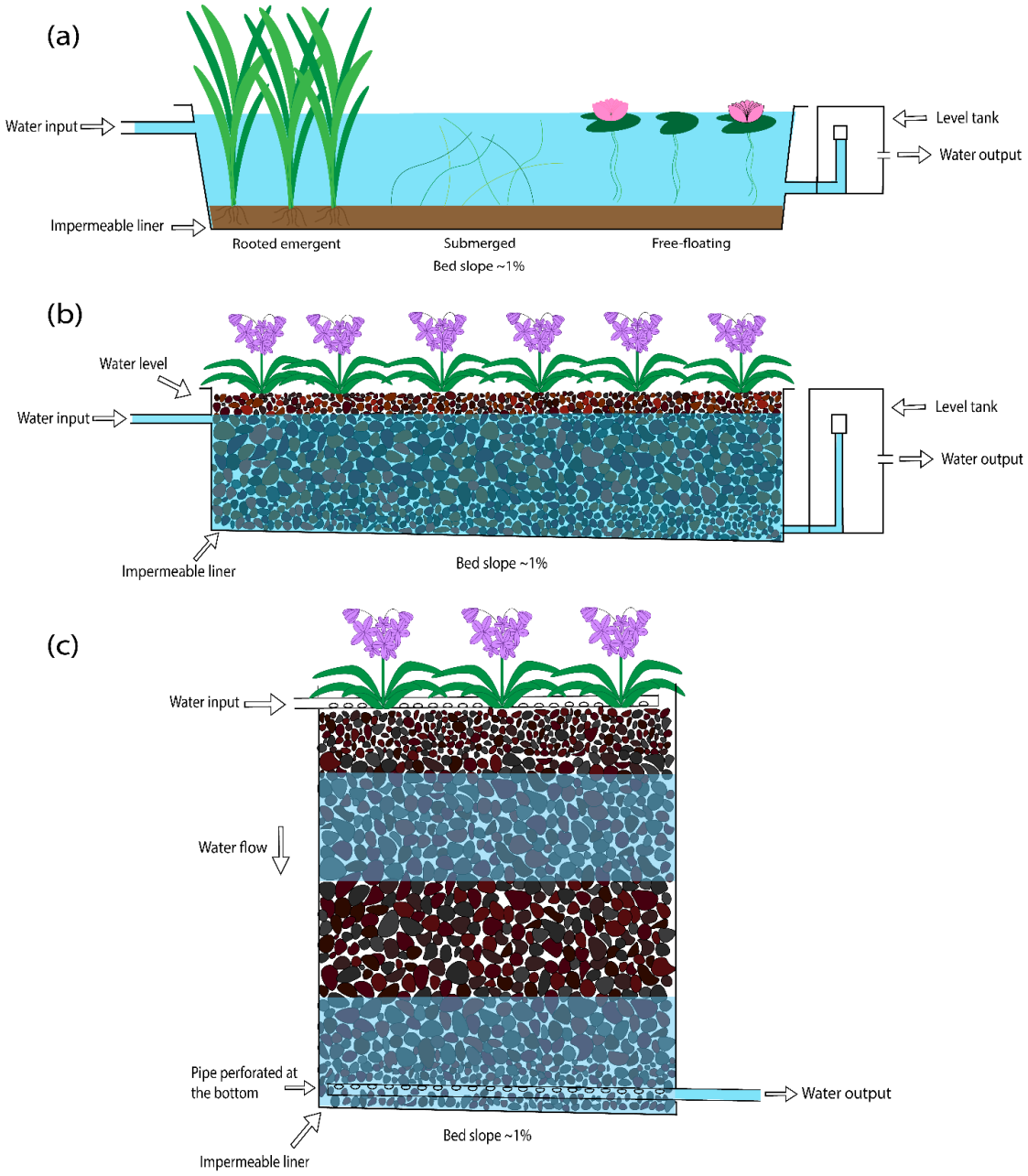

Fig. 1 Constructed wetland configurations: **A**: Surface flow constructed wetland with three types of plants. **B:** Planted horizontal subsurface flow constructed wetland with *Agapanthus africanus.* **C:** Planted vertical subsurface flow constructed wetland.

2.1.3. Hybrid constructed wetland

Hybrid constructed wetlands (HyCW) combine different types of CW (SFCW, HFCW, and VFCW) in series, providing both aerobic and anaerobic conditions to achieve higher wastewater treatment efficiencies. HyCW take advantage of single CW characteristics to improve wastewater treatment, particularly for the removal of nitrogenous compounds [55,87]. HyCW systems that combine VFCW and HFCW are the most common arrangement [88–92] used for municipal wastewater treatment [93,94]. These systems generally consist of several parallel VFCW, followed by various HFCW in series. Higher removal rates of organics (chemical and biological oxygen demand), suspended solids, and total N have been reported in these systems compared to single systems. However, the removal of phosphorus is low in these systems [55,82]. In the first stage (the VFCW), organic matter and suspended solids are removed mainly by filtration, and aerobic conditions are provided for nitrification, while anoxic/anaerobic denitrification conditions are provided in the second stage, as long as sufficient organic matter is available [55,88,90]. In another typical arrangement, the HFCW-VFCW system, nitrification occurs at the end of the process (in the VFCW), and thus the recirculation of the effluent to the HFCW becomes necessary if nitrate removal (by denitrification) is desired. This configuration also allows the use of raw feed as a source of carbon [94].

Table 2.1. Advantages and disadvantages of CW

CW type			References
Surface Flow Constructed Wetland (SFCW)	Advantage	Low construction cost.	[70]
		Avoids clogging at the input and output.	
		Can tolerate an increase in flow due to stormwater without the risk of drowning the plants.	[32]
	Disadvantage	Suitable environment for mosquito reproduction.	[23]
		Human health risk due to pathogens in exposed wastewater.	
		Unpleasant odor.	[95]
Subsurface Flow Constructed Wetland (SSFCW)	Advantage	Odorless.	[95]
		Low energy consumption.	
		Not suitable for mosquitoes and other insect vector reproduction.	[73]
		Minimal risk of public exposure and contact with wastewater.	
		Media provides a greater contact surface for higher treatment performance.	
		Thermal protection for microorganisms.	[74]
	Disadvantage	Limited access for maintenance.	[96]
		Clogging if the elimination of suspended solids in pretreatment is not efficient.	
Horizontal Flow Constructed Wetland (HFCW)	Advantage	May accomplish the removal of emergent, pharmaceutic components	[23]

		of wastewater as a secondary or tertiary treatment.	
		Suitable anoxic-anaerobic conditions for denitrification.	[97]
	Disadvantage	Requires more construction area and design experience.	[98] [99]
		Limitation of oxygen transfer.	
Vertical Flow Constructed Wetland (VFCW)	Advantage	High reduction of suspended solids, pathogens, and biological oxygen demand.	[98]
		Less area required.	
		High oxygen transfer capacity.	[70]
	Disadvantage	Requires design experience.	[100]
		More maintenance is required in comparison with HFCW.	
	Advantage	Higher pollutant removal efficiencies (organic matter and suspended solids).	[55,88,101]
		Enhancement of total nitrogen removal due to nitrification and denitrification conditions (aerobic and anaerobic).	[90]
Hybrid Constructed Wetland (HyCW)	Disadvantage	In the VFCW-HFCW arrangement, denitrification can be affected by organic matter availability in the last unit	[90]
		In the HFCW-VFCW arrangement, it sometimes becomes necessary to recirculate the effluent to remove nitrates.	[94]

2.3. Microbial nutrient removal in constructed wetlands

Hybrid constructed wetlands (HyCW) combine different types of CW (SFCW, HFCW, and VFCW) in series, providing both aerobic and anaerobic conditions to achieve higher wastewater treatment efficiencies. HyCW take advantage of single CW characteristics to improve wastewater treatment, particularly for the removal of nitrogenous compounds [55,87]. HyCW systems that combine VFCW and HFCW are the most common arrangement [88–92] used for municipal wastewater treatment [93,94]. These systems generally consist of several parallel VFCW, followed by various HFCW in series. Higher removal rates of organics (chemical and biological oxygen demand), suspended solids, and total N have been reported in these systems compared to single systems. However, the removal of phosphorus is low in these systems [55,82]. In the first stage (the VFCW), organic matter and suspended solids are removed mainly by filtration, and aerobic conditions are provided for nitrification, while anoxic/anaerobic denitrification conditions are provided in the second stage, as long as sufficient organic matter is available [55,88,90]. In another typical arrangement, the HFCW-VFCW system, nitrification occurs at the end of the

process (in the VFCW), and thus the recirculation of the effluent to the HFCW becomes necessary if nitrate removal (by denitrification) is desired. This configuration also allows the use of raw feed as a source of carbon [94].

2.3.1. Nitrogen removal in CW

Microorganisms make nitrogen removal more efficient [102] by catalyzing the conversion of organic and inorganic compounds [103,104]. Bacteria participates in nitrification, denitrification, ammonification, and anaerobic ammonia oxidation (ANNAMOX), while plants remove nitrogen mainly by assimilation [105,106]. Nitrogen cycle reactions are carried out inside the CW by the interaction between microorganisms and plants, taking advantage of each other's actions to achieve higher removal efficiencies [71].

2.3.1.1. Nitrification

Hybrid constructed wetlands (HyCW) combine different types of CW (SFCW, HFCW, and VFCW) in series, providing both aerobic and anaerobic conditions to achieve higher wastewater treatment efficiencies. HyCW take advantage of single CW characteristics to improve wastewater treatment, particularly for the removal of nitrogenous compounds [55,87]. HyCW systems that combine VFCW and HFCW are the most common arrangement [88–92] used for municipal wastewater treatment [93,94]. These systems generally consist of several parallel VFCW, followed by various HFCW in series. Higher removal rates of organics (chemical and biological oxygen demand), suspended solids, and total N have been reported in these systems compared to single systems. However, the removal of phosphorus is low in these systems [55,82]. In the first stage (the VFCW), organic matter and suspended solids are removed mainly by filtration, and aerobic conditions are provided for nitrification, while anoxic/anaerobic denitrification conditions are provided in the second stage, as long as sufficient organic matter is available [55,88,90]. In another typical arrangement, the HFCW-VFCW system, nitrification occurs at the end of the process (in the VFCW), and thus the recirculation of the effluent to the HFCW becomes necessary if nitrate removal (by denitrification) is desired. This configuration also allows the use of raw feed as a source of carbon [94].

$$NH_4^+ + 1.5\ O_2 \rightarrow 2H^+ + H_2O + NO_2^- \quad (1)$$

$$NO_2^- + 0.5\ O_2 \rightarrow NO_3^- \quad (2)$$

Table 2. Nitrifying microorganisms found in CW identified by molecular techniques

Microorganism	Type of constructed wetland	Pathway	Analysis	Ref.
Xanthomonadaceae Rhodocyclaceae Chitinophagaceae Xanthomonadaceae	HFCW	Nitrification	454 pyrosequencing of the 16S rRNA gene.	[107]
Nitrospira spp. *Nitrosospira* spp. *Nitrosomonas* spp.	VFCW	Nitrification	16S rRNA and *amoA* gene sequences	[108]
Nitrosomonas eutropha *Nitrosococcus mobilis*	HFCW	Nitrification	PCR-DGGE 16S rRNA gene	[109]

Nitrosomonas marina	

580 Genes: *amoA* (ammonia monooxygenase)

581 **2.3.1.2. Denitrification**

582 Denitrification is a reduction reaction with four steps performed by heterotrophic bacteria [110].
583 First, NO_3^- is reduced to NO_2^- by the nitrate reductase enzyme, then NO_2^- is reduced to nitric oxide
584 (NO) by the nitrite reductase enzyme, and then NO is reduced by nitric oxide reductase to nitrous
585 oxide (N_2O) to be reduced by the nitrous oxide reductase enzyme to gaseous N_2 [71] following
586 Eq. 3 [111]. The final electron acceptor is nitrate (NO_3^-), and the donor is organic carbon, where
587 organic carbon can act as a process controller, meaning that its absence inhibits the process
588 [106]. Denitrification is an anaerobic process affected by temperature, pH, oxygen concentration,
589 organic carbon sources, and NO_3^- concentration, among other factors [112].

590 $$0.83CH_3OH + NO_3^- \rightarrow 0.5N_2 + 0.83CO_2 + 1.17H_2O + OH^-$$
591 (3)

592 *Denitratisoma*, *Planctomyces*, *Magnetospira*, *Pseudomonas* spp. and *Dechloromonas* are some
593 of the microorganisms that can carry out the denitrification process in CW [111,113]. Table 3
594 summarizes the denitrifying microorganisms reported in the literature, and the different molecular
595 techniques used to identify them.
596
597 **Table 3.** Denitrifying microorganisms found in CW identified by molecular techniques

Microorganism	Type of constructed wetland	Pathway	Analysis	Ref.
Hyphomicrobiaceae Bradyrhizobiaceae Rhodospirillaceae	HFCW	Denitrification	16S rRNA gene sequencing (V4 region)	[114]
Pseudomonas *Comamonas* *Acinetobacter*	VFCW	Denitrification	qPCR with *amoA*, *nxrA*, *nirS* and 16S rRNA genes	[115]
Pseudomonas *Exiguobacterium* *Thiobacillus*	VFCW	Denitrification	454 pyrosequencing of the 16S rRNA gene	[116]
Thaurea *Dechloromonas* *Candidatus* *Competibacter* *Denitratisoma*	VFCW	Denitrification	High-throughput sequencing of *16S rRNA* gene (*V4* region)	[113]
Rhizobacter Comamonadaceae *Rhizobium*	VFCW	Denitrification	High-throughput sequencing of *16S rRNA* (*V3-V4* region)	[117]
Dechloromonas *Ideonella* *Sulfuritalea* *Cupriavidus*	SFCW	Denitrification	High-throughput sequencing (*nirS* gene)	[118]

Thaurea *Dechloromonas* *Candidatus* *Competibacter* *Denitratisoma*	VFCW	Denitrification	16S rRNA gene sequencing (V4 region)	[113]
Rhizobacter Comamonadaceae *Rhizobium*	VFCW	Denitrification	16S rRNA gene sequencing (V3-V4 region)	[117]
Proteobacteria Actinobacteria Bacteroidetes Cyanobacteria	SSFCW	Denitrification	454 pyrosequencing of the 16S rRNA gene (V3 region)	[119]

Genes: *amoA* (ammonia monooxygenase), *nxrA* (nitrite oxidoreductase), *nirS* (nitrite reductase)

2.3.1.2. Anammox

Anammox is an autotrophic (removal of inorganic N) process carried out by anaerobic ammonium oxidation (anammox) bacteria that oxidize ammonium to gaseous N_2. It is necessary to restrict the NOB and retain the anammox bacteria in symbiosis with AOB for the anammox process to take place [120]. In this process, AOB convert the ammonium present in wastewater to nitrite (Eq. 5), and the anammox bacteria can convert the rest of ammonium and nitrite into N_2 (Eq. 6) [121,122]. NOB should be inhibited because they compete with AOB for dissolved oxygen, ammonium, and nitrite [123]. When organic carbon is available for denitrifying bacteria, the nitrite produced by AOB can be metabolized through denitrification instead of anammox. It has been reported that anammox bacteria outcompete denitrifying bacteria for nitrite at higher chemical oxygen demand concentrations [123]. The anammox process can occur at different temperatures [124–126] and N concentrations [127]. The optimal temperature reported for anammox is between 12°C to 15°C [128], and an optimal pH is between 7.5 to 8.0 [123]. The main challenge related to the anammox process is achieving a high rate process, biomass retention, and a water exit with low N concentrations [127]. Table 4 summarizes the microorganisms that have been reported to be related to the anammox pathway, and the f molecular techniques used to identify them.

$$NH_4^+ + 1.5O_2 \rightarrow NO_2^- + H_2O + 2H^+ \quad (5)$$

$$NH_4^+ + NO_2^- \rightarrow N_2 + 2H_2O \quad (6)$$

Table 4. Anammox microorganisms found in CW identified by molecular techniques

Microorganism	Type of constructed wetland	Pathway	Analysis	Ref.
Candidatus Brocadia fulgida	-	Anammox	Fluorescence *in situ* hybridization (FISH)	[120]
Candidatus Brocadia *Candidatus Kuenenia* *Candidatus Jettenia* *Candidatus Anammoxoglobus*	CW	Anammox	16S rRNA gene sequencing	[129]

Candidatus Scalindua wagner *Candidatus Scalindua marina* *Candidatus Scalindua brodae* *Candidatus Kuenenia stuttgartiensis*	CW	Anammox	16S rRNA gene sequencing	[130]
Candidatus Brocadia caroliniensis *Candidatus Brocadia anammoxidans* *Candidatus Jettenia asiatica* *Candidatus Kuenenia stuttgartiensis* Planctomycetes	SFCW	Anammox	qPCR of 16S rRNA gene	[131]
Proteobacteria Chloroflexi Firmicutes Planctomycetes	HFCW	Anammox	16S rRNA gene sequencing (V4-V5 region)	[132]

2.3.1.3. Comammox

Complete ammonia oxidation (comammox) is a recently demonstrated process through which ammonia is completely oxidated to nitrate in only one step, in contrast to the well-documented nitrification, during which ammonia is oxidized to nitrate in two steps, mediated by two distinct groups of chemolithoautotrophs: AOB and NOB [133,134]. Van Kessel et al. [135] have proven that two chemolithoautotrophic *Nitrospira* species are capable of entirely oxidizing ammonia via nitrite to nitrate (comammox), encoding all the necessary ammonia monooxygenase enzymes. This alternate metabolic pathway has been suggested to save costs on the aeration-energy necessary to carry out nitrification, and to remove the need for external carbon sources for denitrification [134]. Additionally, Pelissari et al. [59] have reported that through the comammox process, *Nitrospira* is unable to produce N_2O, which is an important greenhouse gas. Furthermore, *Nitrosospira* has been reported to perform complete nitrification under low NO_2^- and oxygen concentrations [136], and has been found in environmental samples from natural and engineered ecosystems, agricultural soil, forest soil, and wastewater treatment plants [137]. Table 5 summarizes the microorganisms that have been reported to be related to the comammox pathway and the molecular techniques used to identify them, while Table 6 summarizes the microbiological process related to N removal in each CW type.

Table 5. Microorganisms related to comammox process found through molecular techniques

Microorganism	Type of constructed wetland	Pathway	Analysis	Ref.

Nitrospira	VFCW	Comammox	High-throughput sequencing (*amoA* gene)	[59]
Nitrospira	CW	Comammox	High-throughput sequencing (*amoA* gene)	[137]
N. inopinata *N. nitrosa* *N. nitrificans*	-	Comammox	Sequencing using ABI 3730 x 1 DNA Sequencer (*amoA* gene)	[138]
Thiobacillus *Nitrospira* *Rhodocyclaceae* *Xanthomodales*	HyCW	Related to different pathways of the nitrogen cycle	16S rRNA gene sequencing (V3-V4 region)	[139]

Genes: *amoA* (ammonia monooxygenase)

Table 6. N removal mechanisms in CW types and their associated microorganisms

Removal process	Removal mechanism	Removal depending on CW type	Associated microorganisms (relative abundance)	Ref.
Nitrification	Oxidation of ammonium (NH_4) to nitrate (NO_3) with nitrite (NO_2) as intermediate under aerobic conditions. Occurs in plant rhizosphere by nitrifying bacteria, and is the major pathway for ammonium removal.	In HFCW, the nitrification process is low due to low abundance of nitrifying bacteria in the system, because anaerobic conditions predominate.	*Arthrobacter* (1.57%), *Nitrosomonas (0.03%), Nitrospira* (0.30%)	[74,107,140,141]
		In VFCW, nitrification is the main N removal pathway (73%) due to intermittent feeding, allowing the entrance of air into the system (high oxygenation of porous media).	*Nitrobacter, Nitrospira, Nitrococcus*	[142]
		In SFCW, nitrification occurs in plant roots and sediments.	*Nitrosospira* (20-80%) and *Nitrosomonas (5-70%)*	[143]
Denitrification	Process whereby nitrate (NO_3) is converted into nitrogen (N_2) with nitrite (NO_2), nitric oxide (NO), and nitrous oxide (N_2O) as intermediates under anoxic/anaerobic conditions.	Denitrification rates in HFCW are high due to continuous saturation of the filtration bed with wastewater, creating anoxic/anaerobic conditions.	*Xanthomonadaceae* and *Comamonadacear* (2%)	[64,140,77,107]
		In VFCW, systems remove about 20 to 30 % of total N by denitrification.	*Comamonas, Pseudomonas, Acinetobactes, Bacillus,* Firmicutes, *Exiguobacterium* and *Thiobacillus* (17-46%)	[84,116,144–146]
		Limited denitrification due to aerobic conditions		
		Denitrification in SFCW occurs away from the area of the roots.	*Dechloromonas* (16,2%), Betaproteobacteria (9.7%), *Rohodocyclaceae* (2.1%), and *Rhodanobacter* (1.5%).	[147,148]
Anammox	Transform ammonia to N_2 gas using nitrite (NO_2) to oxidize ammonia under neutral pH and anaerobic conditions.	In HFCW, anammox bacteria are present in the deeper layer of the bed (anoxic/anaerobic environment) 0.5 to 0.6 m.	*Gemmata* (0.5-0.7%), *Planctomyces* (1.1-1.3%), *Pirellula* (0.6-0.8%), and *Isosphaeraceae* (0.4%)	[149]
		In VFCW, anammox bacteria are in the deepest layers of the CW.	Planctomycetes, *Ca. Brocardia, Ca. Kuenenia, Ca. Scalindua, Ca. Anammoxoglobus* and *Ca. Jettenia*	[61,132,150]

		In SFCW, low redox potential and dissolved oxygen provide suitable conditions for anammox. Anammox bacteria are in the inner layer of biofilms.	*Ca. Brocadia, Ca. Jettenia, Ca. Kuenenia* and Planctomycetes	[123,131]
Comammox	Ammonia is completely oxidated to nitrate (NO_3) in one step.	In estuarine tidal flat wetland sediments with a pH of 6.74 to 8.65.	*Nitrospira*	[135,137,138]

2.2.2. Phosphorus removal in CW

P removal within CW is variable and dependent on the CW bed's composition, configuration, and design [151]. P removal in CW is accomplished by different processes, including adsorption by porous media, filtration, sedimentation, precipitation, plant uptake, and bacterial removal [152]. P is present in wastewater in organic and inorganic forms; however, it is most common in the form of free orthophosphates (PO_4^{-3}), which exist in ionic equilibrium [79].

Whereas microbial degradation and plant uptake are related to the removal of phosphates (PO_4^{-3}), adsorption and precipitation processes remove all P forms within the wastewater. In the cases of precipitation and adsorption, P is removed by interaction with minerals such as ferric oxyhydroxide, carbonate, and aluminum in the substrate media [152–154]. Natural and synthetic materials such as zeolite, kaolinite, and red mud have recently been used in CW for P removal [155]. However, natural materials often show smaller adsorption capacities than synthetic materials [156].

Microbial degradation of P is a partly reversible process [73], while microorganisms can assimilate and store P for growth, and only a small portion of the assimilated P is permanently removed [79,154]. Consequently, net microbial P removal is generally very low.

Biological removal of phosphate is accomplished through anaerobic and aerobic pathways. In the former, carbon sources (such as volatile fatty acids) must be available to induce phosphate-removing bacteria to take up acids and release phosphate into the solution. In the latter, phosphate uptake occurs, resulting in overall P removal of 80%-90% [157].

Edwards et al. [158] reported that in an HFCW, P was immobilized in the microbial biomass, reaching a proportion of approximately 25%. Furthermore, these authors reported changes in the solubility of orthophosphate, likely related to different environmental conditions. P is likely to be more soluble under anaerobic conditions in comparison to aerobic microenvironments [158].

2.4. Organic matter degradation in CW

Particulate organic matter is mainly removed by physical mechanisms (filtration and sedimentation), while microbiological degradation processes can remove dissolved organic matter under aerobic and anaerobic conditions [159].

2.4.1. Aerobic degradation

Aerobic degradation of organic compounds is performed by chemoheterotrophic and chemoautotrophic microorganisms [79,160]. The chemoheterotrophic microorganisms metabolize faster the organic compounds and contributes with significant biological oxygen demand reduction [79]. Furthermore, autotrophic bacteria can degrade organic compounds that contain nitrogen [79]. These bacteria utilize oxygen as a final electron acceptor to oxidize organic compounds and produce carbon dioxide (CO_2), as presented by Eq. 7 [160]. The aerobic bacteria in charge of this process display faster metabolic rates than anaerobic bacteria [73]. However, bacterial metabolisms depend on the availability of dissolved organic matter and dissolved oxygen in their immediate environment. The biodegradability of organic matter is an important parameter, which is usually assessed by the biological oxygen demand/chemical oxygen demand ratio, of which values of 0.5 or higher are indicative of readily biodegradable organic matter [161].

$$C_6H_{12}O_6 + 6O_2 \rightarrow 6CO_2 + 6H_2O \quad (7)$$

2.4.2. Anaerobic degradation

Anaerobic degradation of organic compounds in CW is mainly performed by heterotrophic bacteria [74] and can be performed through sulfate reduction, denitrification, and methanogenesis [162]. Table 7 presents the microorganisms related to the degradation of organic matter within CW that have been identified by molecular techniques. Under low oxygen concentrations, nitrate (NO_3^-) becomes the first electron acceptor to decrease [163]. Stepwise anaerobic degradation of organic matter is carried out in the absence of oxygen by acid- or methane-forming bacteria through the biochemical reactions of hydrolysis, acidogenesis, acetogenesis, and methanogenesis [19,164]. First, high molecular weight molecules are hydrolyzed into monomers, e.g., amino acids, glucose ($C_6H_{12}O_6$), and fatty acids. As an example, Eq. 8 shows a hydrolysis reaction where a polysaccharide is broken down into glucose. Second, acidogenic bacteria transform monomers into alcohols, H_2, ammonia, CO_2, and organic acids. Eqs. 9, 10, and 11 show examples of acidogenic reactions where glucose is converted to ethanol (C_2H_5OH), propionate (CH_3CH_2COOH), and acetic acid (CH_3COOH) [165]. Third, acetogenic bacteria convert organic acids and produce hydrogen, acetic acid, and carbon dioxide. Eq. 12 shows the conversion of propionate to acetate. Fourth, methanogenic bacteria consume acetic acid and produce methane CO2 and H_2, as shown by Eq. 13, or by reducing carbon dioxide with hydrogen, as shown by Eq. 14 [19,165].

$$C_{24}H_{40}O_{20}: H_2O + 3H_2O \rightarrow 4C_6H_{12}O_6 \quad (9)$$

$$C_6H_{12}O_6 \rightarrow 2CH_3CH_2OH + 2\ CO_2 \quad (10)$$

$$C_6H_{12}O_6 + 2H_2 \rightarrow 2CH_3CH_2COOH + 2\ H_2O \quad (11)$$

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 2CO_2 + 4H_2 \quad (12)$$

$$CH_3CH_2OH + 2H_2O \rightarrow CH_3COO^- + H^+ + 2H_2 \quad (13)$$

$$CH_3COOH \rightarrow CH_4 + CO_2 \quad (14)$$

$$CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O \quad (15)$$

Table 8 summarizes anaerobic and aerobic organic matter degradation as well as phosphate removal and the associated microorganisms.

Table 7. Microorganisms related to organic degradation process found through molecular techniques.

Microorganism	Type of constructed wetland	Process	Analysis	Ref.
Proteobacteria Actinobacteria Acidobacteria Firmicutes	VFCW	Anaerobic degradation of organic compounds	Anammox 16S rRNA gene sequencing	[166]
Gammaproteobacteria Betaproteobacteria Deltaprotebacteria *Anaerolineae*	SSFCW	Degradation of organic matter	16S rDNA gene sequencing	[167]

Deltaproteobacteria Gammaproteobacteria *Rhodospirillaceae* *Pseudomonas*	CW	Degradation of organic matter	16S rRNA gene sequencing (V3-V4 region)	[168]

Genes: *nirS* (nitrite reductase), *nirK* (nitrite reductase), *nosZ* (nitrous oxide reductase)

77 **Table 8.** Phosphate and organic matter removal mechanisms in CW types and their associated microorganisms

78

Removal process	Removal mechanism	Removal depending on CW type	Associated microorganisms (relative abundance)	Ref.
Phosphate removal	Bacterial removal and plant uptake are in charge of removing P, while precipitation and adsorption by porous media remove all P forms reacting with minerals such as ferric oxyhydroxide and carbonate.	In HFCW and VFCW systems, P removal is carried out by bacteria, plant uptake, adsorption by porous media, and precipitation.	*Flavobacteriaceae, Pseudomonas,* and *Acinetobacter*	[72,107,144,153]
		In SFCW, P removal is done by precipitation, sediment adsorption, plant uptake, and microbial activity.	*Rhodoblastus* (Proteobacteria 39%)	[65,70]
Aerobic degradation of organic matter	Oxidation of organic matter utilizing oxygen as the final acceptor and producing CO_2.	In HFCW systems, aerobic organic matter degradation occurs near the plant's roots and upper layers.	Proteobacteria (33%), *Zobellella* (7.0-37%), *Thauera* (0.07-3.8%), *Pseudomonas* (4.9%) and *Aeromonas* (3.5%)	[72,107,110,162,169]
		In VFCW, intermittent feed mode allows the entrance of air to the system to replace the wastewater, stimulating an aerobic transformation of organic matter.		[98,142]
Anaerobic degradation of organic matter	Multiple sequential step process: hydrolysis, acidogenesis, acetogenesis, and methanogenesis.	HFCW design influences methanogenic activity due to its water depth and anoxic conditions.	*Methanobacteriales* (60.7%), *Methanomicrobiales* (20-71%)	[84,159,162]
		Interaction between denitrifying and methanotrophic bacteria takes place.	*Methanosarcinales (methanosaeta* and *methanosarcina)* (17%)	
		In VFCW occurs between the middle and the bottom layers.		[104,110]

2.5. Factors that influence microbial diversity and community composition

The most abundant bacteria reported in CW are Proteobacteria (31-45%), Bacteroidetes (18-20%), Chloroflexi (7-13%), Firmicutes (4-11%), and Planctomycetes (4-7%) [132]. Proteobacteria represent the most abundant phylum to have been identified in different parts of CW for wastewater treatment [107,114,170–173]. This phylum comprises Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria, Deltaproteobacteria, and Epsilonproteobacteria classes, whose abundance depends on environmental conditions [54]. The members of this phylum can perform nitrification [174], denitrification [175], and sulfate reduction [176,177]. Firmicutes, Actinobacteria, Acidobacteria, Planctomycetes, Chloroflexi, Sinergistetes, Deferribacteres, Nitrospirae, Cyanobacteria, Verrimicrobia, and Archea can be found with lower levels of abundance in CW [54].

A thorough understanding of the composition and ecology of a CW can allow for the stimulation or the inhibition of specific species. This information can be used to optimize pollutant removal performance, but also to plan preventive strategies to avoid failures and poor removal rates. Given that microorganisms are sensitive to environmental factors and prevention is essential as many of these factors are highly variable and unpredictable. Developing well-planned preventive and corrective actions that maintain microbial stability will help reduce costs and extend the system's useful life.

Microbial communities are affected by factors such as temperature, pH, precipitation, dissolved oxygen concentration, organic matter concentration, nutrient concentrations, plants, hydraulic retention time, CW configuration, and geographical location [19,178,179]. Furthermore, CW are subjected to seasonal variations, hence the microbial composition may change, and microbial communities may perform different metabolic pathways [66,170]. Finally, spatial variability in the composition of microbial communities has also been reported throughout CW systems. Microbial activity is generally higher in sites closer to the inlet of the wastewater, and decreases along the course of the constructed wetland [47]. In this section, possible changes in microbial communities in response to environmental, operational, and design factors are discussed.

2.5.1. Environmental factors affecting microbial communities

2.5.1.1. Temperature

Temperature is an essential factor that influences microbial respiration and contributes to forming an adequate wetland microenvironment necessary for microbial nutrient removal. It has been reported that microbial abundance decreases from summer to winter [119]. Increases in water temperature during the summer result in lower oxygen concentrations in the water column, causing hypoxic or anoxic conditions compared to in the winter, when dissolved oxygen increases [119]. In the dissolved oxygen concentration, these temperature changes propitiate changes in bacterial community composition [180]. In the case of SFCW, denitrification rates may decrease during the coldest months and increase during the warm seasons [68]. Interestingly, *Pseudomonas*, which are denitrifying bacteria in CW, have been reported to be more active in the summer than in the winter in natural CW [181,110,180,114]. Although denitrification is generally considered to occur under anaerobic conditions, in a study carried out by Mckenney et al. [181], it was reported that completely anaerobic conditions were not vital for denitrification to occur. Furthermore, Fu et al. [110] have reported that *Pseudomonas* is an efficient aerobic denitrifying bacterial group.

The abundance of commamox bacteria has also been found to change because of temperature variations. Xu et al. [138] found that comammox bacteria's ratio to AOB was highest in the spring and lowest in the winter in a eutrophic lake, suggesting that comammox bacteria, such as *Nitrospira*, thrive under low oxygen concentrations. Similarly, the Proteobacteria phylum has been positively correlated with temperature [182], showing higher levels of abundance in the summer (reported temperature of 25.6°C) than in the winter (reported temperature of 9.2°C) [183].

Higher temperatures in the summer can also cause drought conditions in the sediments. These conditions have been shown to affect microbial communities and reduce rates of nitrification, denitrification, and anammox in estuarine sediments [184]. Furthermore, it has been demonstrated that the composition of nitrifying bacteria changes between cold and warm seasons in HFCW [185]. Research has demonstrated that temperatures below 10°C inhibit the rate of ammonium oxidation in HFCW because plant growth is limited, and therefore poor oxygen transfer in the bed results in lower nitrification rates [186,187]. However, some microorganisms can perform nitrification processes at lower temperatures in HFCW. For example, *Nitrosomonas cryotolerans*, isolated from Alaskan waters, was able to nitrify at -5°C, having an optimum temperature between 22°C and 30°C [109]. In a study performed by He et al. [123], the abundance of anammox bacteria and nitrification bacteria in SFCW did not show significant variations when the temperature varied between 13.8°C and 24.9°C, unless there was a temperature change of more than 6°C.

Methanogenic bacteria in HFCW are also affected by seasonality, specifically radiation changes, because higher temperatures in sediments promote the higher production of exudates by macrophytes' photosynthesis. These exudates are molecules that are readily biodegradable by methanogenic communities [159].

Low temperatures have a negative effect on nitrogen and organic matter removal processes [188]. However, the removal performance of CW is enhanced at warmer temperatures. It has been reported that CW located in subtropical/tropical regions display better removal rates than those located in temperate regions [189]. Similarly, HFCW located in tropical/subtropical region maintain stable conditions for bacterial communities [188]. This is mainly due to the warmer and stable temperatures that occur in these regions throughout the year [190]. Conversely, CW located in temperate climates suffer temperature variations, which can harm treatment performance, as they affect the stability of bacterial communities. Thus, CW are specifically recommended for wastewater treatment in tropical and subtropical countries as they can achieve high removal rates

of pollutants with low operational and construction costs. Furthermore, as warmer temperatures enhance the performance of most bacterial species involved in the various removal processes, design improvements should be focused on the heat retention capability of the system.

2.5.1.2. pH

pH is considered a controlling factor that influences biochemical processes [191]. It is a crucial factor for the establishment of denitrifying, nitrifying, comammox, and anammox microbial communities in the substrates of CW [123,137,143,192]. He et al. [123] reported that SFCW with higher pH values had a higher AOB abundance in comparison with CW with lower pH values, because the optimal pH for AOB growth is between 7.8 and 8.5. In the case of NOB, activity has been reported to be inhibited at pH values above 9.5 [193]. Comammox bacteria have been reported to prefer alkaline environments with optimal pH values between 7 and 8 in natural wetlands [137]. On the other hand, denitrifying bacteria present their highest growth rates at a pH of between 7.0 and 7.5 [194], and denitrification may be affected at pH values lower than 6.0 and higher than 8.0 in SSFCW [74]. Higher pH values have also been suggested to increase ammonia concentrations (2.3 to 10.9 mg L^{-1}) in SFCW, which could inhibit the anammox process [123], with optimal pH values between 7.5 and 8.0 [195]. pH may also negatively affect plants, specifically intracellular metabolic activity, cell growth, and biomass [193]. In the same way, microorganisms related to nitrogen and organic matter removal processes have been observed to be most sensitive to pH variations at the inlet of the HFCW [188].

Controlling the pH in CW is challenging because several factors can affect it (e.g., wastewater composition, rainfall, climate, and substrate media). Even microbial metabolism by-products can affect pH stability and inhibit processes within CW, thus it is crucial to select design features that help maintain pH within an adequate range. One feature that provides important effects on pH is substrate media. Xiao et al. [196] has reported buffer capacities of basalt fiber when it is used as substrate for CW, while Fu et al. [113] has proven that a combination of different materials can provide an adequate pH for nitrogen removal microorganisms. Additionally, monitoring inlet wastewater is recommended to maintain pH stability. Extreme acid or basic shock loads should be detected to prevent its entrance to CW as they can cause a systematic failure.

2.5.1.3. Moisture, rainfall, and water level conditions

CW are vulnerable to seasonal changes in evapotranspiration and precipitation [197]. Water level is known to considerably affect microbial communities' structure, spatial distribution and activity, but not necessarily overall microbial biomass [198,199]. Microbial communities can be sensitive to the dry-wet gradient, as shifts in CW bacterial communities have been reported due to changes in moisture conditions, causing physiological stress [200,201].

Dry conditions are associated with low nutrient availability and favorable oxygen conditions due to the low water table, resulting in lower microbial metabolic activity than in wet conditions [53]. A study performed in artificial and natural wetlands presented by Peralta et al. [191] reported that wetter months (with moisture values from 35% to 49.7%) displayed a less diverse bacterial community, suggesting that these high-moisture conditions cause stress to bacterial communities. This study also found that several phyla, such as Bacteroidetes and Nitrospira, increased slightly, confirming that these bacteria may respond to a gradient of oxic/anoxic conditions as a result of an increase in moisture conditions, in turn affecting the oxygen transfer capacity.

Rainfall runoff can also affect the composition of microbial structure in CW, as it may contain a mixture of contaminants, such as polycyclic aromatic hydrocarbons (PAHs), heavy metals, and nutrients from agricultural, urban and industrial areas [171].

Furthermore, a study by Sgouridis et al. [200] has reported that denitrification rates may be reduced with decreased soil moisture. A study performed in VFCW to evaluate the effect of clogging found that Gemmatimonadetes phylum can be adapted to low soil moisture and probably disappear as the water level increases; in contrast to Desulfobacterales and Syntrophobacterales, sulfur cycle-related bacteria appear due to higher moisture [150]. As discussed previously, research has proven that moisture is a defining factor for microbial community composition. However, further study is needed to evaluate the effect of precipitation-evapotranspiration in CW systems.

Given that moisture and rainfall are highly variable environmental factors, controlling their effect on CW systems can be expensive and impractical. With the help of constant monitoring strategies, it is possible to observe changes in the normal performance of CW, caused by environmental changes in humidity (drought or heavy rain). In such cases, corrective actions may be applied to maintain the stability of bacterial communities, for example water injection in the case of excessive drought or water pumping/deviation in the case of excessive rain.

2.5.2. Microbial variations related with operational and design factors of CW

2.5.2.1. Type of constructed wetland

It has been reported that in HFCW and VFCW, bacteria generally dominate over archaea, with an abundance of 92% and 8%, respectively. However, in SFCW, archaea have been reported to display higher relative levels of abundance (18.9 to 36.4%) in comparison to those displayed in HFCW and VFCW [202]. Wu et al. [202] have experimented on different CW types following the VFCW-SFCW-HFCW sequence, finding that Proteobacteria dominated in all CW types. Additionally, the authors found that Proteobacteria, Firmicutes, Planctomycetes, and Chloroflexi were stable throughout the system's stages (VFCW-SFCW-HFCW). When comparing SFCW and HFCW, Firmicutes and Euryarchaeota were more abundant in SFCW, while Verrucomicrobia and Planctomycetes were more abundant in HFCW. The authors also found that Proteobacteria and Nitrospira were the most abundant phyla in both the VFCW and the HFCW.

Similarly, a study that characterized the microbial community in two different HFCW and VFCW found the presence of Proteobacteria, Bacteroidetes, and Firmicutes in both CW types [104]. However, the higher relative abundance of aerobic microorganisms, such as Firmicutes and *Acinetobacter*, were found in VFCW systems, a result attributed to oxygen saturation [194]. Moreover, Desta et al. [20] reported that Proteobacteria and Firmicutes were the major phyla in a VFCW and Adrados et al. [173] recognized that the Firmicutes phylum was not present in a VFCW. The latter also found that Gammaproteobacteria, Actinobacteria, and Bacteroidetes can be found in both HFCW and VFCW systems, while Firmicutes can only be found in HFCW. It is generally known that VFCW favor aerobic conditions; however, these authors also found that Flavobacterium (a potential denitrifying bacteria) can be found in VFCW [173]. Nevertheless, in other studies a higher abundance of denitrifying bacteria has been detected in the HFCW system [171], because the design of the system allows for an anaerobic environment (with a limited oxygen transfer capacity) [203].

In SFCW, Ibekwe et al. [204] found that Proteobacteria and Cyanobacteria were the dominating phyla. Within the phylum of Proteobacteria, Betaproteobacteria and Gammaproteobacterial were most abundant in the rhizosphere, while Deltaproteobacteria was more abundant in the sediments.

It may be noticed that phyla, such as Proteobacteria, Firmicutes, Bacteroidetes, and Chloroflexi, can be found in the three different CW configurations (SFCW, HFCW and VFCW). However, the dominance of one phylum over others is highly variable and is affected by the CW configurations, mainly because of oxygen transfer properties. In particular, aerobic phyla will dominate under the

aerobic conditions of VFCW. Conversely, anaerobic phyla will have greater abundance in HFCW and SFCW, where oxygen transfer is reduced. The optimal CW configuration is defined by the specific composition of wastewater. However, as reviewed previously, complete nutrient removal requires both aerobic and anaerobic conditions. For this reason, hybrid CW configurations are recommended.

2.5.2.2. Substrate media

The composition of the substrate of CW has been suggested to affect microbial biodiversity and improve pollutant removal, controlling the environmental conditions inside CW [99]. Minerals, marine sediments, rocks, soils, natural and synthetic materials such as zeolite, kaolinite, and red mud have been used as media substrates in CW [75]. It has been reported that Mn ore in VFCW can improve microbial diversity, creating a more oxygenated environment [205]. Tezontle, which is an inert volcanic rock, is widely used in CW because of its suitability for microbial growth, attributed to its neutral pH, physical stability, high porosity, contents of calcium (Ca), iron (Fe) and zinc (Zn), and its lack of nutrients [13,206,207]. Jia et al. [208] have reported that iron (Fe^{2+}) participates as a reactive element in the nitrogen cycle, facilitating nitrification, denitrification, and anammox processes in HFCW. Substrates in VFCW enriched with Fe have been shown to increase microbial communities' diversity and activity [209,210]. Sun et al. [137] have reported that Fe^{2+}, present on the substrate of coastal wetlands, can display a positive effect on nitrification rates and the abundance of comammox *Nitrospira*, as Fe^{2+} increases the enzyme activity related to nitrification.

A substrate called biochar is a carbon-rich product used as a substrate in CW. It contributes to a large surface area and higher cation exchange, enhancing pollutant removal, plant growth and oxygen diffusion, and reducing N_2O emissions. CW with biochar have been reported to have a higher Chao1 index (indicative of species richness) and Shannon's index (indicative of species diversity) [211]. Studies performed in SFCW and SSFCW systems have suggested that biochar can increase the abundance of denitrifying bacteria but not the abundance of nitrifying bacteria; bacteria to improve their relative abundance were *Thauera*, *Candidatus competibacter*, *Dechloromonas*, *Desulfobulbus*, *Chlorobium*, and *Thiobacillus* [167,71]. Given that carbon sources are essential for nitrogen removal, bacterial communities require at least an inlet chemical oxygen demand to a total nitrogen ratio of 20; this value ensures adequate carbon content for denitrification [212]. Another material employed in CW is zeolite, generally used for ammonium removal from wastewater but not to efficiently remove P [213]. A study by Guan et al. [49] performed in a VFCW reported that Chloroflexi was more abundant (4-19.4%) in VFCW filled with gravel and sand, while Cyanobacteria was more abundant (18-19%) in a VFCW filled with zeolite. The same authors reported that zeolite and sand caused remarkable spatial variations of Proteobacterial proportions, suggesting the strong impact of substrate type on the Proteobacterial community structure in an SFCW [49].

Substrate media contain minerals that favor the presence and growth of specific bacterial groups, which in turn enhance processes such as N removal. Wastewater characterization is an essential step for CW design in order to estimate the average loads of pollutants expected, and to define the most convenient type of substrate media. Unusually high concentrations of one specific contaminant, such as N, may be present in wastewater. In such cases, scaling up the system may be a costly alternative and the selection of a substrate media rich in Fe^{2+} could be a feasible solution to remove excessive N.

2.5.2.3. Salinity

High salinity concentrations in wastewater negatively affect the survival of aquatic plants and microorganisms, limiting the pollutant removal capacity of CW [214]. It has been observed that

the abundance of microorganisms may be affected by increasing salt concentration in wastewater [113,215]. Jiang et al. [130] reported a significant shift in the anammox bacterial community in a coastal wetland as the salinity concentration increased (gradient of 0 to 40 gL^{-1}). In this study, a relative abundance of 72% was determined for *Kuenenia* when no salinity was present and a relative abundance of 83% was determined for *Scalindua* (reaching 82%) as the salinity concentration increased (using a salinity gradient of 10 to 40 g L^{-1}).
High salinity concentrations have been reported to inhibit the growth of comammox *Nitrospira* growth (8.80 ppt and 5.92 ppt) [137]. Proteobacteria and Bacteroidetes phyla have been shown to have a high tolerance to salinity, suggesting that they can be used to treat water with high salt concentrations. Likewise, Chloroflexi species are tolerant to salinity stress and water with high concentrations of N and phosphate [115]. The inoculation of CW with these exogenous microorganisms can improve denitrification rates in SSFCW [214].
As discussed above, salinity affects microbial-mediated processes for wastewater treatment, causing lower removal rates. Water can be a scarce resource in coastal zones due to the intrusion of saline water into fresh water sources. For this reason, wastewater treatment plays a crucial role in such regions [216]. Under this scenario, finding microorganisms that are highly resistant to salinity to enhance them in CW is very relevant. In the future, specific CW configurations can be developed to enhance the growth of halophiles to improve treatment efficiency.

2.5.2.4. Wastewater type

Municipal wastewater main contaminants are organic matter, total suspended solids, and nutrients [217]. Chen et al. [45] and Tao et al. [117] have reported Proteobacteria as a major phylum in SSCW and VFCW treating municipal wastewater, following by Chlorobi, Acidobacter, Gemmatimonadetes, and Nitrospirae. Other microorganisms have been reported in SSFCW treating municipal wastewater, such as Spirochaetes[45] and Cyanobacteria [117]. Moreover, Wang and Li [218] have reported that the heterotrophic denitrifying bacteria in an HFCW were inhibited due to low organic carbon and nitrate concentrations in the influent to the HFCW due to a previous pretreatment stage.
Likewise, Bernardes et al. [78] have reported Acinetobacter, Desulfovibrionales, and Synergistales as the most abundant microorganisms in HFCW treating greywater, which is the domestic wastewater coming from showering, dishwashing, and laundry, comprising approximately 70% of domestic sewage [219]. These microorganisms are in charge of lipid degradation, sulfate reduction, and protein degradation, respectively, and may be related to anaerobic degradation [78].
In a system conformed by a VFCW followed by an HFCW treating industrial wastewater containing polycyclic aromatic hydrocarbons (PAHs), Proteobacteria, Bacteroidetes, and Actinobacteria have been found to be the dominant phyla, with a combined relative abundance of approximately 80% [203]. Additionally, the abundance of *Novosphingobium* was found to be exceptionally high (approximately 1.4%) at the bottom of the VFCW system. This genus proved capable of degrading polycyclic aromatic hydrocarbons (PAHs) into smaller compounds available to other microorganisms, thus increasing the biological oxygen demand to chemical oxygen demand ratio and enhancing nitrogen by denitrification [203].
In an SFCW treating swine wastewater with high organic matter concentrations, suspended solids, and nutrients, Firmicutes, Proteobacteria, and Chloroflexi were found to be the three most abundant phyla. However, Proteobacteria decreased their abundance as the concentration of NH_4^+ increased in wastewater [220,221]. Similarly, a study performed in a VFCW treating antibiotic-enriched swine wastewater found that Proteobacteria, Chloroflexi, Planctomycetes, Acidobacteria, and Cyanobacteria were the most abundant in soil samples, while Bacteroidetes, Firmicutes, and Tenericutes increased their abundance in influent and effluent water. Interestingly, Proteobacteria was found to be more abundant in the effluent of the system than in

the influent. These bacteria are dominant in soils, from where they are carried out and transported to treated water [222].
The composition of wastewater to be treated in a specific CW may vary to a certain extent, and thus the composition of municipal wastewater components depends on the geographical and anthropological characteristics of the region where the CW is located and can vary seasonally, although most of these components are easily biodegraded. Industrial wastewater, on the other hand, generally has a more stable composition, but in many cases will have important amounts of recalcitrant compounds. In such cases, more specific treatment process will be needed to eliminate them. Pilot tests can be performed to identify microorganisms that promote the degradation of these recalcitrant compounds, and specific CW designs can be proposed to promote their growth.

2.5.2.5. CW depth

Substrate depth has also been reported to affect the biodegradation (anaerobic and aerobic) of water contaminants. The greater the depth, the higher the possibility of clogging, and the subsequent development of large anaerobic zones [223].
Microbial diversity can vary depending on the CW's depth, being highest at the top of the substrate and decreasing with depth due to different organic matter and nutrient concentration and environmental conditions that facilitate microbial growth [224]. In HFCW, higher microbial diversity has been observed in the upper layers of the substrate compared to in the deeper layers [225]. Krasnits et al. [226] have found that depth in HFCW has a stronger influence on microbial distribution than distance from the wastewater inlet. These authors also reported a decrease in Eubacteria and an increase in Archaea with greater depth. SFCW promote a higher abundance of denitrifiers in comparison to VFCW and HFCW [202]. A water depth of 1.6 m in SFCW has been shown to provide an anaerobic environment, stimulating denitrifier growth as well as the presence of Chloroflexi, which supply energy for denitrifier metabolism [227,228]. This distribution has been attributed to dissolved oxygen concentration, which is lower at the inlet and in deeper zones of SSFCW, and higher in the upper layers and at the outlet [226,229]. It has been reported that the nitrification process requires approximately 1.50 mg/L of DO, while denitrification needs under 0.50 mg/L to achieve nitrogen removal from wastewater [99].
In shallow zones of CW, aerobic conditions predominate, whereas anaerobic zones are found deeper. Previous research conducted on the treatment performance of CW at different depths has concluded that shallow depths are preferred to reach better water quality at the effluent [230,231]. The superior water quality achieved by shallow CW can owe to the prevalence of aerobic conditions, as aerobic bacterial metabolism is faster than anaerobic, achieving higher contaminant removal in shorter times. However, higher biomass production rates can increase the occurrence of clogging events. Studies comparing bacterial communities at different depths of several CW can provide information as to which metabolism (aerobic or anaerobic) prevails. This information can be used to manipulate the design, specifically to promote the development of both aerobic and anaerobic bacterial groups in a single CW.

2.5.2.6. Plant-microbial interactions

Plants are an essential component of the design of CW [232]. Plants in CW promote high microbial diversity and activity because they provide (i) organic compounds (sugars and amino acids) that microorganisms can use as substrates [233], (ii) adsorption sites for bacteria, and (iii) oxygen and root exudates to the rhizosphere that stimulate microbial growth [58,102,234,235]. Plants can improve wastewater treatment as they can directly uptake nutrients for their growth and incorporate them into new tissues. Macrophytes act as a promoter of microbial growth by providing structural support and retaining suspended solids [151], secreting metabolites for

microbial development, and transferring oxygen to the rhizosphere, creating aerobic and anaerobic environments in CW [179,234–236]. Chen et al. [45] has proven that systems with plants possess higher efficiencies of removing nutrients and organics than systems without plants. Therefore, the plant rhizosphere enhances microbial density and activity, providing a root surface for biofilm attachment, carbon sources through root exudates, and an aerobic microenvironment via root oxygen release [143,232]. Plant species, root morphology, and plant developmental stage are important factors that influence plant-microbial interaction and positively affect microbial structure and species richness in HFCW [102]. On the other hand, plant effects can vary depending on the temperature, wastewater type, and season [237].

The interactions between plants and microorganisms accelerate the degradation of contaminants [238]. However, microbial communities may change depending on the plant species used, because each species contributes different amounts of oxygen and carbon sources (developing different rhizosphere characteristics) [103,239,240], leading to different efficiencies in nutrient removal [241]. Multiple studies in different CW types have suggested that plants positively influence the denitrifier community structure [103,240,242,243].

Plant species in each CW are selected depending on the wastewater type. Xu et al. [221] have reported that *Myriophyllum aquaticum* is a ubiquitous macrophyte that can resist high nitrogen concentrations and possesses a significant ability to absorb nutrients. *M. aquaticum* in SFCW has been used to treat swine wastewater with a 3-47% nitrogen removal rate. Meanwhile, Zhang et al. [244] have demonstrated that root extracts of *Thalia dealbata* planted in a SSFCW inhibit cyanobacteria growth. Common reeds such as *Phragmites australis* and *Cyperus malaccensis* have been reported to affect AOB's community structure, but the impact on anammox bacteria and AOB has been reported to be small [138,245]. A study carried out by Chen et al. [45] has reported a higher relative abundance of Actinobacteria (20.7%) in SSFCW planted with *Thypa latifolia* in comparison to that reported for unplanted SSFCW (1.9%), related to the plant's ability to transfer oxygen to the rhizosphere microenvironment.

The interactions between plants and microorganisms positively affect the removal rates of pollutants in CW. Thus, the selection of plant species should be considered a crucial factor in the performance of CW. Climate region, CW design, and wastewater type should be considered to avoid stressful conditions for plants and achieve adequate growth, because plants provide many advantages for microbial communities. Ornamental plants have been used in CW for wastewater treatment [13], as they provide visual and economic value to the system besides playing an important role in microbial community development.

2.6. Conclusions

CW are an appealing alternative to conventional systems for wastewater treatment. The pollutant-removal processes that occur within CW include those that are microbial mediated (nitrification, denitrification, anammox and comammox), plant uptake, and sedimentation. Therefore, microbial structure, diversity, and activity are critical for the proper functioning of CW.

Microbial communities within CW have an evolving nature, strongly linked to their surroundings. These communities are shaped by complex interactions with wastewater, substrate media, plants, and the overall environment. A global understanding of the microbial communities involved in removal processes within CW and their spatial and temporal variation, as well as of the critical factors that alter microbial activity, has proven to be crucial in explaining CW performance.

Molecular techniques and omics technologies have allowed for a deeper understanding of CW's microbial composition and behavior. Research has shown that different environmental, operational, and design factors can shape microbial communities and affect the performance of CW. Further understanding of the critical factors that can be manipulated or controlled to shift the dominance of different microbial groups to enhance microbial activity and improve CW

performance is still needed. Moreover, precise experiments manipulating spatial and temporal variables and assessing microbial behavior, as well as CW performance, could prove useful to develop strategies to optimize CW performance. In addition, mathematical models could become helpful tools to optimize and predict the structure, diversity, and activity of microbial communities within CW.

Chapter 3: Characterization of the spatial variations of microbial communities in a decentralized subtropical wastewater treatment plant using passive methods

Submitted as a research article titled **"Characterization of the spatial variation of microbial communities in a decentralized subtropical wastewater treatment plant using passive methods"** in MDPI Water open access journal:
https://doi.org/10.3390/w13091157

Septic tanks (STs), up-flow anaerobic filters (UAFs), and horizontal-flow constructed wetlands (HFCWs) are cost-effective wastewater treatment technologies especially efficient in tropical and sub-tropical regions. In this study, the bacterial communities within a decentralized wastewater treatment plant (WWTP) comprising a ST, a UAF, and a HFCW were analyzed using high-throughput sequencing of the V3–V4 region of the 16S rRNA gene. Bacterial diversity and its spatial variation were analyzed at the phylum and family level, and principal component analysis (PCA) was applied to nitrogen- and organic-matter-degrading families. The highest percentage of nitrogen removal was seen in the HFCW (28% of total Kjeldahl nitrogen, TKN, and 31% of NH3-N), and our results suggest that families such as Rhodocyclaceae (denitrifying bacteria), Nitrospiraceae (nitrifying bacteria), and Rhodospirillaceae (sulfur-oxidizing bacteria) contribute to such removal. The highest percentage of organic matter removal was seen in the UAF unit (40% of biological oxygen demand, BOD5, and 37% of chemical oxygen demand, COD), where organic-matter-degrading bacteria such as the Ruminococcaceae, Clostridiaceae, Lachnospiraceae, and Syntrophaceae families were identified. Redundancy analysis demonstrated that bacterial communities in the HFCW were more tolerant to physicochemical changes, while those in the ST and the UAF were highly influenced by dissolved oxygen and temperature. Also, pollutant removal pathways carried out by specific bacterial families and microbial interactions were elucidated. This study provides a detailed description of the bacterial communities present in a decentralized WWTP located in a subtropical region.

3.1. Introduction

According to United Nations Sustainable Development Goal Number Six, a substantial increase in water treatment and reuse must be accomplished by 2030 to significantly reduce water scarcity worldwide and to protect the natural environment [246]. Centralized wastewater treatment plants (WWTPs) involve higher maintenance and operational costs than decentralized WWTPs, which are also easier to operate [247]. Anaerobic bioreactors (ARs) and constructed wetlands (CWs) are treatment stages that are commonly used in decentralized systems [13]; these units are classified as passive technologies, since they require low energy consumption and maintenance and operational costs [13,43]. Up-flow anaerobic filters (UAFs) and a septic tank (ST) are examples of ARs that can efficiently remove organic matter and suspended solids. However, the removal of nutrients in these units is often insufficient to comply with water quality regulations [248–250]. On the other hand, CWs are capable of removing nutrients, organic matter, and other pollutants from wastewater [57,58,63]. However, CWs may also have limitations, such as the

possibility of clogging caused by the load of suspended solids in wastewater [251]. Systems consisting of ARs followed by CWs are known to be an efficient combination for wastewater treatment [13,252]. These configurations present advantages such as low energy requirements, low operational costs, and low sludge generation [253]. Furthermore, by incorporating an AR, such as a ST and UAF, as a pretreatment stage before wastewater is fed into a CW can significantly reduce the solid particles and organic matter levels [13,43], while the CW is useful in reducing nutrient loads in wastewater after anaerobic pre-treatment [227].

Microorganisms are a crucial component of both centralized and decentralized wastewater treatment systems because they remove nitrogenous compounds, phosphate, sulfur, and organic matter, among other pollutants [254]. For instance, nitrogen removal within CW is achieved by microbial processes such as nitrification, denitrification, anaerobic ammonium oxidation (anammox), and complete oxidation of ammonia (comammox) [183]. Through anaerobic digestion, organic matter is converted to methane (CH_4) by the action of heterogeneous microbial communities that perform hydrolysis, acidogenesis, acetogenesis, and methanogenesis reactions [19].

The structure of microbial communities in CWs, and the occurrence of several pollutant removal pathways, strongly depend on the occurrence of aerobic and anaerobic microenvironments within them, as well as on many other operational and design factors, such as the presence of macrophytes, the type of substrate media, and hydraulic depth [19,179]. These communities are also affected by environmental conditions and variation (e.g., temperature, moisture, and pH) [66,170]. Spatial variation in the microbial communities within CWs have also been associated with proximity to the wastewater inlet. Higher organic matter and nutrient content occur near to the inlet therefore higher microbial activity has been found near the inlet, and a gradual decrease has been observed towards the outlet [47]. Moreover, differences have been reported in CW microbial diversity when comparing communities attached to plant roots to those attached to the substrate media [54]. Additionally, higher microbial diversity has been found in the upper layers of CWs than in the substrate of lower layers [54,224].

Microbial communities in WWTPs have been analyzed by molecular methods such as denaturing gradient gel electrophoresis (DGGE) [18], fluorescent in situ hybridization (FISH), and terminal-restriction fragment length polymorphism (TRFLP) [255]. The polymerase chain reaction (PCR) method has also been used to investigate the distribution of functional genes and microorganism diversity [54]. However, these methods may underestimate the microbial composition and diversity in these ecosystems because of a lack of sufficient sequences [150], and may fail to capture microbial community complexity [183]. Therefore, high-throughput sequencing is the most widely used method because it enables more extensive and systematic analysis of microbial communities in complex ecosystems [54,103]. More sequences can be obtained through this analysis and, thus, more information is provided, allowing bacterial diversity to be characterized more precisely [150,183].

Desta et al. [28] studied microbial communities of a multi-stage (anerobic/aerobic/VFCW) system treating tannery wastewater in Ethiopia. Bedoya et al. [17] sequenced samples of biosolids from a centralized WWTP treating municipal wastewater located in Colombia. Song et al. [29] studied bacterial diversity of six activated sludge WWTPs located in different climatic regions (tropical, subtropical, and temperate). However, to the best of our knowledge most studies focused on the characterization of microbial communities within wastewater treatment systems have reported predominantly on single treatment stages. Furthermore, very few studies have investigated the effect of physicochemical parameters on the structure of microbial communities within a multi-stage system.

The objective of this study was to characterize the spatial variation in microbial communities between and within the treatment stages of a decentralized WWTP combining a septic tank (ST),

an up-flow anaerobic filter (UAF), and a horizontal-flow constructed wetland (HFCW). Additionally, we investigated the influence of physicochemical parameters on the microbial structure within all three treatment stages. As a contribution to the literature, this work is focused on the study of the microbial communities throughout a multi-stage decentralized treatment system composed of a ST, a UAF, and an HFCW that uses passive methods and has demonstrated to treat domestic wastewater efficiently. This study seeks to gain a greater understanding of the microbial structure and behavior in this WWTP located in a subtropical climate and the microbial response to physicochemical variations within the system. A thorough knowledge of the structure and behavior of microbial communities within the system under study contributes to the development of strategies to enhance microbial removal processes and improve the performance of decentralized WWTPs integrated with anaerobic reactors and constructed wetlands in tropical and subtropical countries. Furthermore, this is one of very few studies to provide a detailed description of the bacterial communities in decentralized treatment plants located in subtropical countries (such as Mexico), where climatic conditions play an essential role in the performance of treatment systems.

3.2. Methodology

3.2.1 Site description

The study site is a decentralized WWTP that combines an ST, a UAF, and an HFCW to treat high strength domestic wastewater generated at a public R&D center located in the municipality of Zapopan (Jalisco, Mexico). The study site is located in a subtropical region with an average annual temperature of 21.8 °C and annual precipitation of 926.4 mm [256]. The WWTP receives a wastewater load of approximately 7.5 m^3/day. Untreated wastewater is received in the pump sump, where it is pumped to the ST. At this stage most particulate solids are removed by sedimentation, and anaerobic conditions promote the anaerobic digestion of organic matter [13,43]. The second treatment stage is the UAF, a tank filled with a porous volcanic rock called tezontle. In this anaerobic unit, wastewater flows from the bottom part of the chamber to the outlet at the top. The porous rocks act as attachment media for the bacteria involved in organic matter degradation. Finally, the wastewater flows to the HFCW (Figure 1), which is a shallow pond (with a hydraulic depth of 60 cm) that is also filled with tezontle which acts as a support medium for the root development of the ornamental plant *Agapanthus africanus* planted at a density of three plants/m^2. The mean hydraulic retention times of the ST, the UAF, and the HFCW were reported as 2.45, 6.4, and 11.75 days, respectively [13,43]. The WWTP at the site has been described in detail by de Anda et al. [13] and mathematically modeled by Fernandez del Castillo et al. [43]. The configuration of the treatment system is shown in Figure 2.

Figure 1. Horizontal flow constructed wetland (HFCW) planted with *Agapanthus africanus*.

3.2.2 Water quality analyses

Four sampling points were established for monitoring water quality (SP1: at the sump pump; SP2 at the UAF inlet; SP3 at the UAF outlet; SP4 at the HFCW outlet), indicated by the blue boxes in Figure 2. Water samples were taken fortnightly for three months (January to March of 2020) to determine biological oxygen demand (BOD_5), chemical oxygen demand (COD), total Kjeldahl nitrogen (TKN), ammonia nitrogen (NH_3-N), organic nitrogen (ON) and total suspended solids (TSS). These determinations were made by the Analytical and Metrological Services Unit (USAM) of the Centro de Investigación y Asistencia Tecnológica del Estado de Jalisco (CIATEJ), following the methods published by the Federation, W. E., and the American Public Health Association [257]. Samples were delivered to the laboratory less than one hour after they were taken. Nitrates (NO_3^-) and nitrites (NO_2^-) were measured by spectrophotometry using multiparametric kits TNT 835 and TNT 839, respectively (DR 5000, HACH, Loveland, CO, USA). System performance was evaluated by calculating the reduction in mass of each pollutant in each treatment stage and in the overall system. Temperature (Temp), pH, electrical conductivity (EC), and dissolved oxygen (DO) were measured at each sampling point using a multi-parameter probe (HI 9828, Hanna) to analyze the influence of physicochemical parameters on microbial communities.

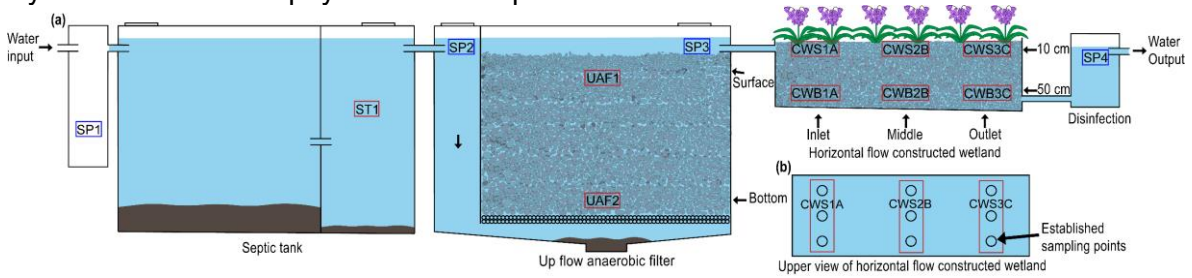

Figure 2. Diagram of sampling points for sequencing and water quality analysis where (**a**) represents a lateral view of the WWTP, sampling points for sequencing analysis are indicated by red boxes and sampling points for water quality analysis (SP1, SP2, SP3, and SP4) are indicated by blue boxes; (**b**) represents an aerial view of the HFCW, individual samples used in the preparation of the composite samples are grouped in red boxes.

3.2.3 DNA extraction and High-throughput sequencing

Nine sampling points were established for DNA extraction and high-throughput sequencing (Figure 2) through the entire treatment system. One sampling point was located in the ST, two sampling points were located in the UAF, and six were located in the HFCW. In the ST and the UAF (the anaerobic stages), samples included water mixed with sediments, while samples taken from the HFCW contained water combined with plant roots, substrate, water, and sediments.
Two sampling campaigns were conducted during 2020, the first in January and the second in March, months which are both within the dry season in Jalisco, Mexico. At each sampling point, and for each sampling campaign, three grab samples, each with three biological replicates, were taken, generating a total of 54 grab samples. Thus, three grab samples (and replicates) were taken from the second chamber of the ST each month (Figure 2; indicated as ST1 inside a red square). In the case of the UAF (Figure 2a), three grab samples (and replicates) were taken at the surface (UAF1), and three grab samples (and replicates) were taken at the bottom (UAF2) each month (adding up to a total of 12 samples for the UAF) (Figure 2; indicated as UAF1 and UAF2 respectively, inside red squares). No composite samples were prepared for the UAF or the ST.
Regarding the HFCW (Figure 2a), samples were taken at a depth of 10 cm (Constructed Wetland Surface – CWS) and at a depth of 50 cm (CWB: Constructed Wetland Bottom) to assess variation associated with depth. Longitudinal distribution was also considered, as samples were taken at the inlet, in the middle, and at the outlet (at the two different depths previously described). The cross-sectional distribution of the microbial community was assessed by taking three grab samples at three points distributed cross-sectionally for each of the inlet, the middle, and the outlet (all at a depth of 10 cm) to prepare composite samples CWS1A, CWS1B, and CWS1C (Figure 2b). Likewise, three grab samples were taken at three points distributed cross-sectionally for each of the inlet, the middle, and the outlet (all at 50 cm depth) to prepare composite samples (CWB1A, CWB2B, and CWB3C) as shown in Figure 2b. The three grab samples taken at CWS1A were used to prepare a (cross-sectional) composite space sample and the same procedure was followed to prepare composite samples for CWS2B, CWS3C, CWB1A, CWB2B, and CWB3C. The resulting composite samples correspond to each depth (10 and 50 cm) and each longitudinal point (inlet, middle, and output). For the HFCW, a total of six composite space samples were prepared (as 18 grab samples were collected) each month (a total of 36 samples).
Samples were stored at 4 °C while they were transported to the laboratory for processing. Once in the laboratory, samples were centrifugated at 800 x g for five minutes to form a sediment pellet and 500 mg of the pellets were placed on a matrix for DNA extraction. Following the manufacturer's specifications, the DNA extraction procedure was performed using the FastDNA Spin Kit for Soil (MP Biomedicals, Solon, OH, USA). DNA extractions were stored at -80 °C until further analysis. High-throughput sequencing of the V3-V4 region of the 16S rRNA gene was performed by Novogene Corporation Inc. (Chaoyang District, Beijing, China) using an Illumina NovaSeq 6000 PE250 (paired-end to generate 250bp paired-end raw reads), obtaining 100k raw reads per sample employing the 341F (CCTAYGGGRBGCASCAG) and 806R (GGACTACNNGGGTATCTAAT) primers.

3.2.4 Bioinformatic analyses

Bioinformatic analyses were performed using QIIME 2.0 (Quantitative Insights Into Microbial Ecology) software [258] following a standard bioinformatic pipeline. Demultiplexed sequences were denoised using DADA2 (p-trunc-len-f 0, p-trunc-len-r 0, p-trim-left-f 0, p-trim-left-r 0). After denoising, two characteristics tables (FeatureData[Sequence] and FeatureData[Taxonomy]) were

constructed using 99% similarity with Greengenes 13_8 99% OTU full-length sequences [259,260]. Afterward, the classifier was trained using the length of the primers by the Naive Bayes classifier method. Finally, the taxonomic classification and the output of DADA2 (denoised sequences) with the trained classifier were aligned with classify-sklearn and the result was visualized as a taxa-barplot [261]. The sequencing run has been uploaded to the Sequence Read Archive of the NCBI with accession number PRJNA700667.

3.2.5 Statistical analysis

The sequencing depth of the 16S rRNA gene was presented using a rarefaction curve performed in R using the rarefy function, which is based on Hurlbert's formulation [262] and the standard errors proposed by Heck [263]. Barplots of relative read abundance were used to understand the composition of the bacterial community for the phylum and selected microbial families related to nitrogen and organic matter removal pathways. The DESeq2 R package was used to normalize read numbers [264]. Microbial groups with relative abundance <1% were grouped as "others" and unclassified families were denoted by "Un".

3.2.5.1 Principal component analysis

Principal component analysis (PCA) was performed for the bacterial families degrading nitrogen and organic matter in each unit (ST, UAF, and HFCW) to evaluate differences between treatment units as well as vertical variation within the UAF and both vertical and longitudinal variation within the HFCW. The aim of this analysis was to transform the original variables (bacterial families) to a new set of variables, the principal components, that are linear combinations of the original variables, which are uncorrelated and are ordered so that the first few of them account for most of the variation within the bacterial families **[265]**. Correlation biplots were used to further interpret bacterial community variation using the first two principal components in each case (variations between and within treatment units). In a biplot: (i) a vector represents a variable (bacterial family) and its length is proportional to the variance of the corresponding variable, (ii) angles between vectors reflect the correlation between the corresponding variables; (iii) points (observations) can be projected perpendicularly onto vectors, and the projection is indicative of the abundances of the families represented in the corresponding observations. The origin represents the average value. Projections in the same direction of the vector indicate values above average while projections in the opposite direction represent values below average **[266]**.

3.2.5.2 Redundancy analysis

The distribution of nitrogen and organic matter degrading bacterial communities between and within treatment units, and the effect of physicochemical parameters on bacterial communities, were analyzed using redundancy analysis (RDA) and the same analysis was performed on the variation in their composition. RDA is an extension of PCA that explicitly models response variables (bacterial families in this case) as a function of explanatory variables (physicochemical parameters in this case) **[266]**. The components in RDA are not only a linear combination of the response variables, but also of the explanatory variables. Correlation triplots were used to extract further information from the RDA results. A correlation triplot consists of two superimposed biplots that include quantitative explanatory and response variables (represented by vectors) and observations (represented by points) **[266]**. The interpretation of correlation triplots is as follows **[267]**: (i) The angles between two response variable vectors, or between two explanatory variable vectors, or between a response variable vectors and an explanatory variable, reflect their

correlations, (ii) Points (representing observations) can be projected perpendicularly onto the response and explanatory variable vectors and indicate their values in the corresponding samples (observations).

3.2.5.3 Principal coordinates analysis

Dissimilarities in the composition of microbial communities between treatment units (ST, UAF, and HFCW) and dissimilarities between these communities at different (longitudinal and vertical) regions within the treatment units (the UAF and HFCW units) were analyzed using principal coordinates analysis (PCoA) based on Bray-Curtis distances. Permutational multivariate analysis of variance (PERMANOVA) ($P < 0.05$) and analysis of similarity (ANOSIM), using the Bray-Curtis dissimilarity index, were applied to evaluate statistically significant differences in the composition of bacterial communities between and within units (including vertical and longitudinal variation within units) [268]. The ANOSIM statistic R (which compares the mean of ranked dissimilarities between groups to the mean of ranked dissimilarities within groups) lies in the interval [-1,1]. A value of 0 indicates a completely random grouping, while positive R values suggest dissimilarity between groups and values below 0 suggest that dissimilarities are greater within groups than between groups [269]. All statistical analyses (PCA, RDA, PCoA, PERMANOVA and ANOSIM) were performed using the R software version 4.0.2, and the scales [270] vegan [271] packages. Graphics were made using ggplot2 package [270].

3.3. Results and Discussion

3.3.1 System performance

The reduction in mass of each pollutant and the content remaining in the system effluent (BOD_5, COD, TKN, NH_3-N, ON, and TSS) are depicted in Figure 3. The system was found to be highly efficient in organic matter removal as it displayed significant mass reduction for both BOD_5 and COD, with average overall values of 548 ± 117 mg/L and 847 ± 181 mg/L, respectively (Figure 3; Table S1), corresponding to average overall reductions of 90% of BOD_5 and 91% of COD. Higher degradation of organic matter occurred in the ST and the UAF, representing a combined reduction of 73% of BOD_5 and 72% of COD (mass reduction: 202 ± 60 mg/L of BOD_5 and 331 ± 93 mg/L of COD in ST; 243 ± 105 mg/L of BOD_5 and 345 ± 137 mg/L of COD in UAF). These results indicate that efficient anaerobic degradation occurs in the anaerobic stages (ST and UAF), in agreement with the DO and temperature levels measured in these stages (Table 1), which are optimal for anaerobic reactors [272]. Degradation of organic matter within the HFCW was also significant, with an average mass reduction of 171 ± 37 mg/L for COD, corresponding to a reduction of 18%. The DO concentrations measured in the HFCW were low but were higher than those observed for the anaerobic stages (Table 1).

Table 1. Average values of physicochemical parameters measured.

	OS (n = 24)	SP1 (n = 6)	SP2 (n = 6)	SP3 (n = 6)	SP4 (n = 6)

DO (mg/L)	0.5 ± 0.5	0.5 ± 0.5	0.3 ± 0.1	0.7 ± 0.5	0.6 ± 0.7
EC (ms/cm)	1.5 ± 0.2	1.4 ± 0.3	1.7 ± 0.2	1.6 ± 0.2	1.5 ± 0.1
pH	7.4 ± 0.7	7.8 ± 0.9	7.2 ± 0.7	7.3 ± 0.7	7.4 ± 0.4
Temperature (°C)	20.4 ± 2.3	21.0 ± 1.7	21.1 ± 2	20.9 ± 2.3	18.5 ± 2.3

DO: dissolved oxygen; EC: electrical conductivity; OS: overall system; SP1: sampling point 1; SP2: sampling point 2; SP3: sampling point 3; SP4: sampling point 4. The mean values of six observations are presented. Measurements were made fortnightly for three months (January to March 2020) at all four sampling points.

TSS removal was high for the system overall, reaching 339 ± 73 mg/L, which represents a reduction of 97% of the inlet load. However, the ST was found to make the highest contribution in comparison to the other treatment stages, with an average mass reduction of 258 ± 65 mg/L (74%), which proved that the ST unit performs its function efficiently, preventing the accumulation of solid particles in the succeeding units (UAF and HFCW) and preventing clogging (obstruction). Regarding nitrogen removal, the overall mass reduction was 173 ± 20 and 67 ± 8 mg/L for TKN and NH_3-N, respectively. These values correspond to overall reductions (the contributions of the three stages combined) of 51% of TKN and 45% of NH_3 from inlet loads. This reduction is significant considering the initial concentration of TKN (337 ± 12 mg/L), as it is comparable to industrial loads; since the wastewater comes from an R&D industrial and biotechnology center, the effluent may have a higher concentration of nitrogen [273]. Similar results were reported by de Anda et al. [13] for this experimental system. As shown in Table S1, the highest reduction in nitrogen mass occurred in the HFCW, with values of 95 ± 26 mg/L (28%), 46 ± 13 mg/L (31%), and 48 ± 13 mg/L (26%) for TKN, NH_3-N, and ON, respectively. Although the concentration of nitrates and nitrites entering the system was low (2.88 ± 1.69 mg/L for NO_3^- and 0.26 ± 0.06 for NO_2), the removal efficiency was high, reaching 93% and 96% for NO_2^- and NO_3^-, respectively. The performance of this experimental system has previously been characterized by Fernández del Castillo [43].

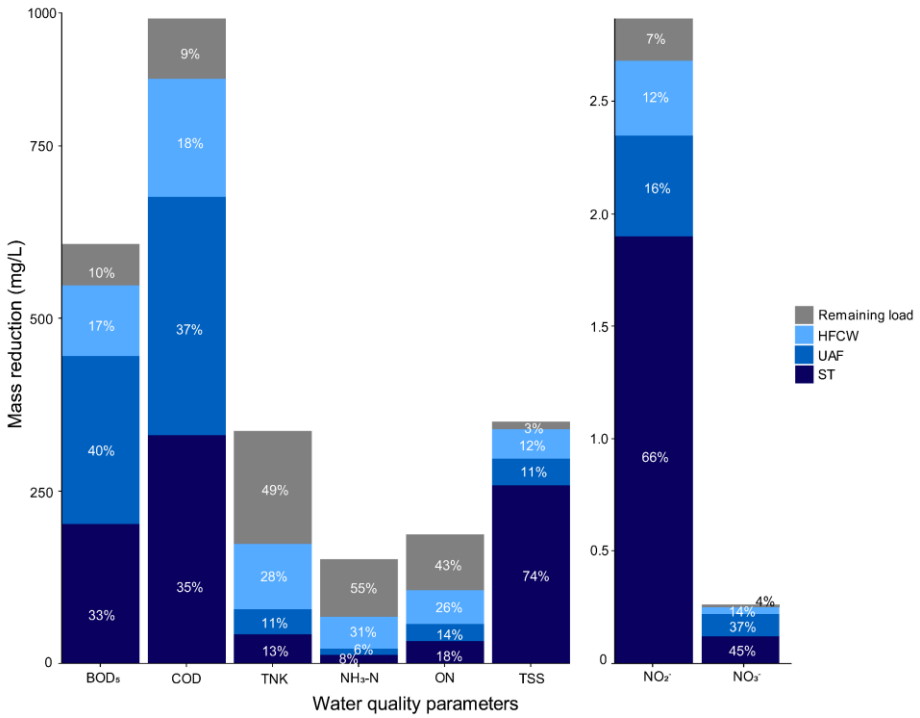

Figure 3. Removal efficiencies for each unit and the system overall. The image represents the mean of six observations taken fortnightly for three months (January to March 2020). ST: septic tank; UAF: up-flow anaerobic filter; HFCW: horizontal-flow constructed wetland; BOD_5: biological oxygen demand; COD: chemical oxygen demand; TNK: total Kjeldahl nitrogen; NH_3-N: ammoniacal nitrogen; ON: organic nitrogen; TSS: total suspended solids; NO_2^-: nitrites, and NO_3^-: nitrates.

3.3.2 Diversity and composition of the bacterial communities

High-throughput sequencing was performed on 52 samples (out of a total of 54 samples), from which 9,512,339 raw reads were obtained from the hypervariable region V3-V4 of the 16S rRNA gene, with a mean length of 420 bp. Sequencing depth was represented by a rarefaction curve of the 16S rRNA gene (Figure S1). Two samples corresponding to the ST (one for each month) were not processed further because the DNA did not meet quality requirements. A total of 5,799,224 (61%) (from the 52 samples) were classified as bacterial employing the Greengenes database, and 3,713,115 (39%) reads were described as unclassified.

Proteobacteria was the most abundant phylum in all the system stages and gradually increased in abundance from one treatment stage to the next (ST<UAF<HFCW), with abundances of 34% in the ST, 38% in the UAF, and 60% in the HFCW (Figure 4a; Table S2). The members of this phylum are involved in a variety of metabolic pathways related to the carbon and nitrogen cycles [104,168,274], which explains their dominance in the whole WWTP. Additionally, Proteobacteria are known to be enhanced by the presence of macrophytes in CWS [275], which explains why their highest relative abundance was in the HFCW and which may also be associated with the high nitrogen mass reduction accomplished in this unit (Figure 3). In addition to Proteobacteria, Firmicutes (26%), Bacteroidetes (14%), and Caldiserica (6%) were the most abundant phyla found in the ST. Firmicutes (21%), Caldiserica (14%), and Bacteroidetes (9%) were the phyla with the highest relative abundance in the UAF (besides Proteobacteria) while Bacteroidetes (9%),

Actinobacteria (8%), and Chloroflexi (5%) presented the most significant abundances in the HFCW. As suggested by these results, the physicochemical conditions of each treatment stage affected its microbial composition. ST and UAF displayed a similar composition, as both are anaerobic units, while the bacterial composition within the HFCW, which contains macrophytes that provide exudates and dissolved organic matter, and transfer oxygen [276], presented more differences.

The higher abundance of Firmicutes found in the anaerobic units in this study, compared to the HFCW, can be explained, as this phylum is known to degrade complex organic molecules [150,277]. The ST is the first unit in the system and is thus expected to receive a higher load of complex organic molecules, which diminished in subsequent stages and, accordingly, the relative abundance of Firmicutes decreases after each stage. Consequently, this phylum may be closely associated with the high rates of BOD_5 and COD mass removal reported for the first two units (the ST and UAF; Figure 3).

Besides Firmicutes and Proteobacteria, Acidobacteria, Actinobacteria, Bacteroidetes, and Chloroflexi phyla were present in all treatment units. These bacterial phyla have previously been reported in anaerobic bioreactors [18]. They are also known to conduct hydrolysis and acidification reactions in HFCW treating effluents with high organic loads, thereby playing an essential role in decomposing organic matter and nitrogen removal [278]. The phyla Gemmatimonadetes and Nitrospirae were only found in the HFCW, where higher levels of nitrogen and phosphate removal occurs. In consequence, previous studies have linked Gemmatimonadetes to nitrogen [279] and phosphate removal [168]. In this study, the phylum Gemmatimonadetes was only present at a depth of 10 cm in the HFCW and was completely absent in the first units (ST and UAF). Cheng et al. [150] reported that the Gemmatimonadetes prefer drier soils and disappear when the water content increases in a vertical subsurface flow CW, which explains why this phylum is present only in the upper layers of the HFCW of our experimental system, where the water content is lower because this is a CW with subsurface flow. Only slight variations in abundance, in terms of phyla, were found in bacterial communities at different depths in the UAF (Figure 4b; Table S3). Regarding the effect of depth on the microbial communities found in the HFCW (Figure 4d; Table S4), the phyla Cyanobacteria, Gemmatimonadetes, and Nitrospirae were present only at 10 cm, which could indicate a dependency on higher oxygen concentrations [280,281]. However, it has been reported that the phylum Nitrospirae includes both aerobic and anaerobic microorganisms related to nitrification (nitrite oxidizers) [66] and methanogenesis [281], indicating that members of this phylum may be found in the upper and lower layers of diverse CW units. However, in the HFCW of our experimental system, bacteria of the phylum Nitrospirae were mainly present at a depth of 10 cm, possibly due to a limited accumulation of nitrite in the lower layers, since nitrite is known to be an unstable intermediate [45]. Significant longitudinal variation in the abundance of phyla was also found in the HFCW, where a decrease in Bacteroidetes and an increase in Actinobacteria were observed from inlet to outlet (Figure 4c; Table S5). The phylum Bacteroidetes is known to degrade complex organic compounds and includes members that can perform denitrification [173]. It is to be expected that the relative abundance of this phylum would decrease as substrate availability decreases from the inlet to the outlet of the HFCW. Furthermore, Gemmatimonadetes were present in the middle and the outlet sections but not near the inlet, which may be attributed to the low concentrations of DO found at the inlet. Some members of the Gemmatimonadetes have been regarded as aerobic heterotrophs capable of assimilating sugars [280,282].

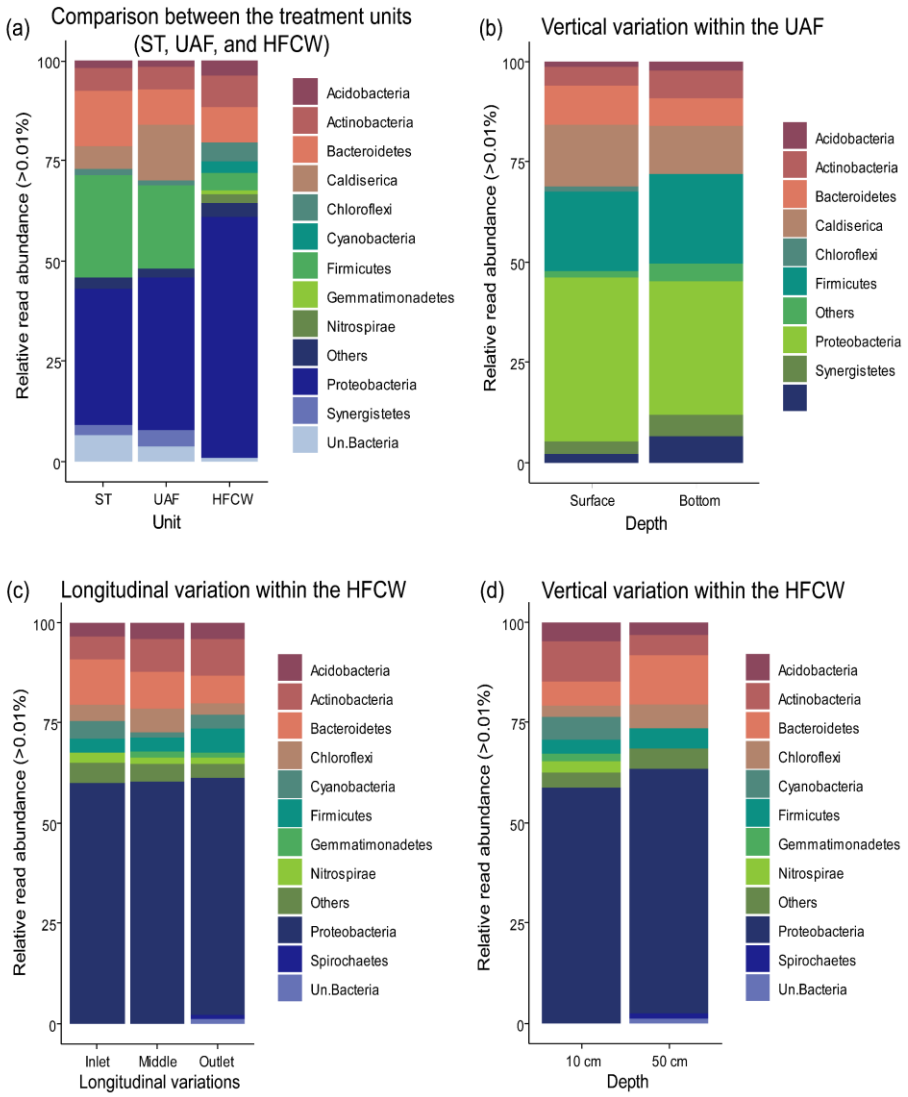

Figure 4. Phylum level taxonomic classification of bacterial sequences, Others refers to bacteria with relative read abundances < 1%, (**a**) Comparison between the treatment units (ST, UAF, and HFCW), (**b**) Vertical variation within the UAF, (**c**) Longitudinal variation within the HFCW, and (**d**) Vertical variation within the HFCW.

3.3.3 Spatial variations of nitrogen and organic matter degrading families

To evaluate the spatial distribution of bacterial communities within the treatment systems, 18 nitrogen degrading families and 29 families that degrade organic matter were selected (Figures 5–8). PCA biplots were developed to analyze the spatial variation in their relative abundance (Figures 6 and 8, only selected bacterial families vectors were plotted for illustration purposes) and a PCoA biplot (Figures S2 and S3) was developed to demonstrate dissimilarities between the treatment units and spatial variation within the system stages. ANOSIM was performed to determine if such dissimilarities were significant (Table S14). As also found at the phylum level, the bacterial communities of the anaerobic stages (ST and UAF) were similar (Figures S2a and S3a). The low levels of oxygen present in the ST and the UAF are necessary for anaerobic degradation and denitrification. In contrast, differences in the composition of the microbial

communities between the HFCW and the anaerobic stages (ST and UAF) may be caused by the development of aerobic microenvironments and symbiotic interactions between bacteria and plants that occur in the HFCW **[283]**.

3.3.3.1 Nitrogen degrading bacterial families

Rhodocyclaceae was the most abundant family in all treatment stages (Figures 5a and 6a), suggesting that the oxygen concentrations found in all units (ST, UAF, and HFCW; Table 1; Table S6) were sufficiently low for their growth. This family has been reported to participate in denitrification, organic matter degradation, and biofilm formation in high strength wastewater treatment systems under anaerobic conditions [250,284,285]. *Comamonadaceae*, *Propionibacteriaceae*, and *Pseudomonadaceae* were present in greater abundances in the ST in comparison to the UAF and the HFCW (Figures 5a and 6a).

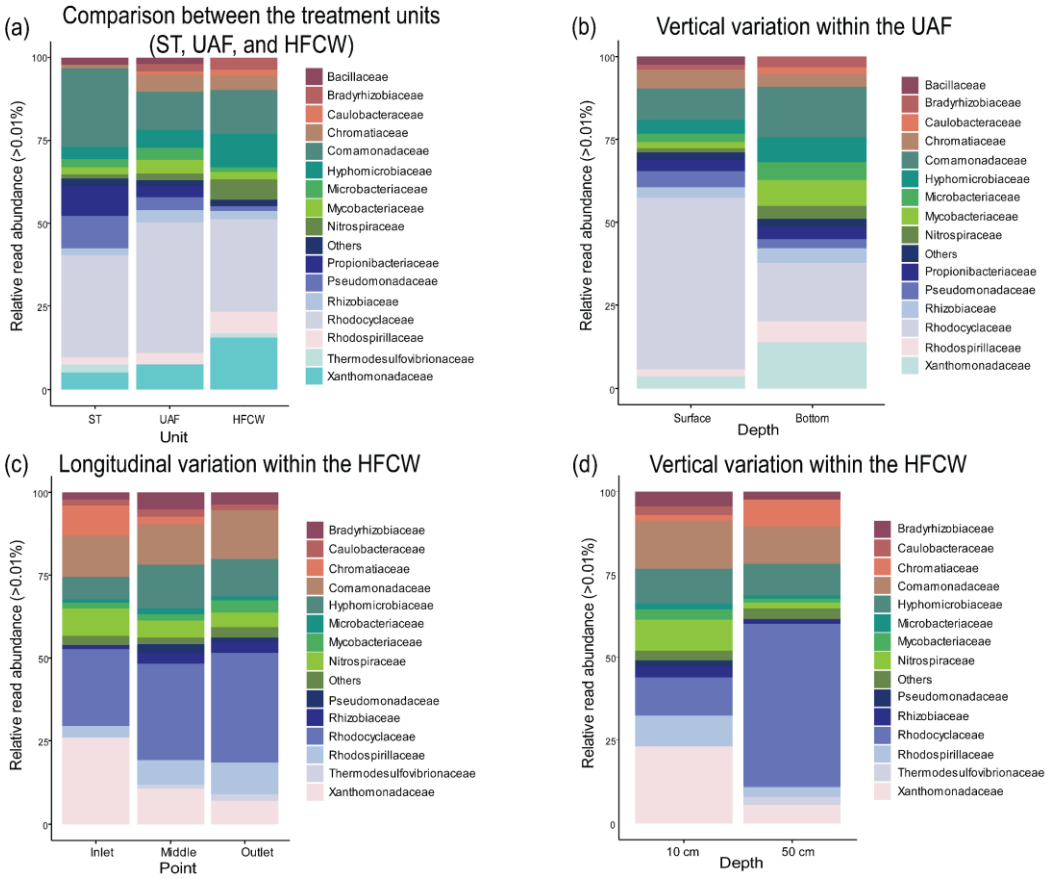

Figure 5. Nitrogen degrading families (**a**) Comparison between the treatment units (ST, UAF, and HFCW), (**b**) Vertical variation within the UAF, (**c**) Longitudinal variation within the HFCW, and (**d**) Vertical variation within the HFCW.

Previous studies have suggested the participation of these families in denitrification is affected by higher concentrations of DO [107,286]. This condition of higher levels of DO can partially be found in the HFCW, since plant roots can infiltrate oxygen to this part of the system [287]. The output of the ST contained the lowest concentration of DO of all the sampling points (0.26 mg/L), which may explain the higher abundance of these families in this treatment stage. The primary function

of ST is to separate the sludge, the effluent, and the scum layer from the wastewater, it removes suspended solids by retention and organic matter by anaerobic digestion [43,288], and reduces pathogen concentrations [13].

The *Chromatiaceae* were found to be present in the UAF at both depths (Figures 5b and 6b). The *Chromatiaceae* are a group of purple sulfur bacteria, commonly found in WWTPs, that utilize sulfide as an electron donor [289] and oxidize it to sulfate under anoxic conditions [290]. Therefore, the presence of this family suggests that denitrification and sulfate removal occur at the bottom of the UAF, and nitrification may occur in the UAF unit's upper layers.

The abundance of the family *Bacillaceae* was higher in the ST and the UAF than in HFCW (Table S7). This family is known to be involved in nitrification under aerobic conditions but under anaerobic conditions members are able to perform denitrification [150,291], suggesting that denitrification occurs in the ST, based on the low levels found in this unit.

In this study the families *Hyphomicrobiaceae*, *Nitrospiraceae*, *Rhodospirillaceae*, *Bradyrhizobiaceae,* and *Xanthomonadaceae* were present at higher abundance at all sampling points within the HFCW unit compared with those in the anaerobic reactors (ST and UAF; Figures 5a and 6a). Aerobic microenvironments may influence this within the HFCW, these being attributed to activity in the plant rhizosphere. Figure 6d shows the vertical variation in the composition of bacterial communities (families) when comparing depths (Figure S2d, ANOSIM R = 0.4933, Significance = 0.001) within the HFCW. The presence of plant roots and the permeation of atmospheric oxygen to a depth of 10 cm enable aerobic bacteria (such as nitrifying bacteria) to grow [292]. In contrast, at a depth of 50 cm anaerobic conditions allow other families to grow, such as denitrifying bacteria [43]. Within the HFCW, the *Nitrospiraceae*, *Rhizobiaceae*, and *Xanthomonadaceae* were the most abundant families at a depth of 10 cm (Figures 5d and 6d). These microorganisms have been regarded as nitrifiers, suggesting that nitrification occurs in the upper layers and near the plant roots, supporting microbial attachment, oxygen transfer to the rhizosphere, and root exudates [47]. At a depth of 50 cm, the *Rhodocyclaceae*, *Chromatiaceae,* and *Bacillaceae* occurred at a much higher abundance (in comparison to the upper layer, Figures 5d and 6d). Similarly, the family *Rhodocyclaceae* was more abundant at the outlet of the HFCW (Figures 5c and 6c).

The family *Rhodospirillaceae* displayed an increasing trend from the inlet to the outlet of the HFCW (Table S9) and was more abundant at a depth of 10 cm than at 50 cm (Figures 5c and 6c). This sulfur oxidizing bacteria is capable of degrading organic compounds under anaerobic conditions and can act as a chemotroph under aerobic conditions [293]. However, Meyer et al. [294] reported a higher abundance of the family *Rhodospirillaceae* (16%) in environments with higher oxygen concentrations during wastewater treatment. These families may be affected when oxygen availability is low, which explains the increase in their abundance in the HFCW, where plants are known to create aerobic microenvironments [233]. Even if the *Rhodospirillaceae* includes anaerobes, our results suggest that aerobic conditions, partially found in the HFCW, are preferred by most family members.

Longitudinal spatial variation within the HFCW were assessed by comparing samples from the inlet, the middle, and the outlet regions of the treatment unit (Figure S2 c; ANOSIM R = 0.2, Significance = 0.001). Variation was found in microbial family abundances from inlet to output, indicating that different metabolic pathways occur in each region of the HFCW. Families *Xanthomonadaceae* and *Nitrospiraceae* exhibited a decreasing trend from the inlet to the outlet (Figures 5c and 6c). These families are known to be aerobic bacteria [295] involved in nitrification [74] in significant symbiotic interactions with plants [296]. This decreasing trend could be related to the ammonia concentration which also decreased along the HFCW. The families *Bradyrhizobiaceae, Hyphomicrobiaceae*, *Pseudomonadaceae, Rhizobiaceae*, and *Rhodospirillaceae* presented higher abundances in the middle section of the HFCW than in the

inlet and outlet (Figures 5c and 6c). The abundance of these bacterial groups may be related to substrate availability as they require carbon sources for growth and their abundance falls as carbon sources are consumed [290,297]. However, a preference for specific carbon sources could be suggested as the highest abundance of these families were observed in the middle part of the HFCW, where the decomposition of complex molecules may generate a significant amount of product, which can in turn be processed by these specific bacterial groups [132,298]. Characterization of the carbon sources along the HFCW could be useful in coming to understand the relationships between the abundance of bacterial families and the availability of specific carbon products and should be considered in future studies.

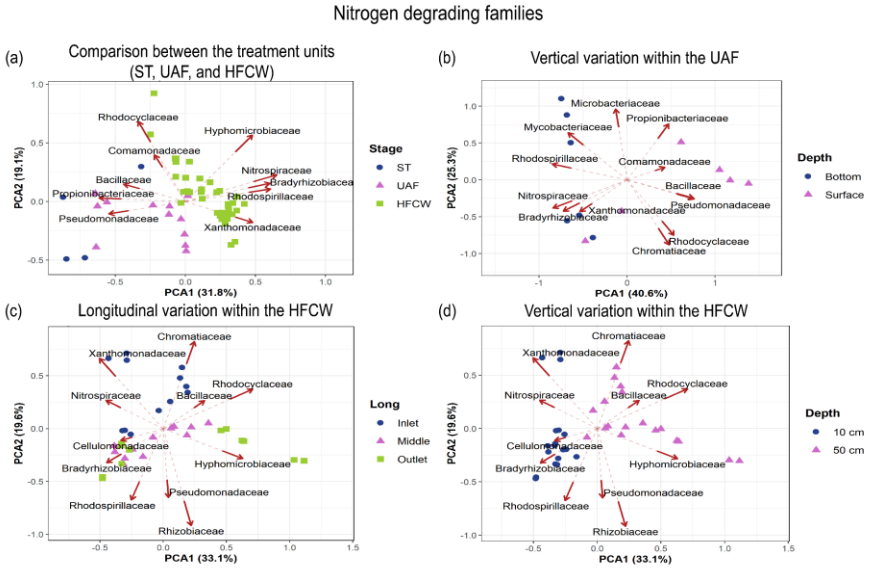

Figure 6. Biplots for nitrogen degrading bacterial families (a) Comparison between the treatment units (ST, UAF, and HFCW), (b) Vertical variation within the UAF. (c) Longitudinal variation within the HFCW (d) Vertical variation within the HFCW. For illustration purposes, only the families that appear in the discussion are shown.

3.3.3.2 Families degrading organic matter

The spatial variation of families that degrade organic matter within and between the treatment stages was analyzed (Figures 7 and 8; Tables S10–S13). The *Ruminococcaceae* was the most abundant family in the ST, followed by the *Bacteroidaceae*, *Lachnospiraceae,* and *Porphyromonadaceae* (Figures 7a and 8a). These families were also more abundant in the ST than in the UAF and HFCW. The most abundant family in the UAF was the *Syntrophaceae*, followed by the *Clostridiaceae* and *Geobacteraceae*. These families exhibited higher abundances in the UAF and were significantly less abundant in the HFCW. The families *Ruminococcaceae* and *Lachnospiraceae* have been related to the hydrolyzation of a variety of polysaccharides [299], acetogenesis [300], and fermentation [280], which are the first phases of degradation of the organic matter [165] present in raw wastewater. Thus, the higher abundance of these families found in the ST indicates that they degrade the more complex organic compounds present in the raw sewage into small molecules, later available for other microbial populations in the treatment units that follow. The *Porphyromonadaceae* has also been reported to be involved in the degradation of organic matter in anaerobic reactors [164,301]. The *Clostridiaceae* are commonly

reported to be highly abundant in the effluent of anaerobic reactors such as the up flow anaerobic sludge blanket reactor [302,303].
Desulfovibrionaceae, *Enterobacteriaceae*, *Mogibacteriaceae*, and *Moraxellaceae* were present in both the ST and the UAF (the anaerobic stages). In contrast, *Acetobacteraceae, Methylococcaceae*, and *Syntrophobacteraceae* were only found in the UAF and the HFCW, and have been referred to as anaerobic fermentative bacteria [304] and have previously been found in other HFCW [305]. Similarly, the families *Chitinophagaceae*, *Cytophagaceae*, *Methylophilaceae*, *Saprospiraceae*, and *Sphingomonadaceae* were found exclusively in the HFCW (Figures 7a and 8a).

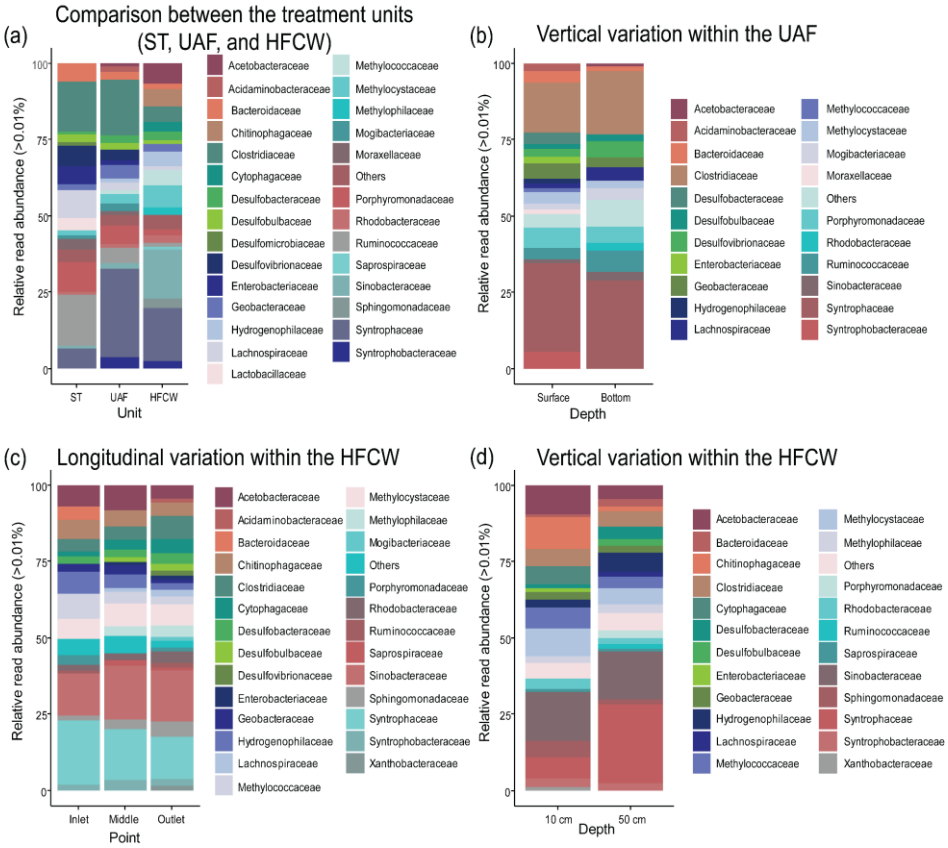

Figure 7. Organic matter degrading families (**a**) Comparison between the treatment units (ST, UAF, and HFCW), (**b**) Vertical variation within the UAF, (**c**) Longitudinal variation within the HFCW, and (**d**) Vertical variation within the HFCW.

Van Lier et al. [306] reported that hydrolysis is usually the first and limiting step in the removal of organic matter, as it converts complex substrates into monomeric and dimeric compounds that form the substrates fed to the reactors that follow, and thus hydrolysis determines the overall removal of organic matter in the WWTP [306]. Similarly, Rajagopal et al. [307] reported that a pre-treatment process, such as the ST, is necessary to carry out hydrolysis since the degradation of particulate organic matter is slow and affects the performance of the following processes. The results presented here suggest that the hydrolysis step is performed within the ST, producing substrates that are available for the treatment units that follow, where fermentative and methanogenic bacteria were found.
The presence of Betaproteobacteria class members, such as *Desulfobacteraceae* and *Desulfobulbacteraceae*, was observed in all three units, with similar abundance (Figures 7a and

8a). Members of the phylum Proteobacteria, such as Betaproteobacteria and Deltaproteobacteria, have been reported to participate in the sulfur cycle in CW [54] and anaerobic digestion processes [294,308]. In addition, Meyer et al. [294] found that the presence of sulfur, potassium, manganese, total nitrogen, and total phosphorus in the wastewater contributed to variation in the structure and composition of families related to the sulfur cycle. The presence of these families in our experimental system indicates the occurrence of the sulfur cycle and the reduction of inorganic sulfur compounds [305].

The microbial composition differed between the upper (output) and the lower (input) layers of the UAF (Figure S3b; ANOSIM R = 0.3056, Significance = 0.003). Families of obligate anaerobes were found in the lower layers of the UAF and facultative anaerobic bacteria were found in the upper layers. The abundance of families affected by oxygen, such as *Clostridiaceae*, *Desulfovibrionaceae*, *Mogibacteriaceae*, and *Sinobacteraceae,* decreased from the bottom to the surface (input to output) (Figures 7b and 8b). Other families, like the *Bacteroidaceae*, *Desulfobacteraceae*, *Geobacteraceae*, and *Methylocystaceae*, were more abundant at the surface (Figures 7b and 8b), indicating that they may include some facultative anaerobic species, the abundance of this families can be enhanced by a slight increase in oxygen levels. In addition to oxygen availability, it has been suggested that substrate availability and competitive interactions between microbial populations shape the distribution of the bacterial communities from inlet to outlet in anaerobic treatment units such as up flow anaerobic sludge blanket reactors [309,310].

Regarding vertical variation within the HFCW (Figures 7d and 8d), the families *Acetobacteraceae*, *Geobacteraceae, Hydrogenophilaceae*, and *Syntrophobacteraceae* presented a higher abundance at a depth of 10 cm than at a depth of 50 cm (Figure S3d, ANOSIM R = 0.4575, Significance = 0.001). In contrast, the families with a greater abundance at a depth of 50 cm were the *Clostridiaceae*, *Cytophagaceae*, and *Sphingomonadaceae* (Figure 8d). As previously mentioned, these changes may be related to the presence of root exudates and higher oxygen availability, which are more available at a depth of 10 cm. In the same vein, Krasnits et al. [311] reported that methanogenic bacteria and archaea were more strongly influenced by depth than by distance from the inlet in a study of the distribution of microbial communities in an HFCW. These authors attribute this tendency to the oxidation-reduction potential (ORP) within the HFCW. The ORP parameter was not considered in this study, but an examination of this parameter is suggested for further studies.

Liu et al. [312] reported an increasing trend for the family *Sphingomonadaceae* along the flow path of an HFCW and a decreasing trend along the flow path of a VFCW, attributing this behavior to the direction of water flow, and highlighted the influence that the type of CW exerts on the structure of the bacterial community. This fact is consistent with our results (Figures 7c and 8c) where *Sphingomonadaceae* increased in abundance from inlet to outlet along the HFCW. However, in this study, few longitudinal differences were found in the HFCW with respect to the families related to organic matter removal (Figure S3c, ANOSIM R = 1996, Significance = 0.001).

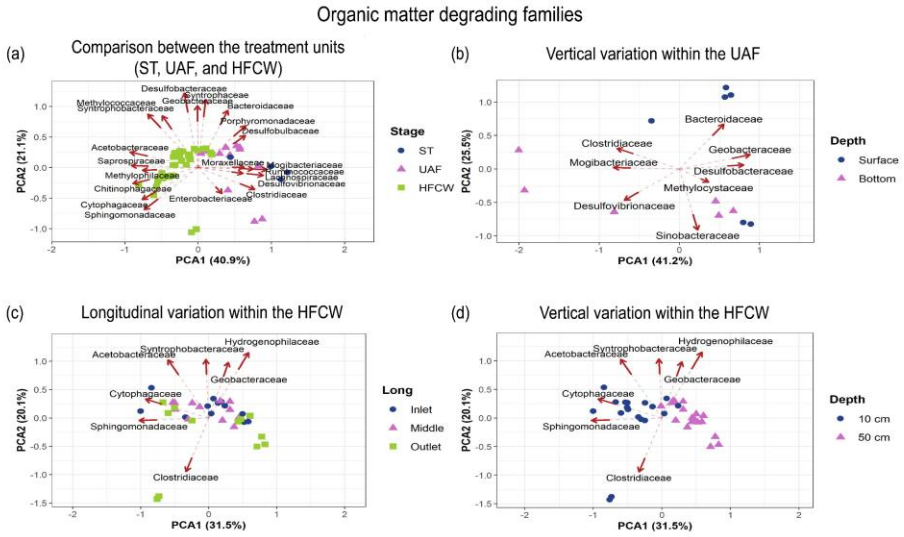

Figure 8. Biplots for organic matter degrading families (a) Comparison between the treatment units (ST, UAF, and HFCW), (b) Vertical variation within the UAF, (c) Longitudinal variation within the HFCW, (d) Vertical variation within the HFCW. For illustration purposes, only the families that appear in the discussion are shown

3.3.4 Effect of physicochemical parameters on bacterial communities

An RDA was performed to analyze the correlation between microbial families and the physicochemical parameters measured in the treatment stages (BOD_5, COD, TKN, NH_3-N, ON, TSS, NO_2^-, temperature, DO, pH, EC; NO_3^- was omitted to avoid redundance in the analysis as it presented collinearity with NO_2^-). The two main redundancy components explained 61% of the total variability in the bacterial community of nitrogen degrading families (a set of 18 families), while the two main redundancy components explained 70% of the total variability in the case of the families degrading organic matter. Figure 9 shows the RDA correlation triplot that explains the correlation between the physicochemical parameters, and selected bacterial families represented by a number (Table 2).

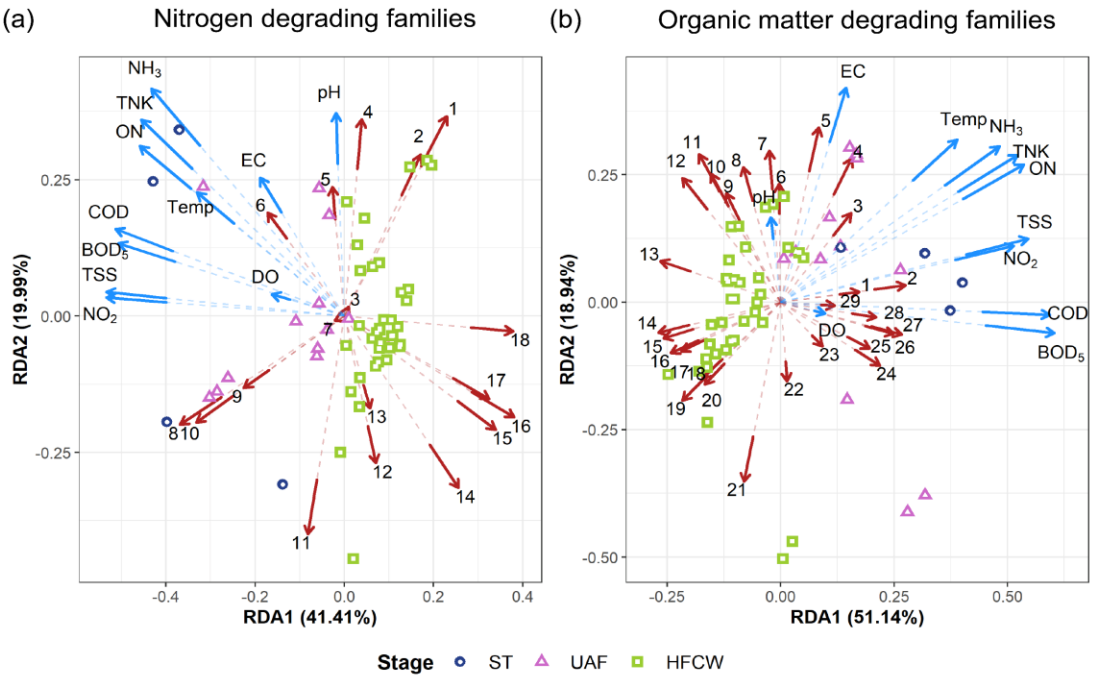

Figure 9. Redundancy analysis (RDA) showing correlations between physicochemical parameters and selected bacterial families (**a**) RDA for nitrogen degrading families, (**b**) RDA for organic matter degrading bacteria families. Each blue vector represents a physicochemical parameter and each red vector represents a bacterial family. Table 2 presents the bacterial families assigned to each number.

Table 2. Numbers assigned to bacterial families.

A) Nitrogen Degrading Families		**B) Organic Matter Degrading Families**			
Number	**Family**	**Number**	**Family**	**Number**	**Family**
1	*Xanthomonadaceae*	1	*Desulfobulbaceae*	19	*Methylophilaceae*
2	*Caulobacteraceae*	2	*Ruminococcaceae*	20	*Sphingomonadaceae*
3	*Comamonadaceae*	3	*Moraxellaceae*	21	*Rhodobacteraceae*
4	*Chromatiaceae*	4	*Porphyromonadaceae*	22	*Xanthobacteraceae*
5	*Mycobacteriaceae*	5	*Bacteroidaceae*	23	*Desulfomicrobiaceae*
6	*Microbacteriaceae*	6	*Syntrophaceae*	24	*Lachnospiraceae*
7	*Rhodocyclaceae*	7	*Geobacteraceae*	25	*Mogibacteriaceae*
8	*Pseudomonadaceae*	8	*Desulfobacteraceae*	26	*Clostridiaceae*
9	*Bacillaceae*	9	*Acidaminobacteraceae*	27	*Desulfovibrionaceae*
10	*Propionibacteriaceae*	10	*Syntrophobacteraceae*	28	*Lactobacillaceae*
11	*Rhizobiaceae*	11	*Hydrogenophilaceae*	29	*Enterobacteriaceae*
12	*Cellulomonadaceae*	12	*Methylococcaceae*		

13	*Thermodesulfovibrionaceae*	13	*Acetobacteraceae*
14	*Paenibacillaceae*	14	*Sinobacteraceae*
15	*Rhodospirillaceae*	15	*Saprospiraceae*
16	*Hyphomicrobiaceae*	16	*Chitinophagaceae*
17	*Bradyrhizobiaceae*	17	*Methylocystaceae*
18	*Nitrospiraceae*	18	*Cytophagaceae*

As mentioned above, the angles between the vectors of the bacterial families represent correlations between the families and angles between the vectors of water quality parameters represent correlations between these parameters (Figure 9). Likewise, angles between the vectors of bacterial families and the vectors of water quality parameters represent correlations between them. Additionally, points representing each observation (sequenced sample) can be projected perpendicularly on the vectors of families or the vectors of the physicochemical parameters and give an indication of their corresponding values in such observations. The origin represents the mean value, projections in the same direction as the vector indicate values above average, and projections in the opposite direction represent values below average.

For both nitrogen and organic matter degrading bacterial families, DO, EC, and temperature displayed a higher influence on the bacterial communities in the ST and the UAF than on the communities present in the HFCW (Figure 9a,b). Organic matter removal is performed mainly by ammonification and methanogenesis in the ST and the UAF. The RDA results suggested that temperature affected the nitrogen removal related families within the ST and the UAF. It has been reported that nitrification processes can occur within a wide temperature range (16.5 to 32.5 °C) with an optimal range of 20 to 25 °C [74]. As shown in Table 1, the temperatures recorded at the output of the ST and the UAF were 21.06 ± 1.97 °C and 20.91 ± 2.31 °C, respectively. Although the temperature of the anaerobic stages is within the optimal range for denitrification, slight temperature changes may cause variations in the composition of the communities related to nitrogen removal. Temperature is also essential for anaerobic digestion, especially for the hydrolysis of complex organic compounds, whose breakdown is highly sensitive to temperature [313]. Methanogenic activity is reduced 10-20 times at low temperature (<15 °C) in comparison to activity at 35 °C [314]. Advantageously, wastewater treatment systems in tropical or subtropical regions are less affected by this issue, since wastewater temperature remains stable at above 25 °C throughout the year [315].

EC is used to measure the number of ions relative to salinity, a characteristic of wastewater that significantly affects bacterial communities in treatment systems [316]. Figure 9b shows how EC affects the bacterial families related to organic matter degradation in specific samples of the UAF. The most abundant families in the UAF (of those related to organic matter degradation) were the *Syntrophaceae*, *Clostridiaceae, Sinobacteraceae*, *Geobacteraceae,* and *Ruminococcaceae* (Figure 7a)*,* suggesting that these bacteria are more sensitive to variation in the salinity of wastewater. All these families have been identified in high salinity environments. The families *Syntrophaceae* and *Clostridiaceae* have been found in an acclimated marine sediment-derived culture used for biomethane production under high salinity conditions [317]. The presence and overgrowth of *Sinobacteraceae* has been related to high salinity conditions in shrimp culture enclosure ecosystems [318]. Further, the member *Glk. subterraneus* of the family *Geobacteraceae,* is a halotolerant bacterium that has been associated with high current generation (> 1 A m^{-2}) in electroactive biofilms used for microbial fuel cell technology [319]. Finally, the relative abundance of *Ruminococcaceae* is known to increase in salt stress conditions

in a UAF used to produce methane from molasses wastewater [320]. The results found here suggest that our experimental system is tolerant of high salinity conditions, increasing its viability and applicability for treating different wastewater types.

Buffer capacity has been reported in anaerobic digestion processes, which explains why pH does not significantly affect bacterial communities in the anaerobic phases [321]. Higher nitrification rates have been reported in CW with pH ranges of 7.0–7.5 [321,322], while denitrification processes are optimal at a pH of 7.5 [323]. The pH measured at the outflow of the HFCW was 7.4 ± 0.4, which is within the optimal range for nitrification and methanogenic bacteria. The results also suggest that pH has a higher influence on communities in the HFCW than on those in the anaerobic stages (Figure 9; Table 2; Table S15 and S16). Variations in the pH of wastewater can cause stress in bacterial communities, as the intracellular pH of most microorganisms is close to neutral [324]. All the pH measurements recorded in this study (Table 1) were close to 7. However, variations in pH may occur inside the HFCW in specific regions (further pH measurements across the HFCW are required to prove this statement). Interestingly, bacterial communities at the inlet of the HFCW were the most sensitive to pH variation. The most abundant nitrogen degrading families were the *Rhodocyclaceae*, *Xanthomonadaceae*, *Chromatiaceae* and *Comamonadaceae* and the most abundant organic matter degrading families were *Syntrophaceae*, *Methylococcaceae*, *Bacteroidaceae*, and *Sinobacteraceae.* The *Rhodocyclaceae* and *Comamonadaceae* are denitrifying bacteria that use nitrate or oxygen as electron acceptors and short-fatty acids as electron donors [325], and these families have been reported to be the most abundant in other wetland systems [107,326]. Additionally, the *Xanthomonadaceae* is involved in forming microbial biofilm and granules as they participate in the production of extracellular polymeric substances [327]. Consequently, as these families are affected by pH variation, it is plausible that this parameter is a determining factor for nitrogen removal and the formation and stability of the wetland microenvironment.

It is reported that the *Syntrophaceae* family in syntrophic partnership with methanogens (*Methylococcaceae*) can degrade organics to H_2 and CO_2 to methane in methanogenic environments [328,329]. Therefore, manipulation of these families may prove useful in reducing the amount of methane produced by the system. However, further investigation is needed to determine the specific species involved in methane oxidation and the specific conditions required to favor their prevalence.

It can be observed in Figure 9 that vectors representing the concentrations of pollutants (BOD_5, COD, TKN, NH_3-N, ON, TSS, NO_3^-, NO_2^-) are grouped in one single quadrant (quadrant II for nitrogen degrading families and quadrant I for organic matter degrading families). The observations made in the ST can be projected perpendicularly onto the vectors of the physicochemical parameters and give an indication of their corresponding values in such observations. In this case, greater concentrations were always observed at the initial stage (ST) and lower concentrations are found the next two treatment stages (UAF and HFCW).

Temperature, closely linked to climatic variation, is located in the same quadrant as the vectors of the physicochemical parameters. Thus, the vectors of the bacterial families that point in the same direction or in the opposite direction to the vectors of the water quality parameters and temperature (forming small angles or close to plane angles) are highly influenced by the pollutant inlet concentrations and variations in climate. Conversely, the vectors of bacterial families that form perpendicular angles with the vectors of the water quality parameters indicate that these families are barely influenced by variations in the water quality parameters. The families related to nitrogen degradation that were barely influenced by the physicochemical parameters comprised the *Pseudomonadaceae*, *Bacillaceae*, *Propionibacteriaceae, Xanthomonadaceae, Caulobacteraceae,* and *Chromatiaceae*. Similarly, the *Syntrophaceae, Geobacteraceae, Desulfobacteraceae, Acidaminobacteraceae, Syntrophobacteraceae, Hydrogenophilaceae,*

Methylococcaceae, Xanthobacteraceae and *Desulfomicrobiaceae* were the organic matter degrading families that were mostly unaffected by water quality. It is important to note this group of bacteria can be considered to provide robustness to the treatment system.
Finally, correlations between specific bacterial families can be analyzed using the data presented in Figure 9. For example, a correlation between the *Sinobacteraceae* and the *Saprospiraceae* can be observed in Figure 9b, as the angle between them is close to zero. Similarly, these families are negatively correlated to nitrogen concentration (TKN, ON, and NH_3-N), suggesting the existence of a mutualistic interaction between them by which nitrogen degradation is enhanced, however, this type of interaction must be further investigated. Furthermore, the families *Sinobacteraceae* and *Saprospiraceae* have been reported to be consumers of organic matter.
In addition, it can be deduced from Figure 9a that the occurrence of the *Hyphomicrobiaceae* and the *Bradyrhizobiaceae* is positively correlated, and they are negatively correlated with COD and BOD_5 concentrations. These correlations prove the interconnection between biological pathways for pollutant degradation. For instance, nitrogen and organic matter degradation are intimately related in the denitrification pathway, through which an electron is transferred from carbonaceous compounds to gaseous nitrogen [330]. Additionally, proteins contained in organic matter represent a significant amount of organic nitrogen. Protein degradation causes the accumulation of different nitrogenous compounds in wastewater [331]. Future studies should focus on the complex interconnections of pollutant removal pathways and microbial interaction networks.

3.3.5 Bacterial communities in multi-stage WWTPs located in subtropical regions

To the best of our knowledge, very few studies have focused on analyzing the structure and diversity of microbial communities in multi-stage decentralized WWTPs treating domestic wastewater in tropical and subtropical regions. Bedoya et al. [17] reported the microbial communities within the biosolids of a centralized WWTP treating municipal wastewater in Colombia. This plant uses an activated sludge system to treat wastewater generated by approximately 500,000 people (influent ~1.8 m^3/s). The authors found that Proteobacteria (66%) was the major phylum, followed by Actinobacteria, Firmicutes and Bacteroidetes. Similar results were obtained in the present study, in which Proteobacteria were the most abundant in all three treatment units of the system (34% in the ST; 38% in the UAF; 60% in the HFCW). Bacteroidetes and Firmicutes were also some of the most abundant phyla in anaerobic reactors (ST and UAF); additionally, Bacteroidetes and Actinobacteria were highly abundant in the HFCW.
The study of Desta et al. [28] reported the microbial communities within a multi-stage system treating tannery wastewater located in Modjo, Ethiopia. The system was integrated with two anaerobic reactors, followed by one aerobic reactor and a vertical-flow constructed wetland (VFCW) planted with *Phragmites australis* as a final step. These authors reported a relative abundance of 53% for Firmicutes and 24% for Proteobacteria in the aerobic reactor, a relative abundance of 52% for Firmicutes and 14% for Proteobacteria in the anaerobic reactor, and 44% for both Firmicutes and Proteobacteria in the VFCW [28]. In contrast, in this study, Firmicutes presented a lower relative abundance in the ST (26%) and the UAF (21%), while Proteobacteria showed a higher relative abundance in the ST (34%) and the UAF (28%). Although the system reported by Desta et al. [28] and that described in the present study are both in a subtropical region, the differences in abundance of these important phyla could be attributed to the wastewater characteristics and differences in system configuration, since in Desta et al. [28] the system described included an aerobic reactor that functioned as a pretreatment for the VFCW.
Song et al. [29] studied six activated-sludge WWTPs located in different climatic regions (tropical, subtropical, and temperate) and reported that Proteobacteria, Bacteroidetes, Chloroflexi, Acidobacteria and Nitrospirae were the major phyla in the WWTPs analyzed. However,

Actinobacteria, Bacteroidetes, and Chloroflexi were more abundant in subtropical and temperate WWTPs compared to those in tropical regions. In the present study, Proteobacteria, Firmicutes, Bacteroidetes and Caldiserica were the most abundant phyla in the ST and the UAF, while Proteobacteria, Bacteroidetes, Actinobacteria and Chloroflexi were the most abundant in the HFWC. Based on these results, the microbial composition of the HFCW studied here is the most similar to that of the subtropical WWTPs studied by Song et al. [29]. Activated sludge systems and HFCWs are open processes highly affected by climate variations, while ST and UAF are confined spaces where more stable conditions can be maintained even with climate variability. In general, closed systems can present better conditions for bacteria susceptible to climatic variability. In the same study [29], the Nitrospirae phylum presented a higher abundance in moderately high temperatures, reaching higher nitrogen removal rates in tropical regions than in subtropical and temperate regions. This fact can be considered when improving the nitrogen removal rates in the decentralized system studied here. For example, a greenhouse could be installed to house the HFCW to provide higher temperatures inside the system, which could also enhance plant growth and allow for the cultivation of a wider variety of plants.

According to the RDA (Figure 9), family members of *Mycobacteriaceae*, *Microbacteriaceae, Moraxellaceae,* and *Porphyromonadaceae* are positively influenced by temperature. Conversely, temperature showed no significant effect on the families *Rhizobiaceae*, *Nitrospiraceae*, *Methylococcaceae* and *Desulfomicrobiaceae*. Previous studies have reported that the members of the family *Nitrospiraceae* are involved in nitrification, a process that may be affected by temperature changes in WWTPs located in regions with temperate climates. However, in tropical and subtropical regions, as in the case of the present study, *Nitrospiraceae* are not affected significantly by temperature, as the weather is warmer and more stable throughout the year. Accordingly, open systems, such as HFCWs, located in tropical/subtropical regions are adequate in maintaining more stable conditions for microbial communities throughout the year to enhance the wastewater bioremediation processes.

In general, a dominance of Proteobacteria, Actinobacteria, Bacteroidetes, Chloroflexi and Firmicutes can be found in tropical and subtropical regions, based on the studies of WWTPs located in these regions. However, the dominance of a specific phylum in these systems cannot be generalized, as their abundances vary widely from one study to another. Further studies in subtropical regions are required to generalize the microbial communities that may be expected in a WWTP, but these must also consider other factors that affect bacterial distribution, such as the type of wastewater, process features and design, and seasonality, among others. Detailed studies can also be useful in finding bacterial species that are favored in these regions and in developing strategies to use them to enhance the performance of decentralized technologies

3.4. Conclusions

Passive wastewater treatment technologies are especially appropriate for tropical and sub-tropical regions as the climatic conditions facilitate adequate and stable performance throughout the year. Furthermore, the cost and infrastructure required to implement these systems in developing countries are acceptable. This study's contribution lies in the detailed description of the presence and distribution of the bacterial communities throughout a complete wastewater treatment system composed of ST, UAF, and HFCW. The pollutant removal pathways implemented by specific bacterial families within the different treatment stages were elucidated. Additionally, we described possible microbial interactions that enhance the removal of specific pollutants, as well as the influence of physicochemical parameters on the composition of the bacterial communities.

The characterization of the spatial variation in the microbial communities in our experimental system provides an in-depth understanding of the complexity of bacterial communities in a subtropical WWTP that combines an ST, a UAF, and an HFCW, and lays the foundation for future studies where manipulation of these microbial communities can result in better WWTP performance. Future studies should also focus on the complex interconnections of pollutant removal pathways and microbial interaction networks.

Chapter 4: Conclusions and future perspectives

The decentralized system treating domestic wastewater achieves organic matter removal efficiencies of 90% for COD and BOD_5 and total nitrogen removal of 51%. The higher removal efficiencies could be attributed to the presence of bacteria related to organic degradation and nitrogen removal through the system. Besides, significant differences in the microbial community's composition were found between anaerobic reactors (ST and UAF) and HFCW. On the other hand, communities within anaerobic reactors show to be affected by physicochemical parameters compared to CW. However, it is essential to understand microbial communities' behavior in response to abiotic and biotic factors to completely understand wastewater treatment.

A deeper understanding of the critical environmental, operational, and design factors of CW can be useful to develop strategies to manipulate or control the structure of microbial communities and to shift the dominance of different microbial groups and enhance their activity to improve the performance of decentralized WWTP. Precise experiments manipulating these critical factors and assessing microbial behavior as well as the WWTP performance could prove useful to encounter key strategies to optimize WWTP efficiency. Future research should also focus on developing mathematical models able to predict the structure, diversity, and activity of microbial communities as useful tools to optimize the functioning of WWTP. This work provides an in-depth understanding of bacterial communities of a WWTP and may lead to future studies where manipulation of these microbial communities can result in optimal WWTP performance.

Further investigation is needed on how iron (Fe_2^+) contained in the tezontle or other types of substrate media may influence the behavior of microbial communities during wastewater treatment since the literature reports that iron can enhance the activity of bacteria related to the removal of nitrogen. In the same way, more experiments are needed to determine the adequate iron form that microorganisms can uptake and use, as well as if this form is found in the common types of substrate media already used for wastewater treatment.

Additionally, more research is needed to find out if CW can remove pathogenic microorganisms from treated wastewater and how CW are able to remove them without the aid of chlorination or physical mechanisms as a final treatment stage.

Appendix A: Analytic techniques for water quality analysis

A1. Biological oxygen demand (BOD_5)

Analysis of BOD_5 was performed according to NMX-AA-028-SCFI-2001.

Solutions:

1. Phosphate buffer: 8.5 g of monobasic potassium phosphate (KH_2PO_4), 21.5g of dibasic potassium phosphate (K_2HPO_4), 33.4g of dibasic sodium phosphate heptahydrate (Na_2HPO_4 $7H_2O$) and 1.7 g of ammonium chloride (NH_4Cl) dissolved at 1L. The pH of the solution should be 7.2
2. Magnesium sulfate solution: 22.5 g of magnesium sulfate heptahydrate ($MgSO_4$ $7H_2O$) dissolved in 1L of water.
3. Calcium chloride solution: 27.5 g of anhydrous calcium chloride ($CaCl_2$) dissolved in 1L of water.
4. Ferric chloride solution: 0.25 g of ferric chloride hexahydrate ($FeCl_3$ $6H_2O$) dissolved in 1L of water.
5. Sulfuric acid solution (0.1N): 2.8 mL of concentrated sulfuric acid (H_2SO_4) added in 500 mL of water, mixed and diluted to 1L.
6. Sodium hydroxide solution (0.1N): 4.0 g of sodium hydroxide (NaOH) dissolved in 1 L of water.
7. Sodium sulfite solution: 1.5 g of sodium sulfite (Na_2SO_3) dissolved in 1 L of water.
8. Glutamic acid-glucose solution: glucose and glutamic acid were dried at 103°C for one hour. 150 mg of glucose ($C_6H_{12}O_6$) and 150 mg of glutamic acid ($C_5H_9NO_4$) and added to 1L of water. This solution has a BOD5 of 198 mg/L.
9. Ammonium chloride solution: 1.15 g of ammonium chloride (NH_4Cl) dissolved in 500 mL of water. The pH was adjusted to 7.2 with a sodium hydroxide solution and it was measured at 1L. Contains 0.3 mg N/mL.
10. Water for dilution: For each liter of water add 1 mL of each of the following solutions: magnesium sulfate solution (2), calcium chloride solution (3), ferric chloride solution (4) and phosphate buffer solution (1).

Process:

Sampling and storage: Samples was stored at 4°C for no more than 24 hours.

pH control: pH must be adjusted between 6.5 to 7.5 with sulfuric acid or sodium hydroxide of adequate concentrations that does not dilute the sample more than 0.5%.

Inhibition of nitrification: Samples from biological treatment effluents, it is necessary to inhibit nitrification by adding 2-chloro-6-(trichloromethyl) pyridine at concentration of 10 mg/L to the sample.

Inoculum control: It is necessary to have a population of microorganisms capable of oxidizing the biodegradable organic matter in the sample. In some cases, samples do not contain sufficient microbial communities to oxidize organic matter. In these cases, inoculate dilution water (solution 10) with population of microorganisms. Determine the BOD5 of the inoculum as for any other sample, this is an inoculum control. From this value and a value known from the dilution of the inoculum (in water dilution, solution 10) determine the DO consumption of the inoculum. Ideally, make such solutions of the inoculum that the greatest amount of the results presents a decrease of at least 50% of the DO. The representation of the decrease in DO (mg/L) with respect to

milliliters of inoculum, a straight line whose slope corresponds to the decrease in DO per milliliter of the inoculum. The intersection of the abscissa axis (DO) represents the oxygen consumption caused by the dilution water and must be less than 0.1 mg/L. To determine the DO consumption of a sample, DO consumption of the inoculum is subtracted from the total DO consumption. The total DO uptake of the inoculated dilution water should range between 0.6 mg/L to 1 mg/L.

Dilution: dilutions that result in a residual DO greater than 1mg/L and OD uptake of at least 2 mg/L after 5 days of incubation produce the most reliable results. Make several dilutions (at least 3) for duplicates of the prepared sample to obtain a DO in this range. Recommendation: 1% to 5% for settle and raw wastewater, 5% to 25% for biologically treated effluent. Make dilutions in individual 300 mL Winkler flasks, using the dilution water as eluent.

Blank of dilution water: use a dilution water blank as a rough control of the quality of the dilution water without inoculum and the cleanliness of the incubation flasks. Along with each batch of samples, incubate a flask with dilution water. Determine the initial and final DO. DO consumption should not be greater that 0.2 mg/L and preferably not less than 0.1 mg/L.

Control of glucose-glutamic acid: check in each analytical batch the quality of the dilution water, the effectiveness of the inoculum and the analytical technique by BOD5 determinations in standard samples of known concentration. Determine the BOD5 of a 2% solution of the glucose-glutamic acid standard control solution using the same incubation process as for the samples and the blank.

Incubation and Do measurement: incubate BOD5 bottles containing blank, diluted samples, inoculum control and glucose-glutamic acid control for 5 days at 20 C. The DO of the samples should be measured with a membrane electrode before the incubation period starts and after the incubation period is finished, immediately after opening the bottles to avoid absorption of oxygen from the air by the sample.

Calculations:

Inoculum or dilutions are not used:

$$DBO_5 = OD_i - OD_5$$

Where: OD_i is the initial dissolved oxygen in mg/L, OD_5 is the dissolved oxygen on the fifth day in mg/L, BOD_5 is the resulting biological oxygen demand

Using dilution:

$$DBO_5 = \frac{OD_i - OD_5}{\%\ de\ dilución\ en\ decimales}$$

When using inoculum:

$$DBO_5 = (OD_i - OD_5) - \frac{C_1(B_1 - B_2)(V_t)}{C_2(V_m)}$$

Where: B_1 is the DO of the inoculum before incubation (mg/L); B_2 is the DO of the inoculum after incubation (mg/L); C_1 is the volume of inoculum in the sample; C_2 is the volume of the inoculum in the control inoculum; V_t is the total volume in the Winkler flask and V_m is the volume of inoculum used to inoculate.

A2. Chemical oxygen demand (COD)

Analysis of COD were performed according to NMX-AA-030/1-SCFI-2012.

Solutions:

1. Sulfuric acid solution (H_2SO_4) concentration of 4 mol/L: add approximately 500 mL of water, 220 mL of sulfuric acid (p= 1.84 g/mL) slowly and with caution. Let cool and dilute to 1 L.
2. Silver sulfate solution: add 10 g of silver sulfate (Ag_2SO_4) to 35 mL of water and dilute 965 mL of sulfuric acid (p= 1.84 g/mL) in portions. Leave 1 or 2 days for dissolution. Solubilization is facilitated by stirring.
3. Potassium dichromate solution (high concentration; $K_2Cr_2O_7$ = 0.04 mol/L), reference material solution, certified (when applicable). Add 100 mL of sulfuric acid (p=1.84 g/mL), allow cool and add 11.768 g potassium dichromate, dried at 105°C ± 2°C for 2 h and dissolve. Transfer the solution to a volumetric flask and dilute to 1 L. The solution is stable for at least 12 months.
4. Ferrous ammonia sulfate (FAS), standard solution, $[(NH_4)2Fe(SO_4)2{\cdot}6H_2O \approx 0.12$ mol/L. Dissolve 47 g of ferrous ammonia sulfate hexahydrate in water. Add 20 mL of sulfuric acid (p= 1.84 g/mL). Cool and dilute with water to 1 L. These solutions must be evaluated prior to their use, as follows:
    a. Diluir 10 mL de disolución de dicromato de potasio ($K_2Cr_2O_7$) = 0.04 mol/L or ($K_2Cr_2O_7$) = 0.004 mol/L with approximately 100 mL of 4 mol/L sulfuric acid. Titrant the solution with ammoniacal ferrous sulfate to be validated using 2 or 3 drops of ferroin as indicator
5. Potassium hydrogenphthalate (potassium biphthalate), reference material solution, c($KC_8H_5O_4$) =0.0021 mol/L. Weigh and dissolve 0.425 g of potassium hydrogen phthalate, dried at 105°C ± 2°C, in water and dilute to 1L. The solution has a theoretical γ(COD) of 500 mg/L. This solution is stable for at least six months when stored at approximately 4°C ± 2°C. Discard if crystallization or turbidity is observed.
6. Ferroin, indicator solution. Dissolve 0.7 g of iron sulfate heptahydrate (II) ($FeSO_4$ $7H_2O$) or 1 g of ferrous ammonium sulfate hexahydrate (NH_4 $2Fe$ $(SO_4)2$ H_2O) and stir until dissolved. Dilute to 100 mL. This solution is stable for several months when stores protected from light.

Procedure:

Measurement

Transfer 10 mL of the sample (dilute if required) to the reaction flask, add 0.4 g of mercury (II) sulfate, and add 5 mL of the potassium dichromate solution. Add boiling beads to the test portion (always 10 mL) and mix well. Analyze a test blank.

Slowly add 15 mL of the silver sulfate-sulfuric acid mixture and immediately then insert the flask into the condenser.

Bring the reaction mixture to a boil within 10 min and continue to boil for another 110 min ± 5 min.

Let the flask cool and rinse the condenser with a small amount of water. Separate the condenser and dilute the reaction mixture to approximately 75 mL and allow to cool to room temperature.

Titrate the excess of potassium dichromate with ferrous ammonia sulfate adding 1 or 2 drops of Ferroin as an indicator.

NOTE 1: The reaction mixture has to boil slightly without any flash evaporation. Flash bubbling indicates local overheating in the solution, which can lead to erroneous results. Flash evaporation can be caused by intense heating or inefficient boiling beads.

NOTE 2: Although the amount of ferroin added is not critical, it should be kept constant as much as possible. Consider the first sustained color change from blue-green to reddish brown as the end point, even though the blue-green color may reappear after a few minutes.

Calculations:

The chemical oxygen demand, COD, expressed as mass concentration (mg / L), is given by the following equation:

$$\gamma(COD) = \frac{\left(V_{FAS_b} - V_{FAS_m}\right) \cdot c_{FAS} \cdot M_O \cdot v_o}{V_m}$$

A3. Total Suspended Solids (TSS) and Total Dissolved Solids (TDS)

Analysis of TSS and TDS were performed according toNMX-AA-034-SCFI-2015

Reagents:

• Water: maximum conductivity 5.0 μS / cm at 25 ° C, and pH: 5.0 to 8.0.

Process:

Preparation of crucible: Place the crucibles in the oven at a temperature of 105 ° C for at least 20 min. Transfer the crucibles to the desiccator and let it cool for at least 20 min. NOTE: The handling of the crucibles during the analysis must be carried out at all times with the forceps. Weigh the crucibles and repeat the oven-desiccator cycle until obtaining a difference ≤ 0.0005 g in two consecutive weighing's. Record as m1, considering the last mass value for calculations.

Preparation of filtration device and / or drying supports: Use a 1.5 μm porosity fiberglass filter that adapts to the filtration and / or drying device and / or aluminum tray, with the help of tweezers, place it with the rough face up on the drying and / or filtration device. NOTE: Wet the filter with water to ensure that it adheres perfectly, only in case of using Gooch crucible. The drying support with the filter is placed in the oven at 105 ° C for at least 20 min, after which time it is transferred to a desiccator. Weigh the filter device and / or drying supports and repeat the oven-desiccator cycle until obtaining a difference ≤ 0.000 5 g in two consecutive weighing´s. Record as m2, considering the last mass value for calculations. Sample Preparation - Samples must be at room temperature when testing. Shake the samples to ensure homogenization.

Measurement of total dissolved solids (TDS): It is recommended to select the sample volume in such a way that the dry residue on the crucible is in a mass range of 2.5 mg to 200 mg. In the crucible previously brought to constant mass m1, filter an aliquot of the sample through a glass fiber filter in the crucible or filter device. Pour the aliquot into a prepared capsule and evaporate to dryness in the drying oven at 105 ° C or evaporate almost to dryness without boiling the sample,

on a hot plate. Place the crucible with the sample in the oven at 105 ° C for at least 1 h. Transfer the crucible to the desiccator to bring it to a constant mass, register as m5. NOTE: If after 1-hour moisture or liquid is still observed in the crucible, continue drying in the oven.

Measurement of total suspended solids (TSS): It is recommended to select the sample volume according to the characteristics of the sample. Homogenize the sample by vigorous shaking of the container, transfer immediately, and in one step, an adequate volume of sample to a graduated cylinder. Filter the sample: a) Through the filter placed in the Gooch crucible or b) Through the filter that is taken from the aluminum tray and placed in the filtration equipment with the help of tweezers m2. Rinse the cylinder with enough volume to carry out the solids and pour into the filter. NOTE: Some types of water contain materials that block the filter pores or reduce their diameter. This increases the filter time and the results are related to the volume of the sample. If such filter blockage is observed, the measurement should be repeated with a lower volume. The results should be interpreted considering the above. Insert the drying support with the filter in the oven at 105 ° C for at least 1 h, in case of using a drying support other than the Gooch crucible, carefully remove the filter from the filter equipment using tweezers. Then carry out a constant mass and record the mass obtained as m6.

Calculations:

$$TDS = \frac{(m_5 - m_1)}{V} 1\,000\,000$$

Where TDS are the total dissolved solids, in mg / L; m1 is the mass of the empty capsule, in g; m5 is the mass of the capsule with the dry residue of the filtered sample, in g, and V is the sample volume, in mL.

$$TSS = \frac{(m_6 - m_2)}{V} 1\,000\,000$$

TSS is the total suspended solids, in mg / L; m2 is the mass of the drying support with the filter before filtration, in g; m6 is the mass of the drying support with the filter, in g; V is the sample volume used in mL.

A4. Total Kjeldahl Nitrogen (TKN), Organic Nitrogen (ON) and Ammoniacal Nitrogen (NH_3-N)

Analysis of TKN, ON and NH_3-N were performed according to NMX-AA-026-SCFI-2010

Solutions:

• Sodium hydroxide-thiosulfate solution: weigh approximately 500 g of sodium hydroxide and 25 g of sodium thiosulfate pentahydrate, dissolve in water; allow to cool to room temperature and dilute to 1 L with water.

• Borate buffer solution: add 88 mL of 0.1 mol / L NaOH solution to 500 mL of 0.025 mol / L sodium tetraborate solution and dilute to 1 L with water.

• Sodium tetraborate solution (0.025 mol / L): weigh approximately 9.5 g of sodium tetraborate decahydrate and dilute to 1 L with water.

• Boric acid indicator solution: weigh approximately 20 g of boric acid, dissolve in 500 mL water, add 10 mL of the indicator mixture and dilute to 1 L. Store the solution in a plastic container or in a boron-free container. prepare monthly.

• Mix of indicators: weigh approximately 200 mg of methyl red indicator dilute to 100 mL with alcohol. Weigh out approximately 100 mg of methylene blue indicator and dilute to 50 mL with alcohol. Mix the two solutions in a glass bottle. Prepare monthly.

• Titrated sulfuric acid solution (≈ 0.006 mol / L): dilute 200 mL of the 0.03 mol / L sulfuric acid solution in 1 L of water. Titrate the sulfuric acid solution obtained with a solution of 30 mL of carbon dioxide-free water and 0.0318 g of anhydrous sodium carbonate, previously dried for 1 h at 140 ° C, and 2 drops of the methyl orange indicator; Titrate this solution with sulfuric acid until the indicator turns from yellow to tan. Calculate the exact mass concentration of the solution (1 mL = 0.28 mg of N-ammonia or organic).

• Reagent for digestion: weigh approximately 134 g of potassium sulfate and 7.3 g of anhydrous copper (II) sulfate, dissolve in 800 mL of distilled water, carefully add 134 mL of sulfuric acid. Allow to cool to room temperature and dilute the mixture to 1 L with water. Store the solution at a temperature of 20 ° C to avoid crystallization.

• Sodium hydroxide-thiosulfate reagent solution: weigh approximately 500 g of sodium hydroxide and 25 g of sodium thiosulfate pentahydrate, dissolve in water; allow to cool to room temperature and dilute to 1 L with water.

Procedure:

Clean the distillation equipment before use, distilling a mixture (1: 1) water + sodium hydroxide-thiosulfate solution until the distillate is free of ammonia. This operation must be carried out every time the appliance is out of service.

Ammoniacal nitrogen: Determine the volume of the sample according to Table 1A, if necessary, adjust the volume to approximately 500 mL and neutralize to pH 7, with sodium hydroxide 12.5 mol / L or sulfuric acid 5 mol / L. Place the measured sample in an 800 mL Kjeldahl flask.

Table 1A. Sample Volume Section.

Mass concentration of nitrogen in the sample (mg/L)	Sample Volume (mL)
0-1	500
1-10	250
10-20	100
20-50	50
50-100	25

Add 25 mL of the borate buffer and adjust the pH to 9.5 with 6 mol / L sodium hydroxide solution using a potentiometer or indicator paper to verify. Add a few glass beads or boiling beads to the Kjeldahl flask. Connect the Kjeldahl flask to the condenser, distill the sample taking care that the

condenser temperature does not exceed 302 K (29 ° C). Collect the condensate in a container containing 50 mL of the boric acid indicator solution, submerging the tip of the condenser or an extension thereof below the surface of the liquid. Distillation is complete when approximately 300 mL of distillate has been collected, including 50 mL of the boric acid indicator solution. Remove the collecting flask and titrate with 0.006 mol / L sulfuric acid titrant until the indicator turns from emerald green to purple. Record the spent volume of acid as volume A.

Organic Nitrogen: cool the residue contained in the Kjeldahl flask. Digestion: Carefully add 50 mL of digestion reagent to the Kjeldahl flask and mix thoroughly. Add a few glass beads or boiling stones. Mix and connect to the Kjeldahl team; allow the sample to boil until the volume of the solution is reduced to approximately 25 mL to 50 mL and a large release of white vapors is observed (these vapors may darken when the sample contains large amounts of organic matter). NOTE: If the sample contains a significant amount of suspended material, add an additional 50 mL of Digestion Reagent. Continue digestion for a further 30 min. During this period, the solution changes from cloudy until it is clear and colorless or slightly pale yellow. During digestion, the Kjeldahl flask must remain tilted. Cool the flask and its contents, dilute to 300 mL with water, and mix. Carefully add 50 mL of the sodium hydroxydothiosulfate solution to form an alkaline layer at the bottom of the flask. Mix vigorously and verify with test strips that the pH of the solution is greater than 11 pH units. Connect the Kjeldahl flask to the condenser, distill the sample taking care that the condenser temperature does not exceed 302 K (29 ° C). Collect the condensate in a container containing 50 mL of the boric acid indicator solution, submerging the tip of the condenser or an extension of it below the surface of the liquid. Remove the collecting flask and titrate with 0.006 mol / L sulfuric acid titration solution until the solution turns from emerald green to purple. Record the spent volume of acid as volume C.

Blank: use 500 mL of distilled water instead of the sample and carry out all the steps of the procedure. The volume spent on the titration of the blank, record it as volume B.

Calculations:

Calculate the mass concentration of ammonia nitrogen, in mg / L in the sample as follows:

$$\gamma_{(N-NH_3)} = (V_A - V_B) \cdot c(H_2SO_4) \cdot A_r(N)/V_m$$

Where, $\gamma_{(NH3-N)}$ is the mass concentration of ammonia nitrogen; V_A is the mL of sulfuric acid spent in the titration of the sample; V_B is the mL of sulfuric acid spent on the blank; c (H2SO4) is the concentration of sulfuric acid in mol / L; Vm are the mL of sample, and Ar (N) are the atomic mass of nitrogen.

Calculate the mass concentration of organic nitrogen, in mg / L in the sample as follows:

$$\gamma_{(NOrg)} = (V_C - V_B) \cdot c(H_2SO_4) \cdot A_r(N)/V_m$$

Where, VC are the mL of sulfuric acid spent in the titration of the sample after the digestion process.

Calculate the mass concentration of total Kjeldahl nitrogen, in mg / L in the sample as follows:

$$\gamma_{(NTK)} = \gamma_{(N-NH_3)} + \gamma_{(NOrg)}$$

Where, $\gamma_{(NTK)}$ is the mass concentration of total nitrogen Kjeldahl.

A5. Total Phosphorus (TP)

Analysis of TP were performed according to NMX-AA-029-SCFI-2001

Reagents:

• Phosphate stock solution: weigh 219.5 mg of anhydrous monobasic potassium phosphate previously dried at 105 ° C for two hours, make up to 1 L with water; 1.0 mL = 50.0 µg of P as PO43-.

• Strong acid solution: carefully add 300 mL of concentrated sulfuric acid to approximately 600 mL of water. Allow to cool and add 4 mL of concentrated nitric acid and make up to 1 L with water.

• Solution A. Accurately weigh 25 g of ammonium heptamolybdate and dilute in 300 mL of water.

• Solution B. Accurately weigh 1.25 g of ammonium metavanadate and dilute in 300 mL of distilled water, heat to boiling. Cool and add 330 mL of concentrated hydrochloric acid. Let cool to room temperature.

• Vanado-molybdate reagent solution: Add solution A to solution B, mix and make up to 1 L.

Procedure:

Sample collection, preservation and storage: Take a minimum of 500 mL of sample in plastic containers. Store in the refrigerator at 4 ° C and in the dark. The maximum storage time prior to testing is 28 days.

Calibration: A calibration curve is made for a working range between 1.0 mg / L to 20.0 mg / L: Prepare the standard solutions of the phosphate stock solution with a minimum of 4 points in the range from 1.0 mg / L to 20.0 mg / L in 100 mL volumetric flasks and develop the color as indicated below.

Sample digestion: Use 50 mL or the appropriate portion of the sample well mixed. Add a drop of phenolphthalein. If a red color appears, add sulfuric acid drop by drop until the color disappears. Subsequently add 1 mL of strong acid solution and 0.4 g of ammonium persulfate or 0.5 g of potassium persulfate. Heat until boiling and keep it on the heating plate, for 30 min or 40 min or until the final volume reached is 10 mL. Organophosphate compounds may require 1.5 to 2 hours for complete digestion. Cool and dilute to 30 mL with water, add a drop of phenolphthalein, and neutralize until fading to a pale pink color with the sodium hydroxide solution (1N). Make up to 100 mL with distilled water. In some samples a precipitate may form at this stage but should not be filtered. Mix well for any subdivision of the sample. The precipitate (possibly calcium phosphate) is resolved under the acidic conditions of the colorimetric test for phosphorus.

Vanadomolybdophosphoric acid method: Adjust the pH of the sample. If the sample has a pH greater than 10, add a drop of phenolphthalein to 50 mL of the sample and then remove the pink color with a hydrochloric acid solution (1: 1), before diluting to 100 mL. Remove excess color in the samples by adding 200 mg of activated carbon to a 50 mL sample in an Erlenmeyer flask and shake for 5 min, then filter to remove the activated carbon. Check the phosphates of each batch of activated carbon. Take an aliquot containing from 0.05 mg to 1.0 mg of phosphorus, in a 50 mL volumetric flask. Add 10 mL of the vanado-molybdate reagent solution and dilute to the mark with water (50 mL). Prepare a blank using an amount of water equal to the sample aliquot. After

10 min or more, measure the absorbance of a sample against a blank at a wavelength of 400 nm to 490 nm, depending on the desired sensitivity (Table 2A).

Table 2A. Wavelenght of phosphate range

Range of P in mg/L	Wavelenght (nm)
1.0 – 5.0	400
2.0 – 10	420
4.0 - 20	470

The color is stable for days and its intensity is not affected by variations in ambient temperature.

Calculations:

Calculate the concentration of the sample by means of the equation obtained from the calibration curve and which is represented by the following equation:

$$Y = mX + b$$

Where, m is the slope; b is the ordinate to the origin; Y is the absorbance, and X is the concentration (mg P / L). In case of dilution of the sample throughout the development of the method (digestion and sample aliquot), use the following equation:

$$mg\frac{P}{L} = concentration \cdot Dilution\ Factor$$

Report the results in mg P / L to two tenths, with the corresponding precision.

Appendix B: Rarefaction curve and statistical analysis

Table S1. Removal efficiencies of the overall system and for stage

	Removal efficiency (%)				Mass reduction (mg/L)			
Parameter	ST	UAF	HFCW	OS	ST	UAF	HFCW	OS
BOD_5	33 ± 07	57 ± 17	64 ± 08	90 ± 05	201.6 ± 60.2	243.2 ± 105.1	103.3 ± 22.1	548.1 ± 117.0
COD	35 ± 05	55 ± 16	67 ± 10	90 ± 05	330.8 ± 92.8	345.2 ± 136.9	171.0 ± 37.0	847.1 ± 181.0
TSS	73 ± 04	41 ± 15	78 ± 05	97 ± 00	257.7 ± 65.2	38.8 ± 15.0	42.5 ± 11.4	339.0 ± 73.3
TNK	12 ± 11	12 ± 03	36 ± 07	51 ± 05	42.3 ± 37.7	36.2 ± 8.4	94.6 ± 25.9	173.0 ± 20.3
NH_4	07 ± 10	08 ± 04	35 ± 08	45 ± 04	11.4 ± 15.7	10.9 ± 6.9	46.3 ± 13.5	68.5 ± 7.2
ON	17 ± 13	17 ± 03	37 ± 07	57 ± 08	32.7 ± 25.6	25.3 ± 2.4	48.3 ± 12.8	106.2 ± 17.0
NO_2	53 ± 32	44 ± 15	63 ± 08	91 ± 05	1.90 ± 1.82	0.45 ± 0.22	0.33 ± 0.08	2.7 ± 1.7
NO_3	44 ± 15	67 ± 14	74 ± 13	95 ± 02	0.12 ± 0.05	0.10 ± 0.04	0.03 ± 0.02	0.2 ± 0.1

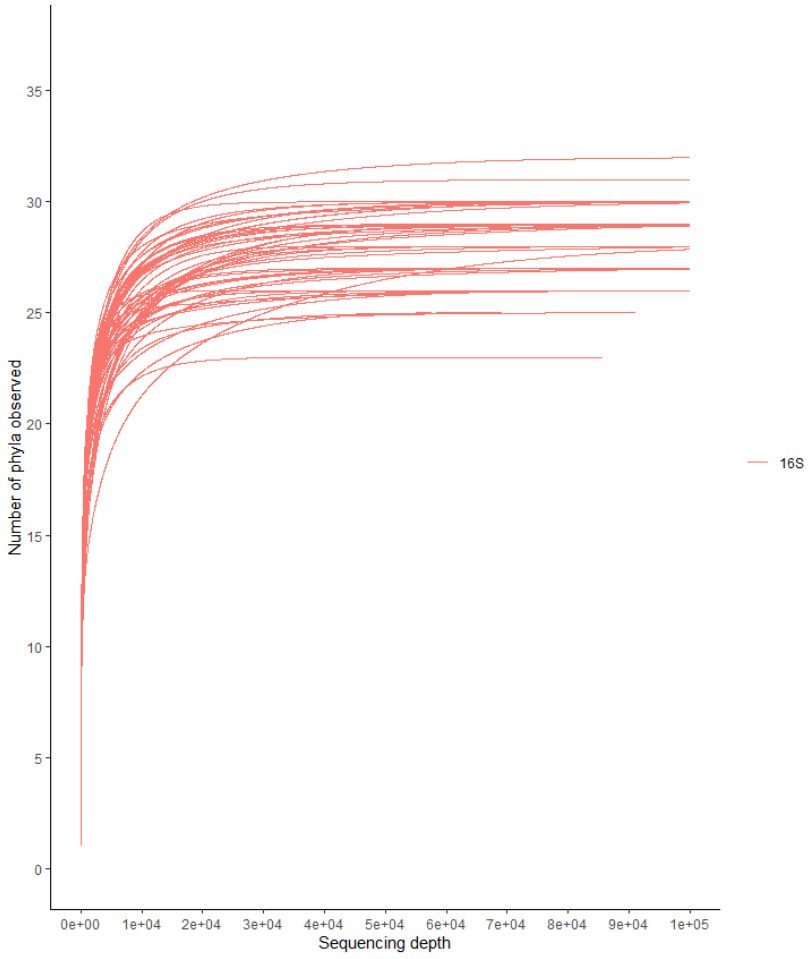

Figure S1. Rarefaction curve of 16S rRNA gene samples.

Table S2. Percentage of reads classified as bacterial phyla > 1% using Greengenes 13_8. Comparison between the three treatment stages (ST, UAF and HFCW) for nitrogen degrading families.

	Greengenes %		
Phyla > 1%	**ST**	**UAF**	**HFCW**
Proteobacteria	33.92%	37.81%	59.85%
Acidobacteria	2.05%	1.78%	3.96%
Actinobacteria	5.68%	5.41%	7.61%
Bacteroidetes	13.66%	8.81%	9.04%
Caldiserica	5.69%	13.97%	-
Chloroflexi	1.62%	1.12%	4.45%
Cyanobacteria	-	-	3.06%
Firmicutes	25.49%	20.81%	4.23%
Synergistetes	2.51%	4.05%	-
Gemmatimonadetes	-	-	1.15%
Nitrospirae	-	-	1.98%
Un Bacteria	6.59%	3.93%	1.01%
Others	2.78%	2.32%	3.66%

Table S3. Percentage of reads classified as bacteria phyla > 1% using Greengenes 13_8. Vertical variations within the UAF.

	Greengenes %	
Phyla > 1%	**Surface**	**Bottom**
Proteobacteria	40.79%	33.27%
Acidobacteria	1.35%	2.43%
Actinobacteria	4.57%	6.68%
Bacteroidetes	10.02%	6.96%
Caldiserica	15.31%	11.93%
Chloroflexi	1.31%	-
Firmicutes	19.73%	22.44%
Synergistetes	3.16%	5.41%
Un Bacteria	2.21%	6.53%
Others	1.54%	4.34%

Table S4. Percentage of reads classified as bacteria phyla > 1% using Greengenes 13_8. Vertical variations within the HFCW.

	GreenGenes %	
Phyla > 1%	**10 cm**	**50 cm**
Proteobacteria	58.65%	61.03%
Acidobacteria	4.75%	3.18%
Actinobacteria	10.02%	5.20%
Bacteroidetes	6.00%	12.07%
Chloroflexi	2.81%	6.08%
Cyanobacteria	5.83%	-
Firmicutes	3.37%	5.08%
Gemmatimonadetes	1.70%	-
Nitrospirae	2.97%	-

Spirochaetes	-	1.21%
Un Bacteria	-	1.20%
Others	3.89%	4.94%

Table S5. Percentage of reads classified as bacteria phyla > 1% using Greengenes 13_8. Longitudinal variations within the HFCW (Inlet, Middle and Outlet).

	GreenGenes %		
Phyla > 1%	**Inlet**	**Middle**	**Outlet**
Proteobacteria	60.08%	60.28%	59.15%
Acidobacteria	3.48%	4.30%	4.15%
Actinobacteria	5.85%	8.07%	9.05%
Bacteroidetes	11.02%	9.08%	6.85%
Chloroflexi	4.26%	5.98%	3.12%
Cyanobacteria	4.47%	1.27%	3.35%
Firmicutes	3.45%	3.38%	5.94%
Gemmatimonadetes	-	1.55%	1.22%
Nitrospirae	2.56%	1.65%	1.67%
Spirochaetes	-	-	1.00%
Un Bacteria	-	-	1.14%
Others	4.84%	4.43%	3.37%

Table S6. Percentage of reads classified as bacteria family > 1% using Greengenes 13_8. Comparison between the three treatment stages (ST, UAF and HFCW) regarding the nitrogen degrading families.

	GreenGenes %		
Family > 1%	**ST**	**UAF**	**HFCW**
Microbacteriaceae	2.40%	3.58%	1.35%
Mycobacteriaceae	2.20%	4.11%	2.35%
Propionibacteriaceae	9.15%	3.41%	-
Bacillaceae	2.04%	1.96%	-
Nitrospiraceae	1.19%	2.12%	6.10%
Bradyrhizobiaceae	-	2.07%	3.55%
Thermodesulfovibrionaceae	2.45%	-	1.28%
Hyphomicrobiaceae	3.59%	5.37%	10.15%
Caulobacteraceae	-	1.18%	1.79%
Rhizobiaceae	1.86%	3.76%	2.46%
Rhodospirillaceae	2.10%	3.60%	6.55%
Comamonadaceae	23.85%	11.64%	13.05%
Rhodocyclaceae	30.96%	39.23%	28.00%
Pseudomonadaceae	9.76%	4.03%	1.53%
Xanthomonadaceae	5.09%	7.41%	15.49%
Chromatiaceae	1.07%	4.97%	4.51%
Others	2.27%	1.56%	1.85%

Table S7. Percentage of reads classified as bacteria family > 1% using Greengenes 13_8. Vertical variations of nitrogen degrading families within the UAF.

Family > 1%	**GreenGenes %**

	Surface	Bottom
Microbacteriaceae	2.48%	5.50%
Mycobacteriaceae	2.04%	7.69%
Propionibacteriaceae	3.26%	3.68%
Bacillaceae	2.51%	-
Nitrospiraceae	1.03%	4.00%
Bradyrhizobiaceae	1.41%	3.20%
Hyphomicrobiaceae	4.18%	7.42%
Caulobacteraceae	-	1.84%
Rhizobiaceae	3.26%	4.63%
Rhodospirillaceae	2.10%	6.20%
Comamonadaceae	9.53%	15.30%
Rhodocyclaceae	51.72%	17.62%
Pseudomonadaceae	4.91%	2.52%
Xanthomonadaceae	3.65%	13.93%
Chromatiaceae	5.55%	3.96%
Others	2.37%	2.52%

Table S8. Percentage of reads classified as bacteria family > 1% using Greengenes 13_8. Vertical variations of nitrogen degrading families within the HFCW.

	GreenGenes %	
Family > 1%	**10 cm**	**50 cm**
Microbacteriaceae	1.58%	1.04%
Mycobacteriaceae	3.20%	1.25%
Nitrospiraceae	9.47%	1.71%
Thermodesulfovibrionaceae	-	2.27%
Bradyrhizobiaceae	4.46%	2.35%
Hyphomicrobiaceae	10.67%	9.49%
Caulobacteraceae	2.51%	-
Rhizobiaceae	3.07%	1.67%
Rhodospirillaceae	9.30%	2.96%
Comamonadaceae	14.41%	11.27%
Rhodocyclaceae	11.71%	49.20%
Pseudomonadaceae	2.02%	-
Xanthomonadaceae	23.08%	5.61%
Chromatiaceae	1.74%	8.11%
Others	2.77%	3.08%

Table S9. Percentage of reads classified as bacteria family removal > 1% using Greengenes 13_8. Longitudinal variations of nitrogen degrading families within the HFCW (Inlet, Middle and Outlet).

	GreenGenes %		
Family > 1%	**Inlet**	**Middle**	**Outlet**
Microbacteriaceae	1.12%	1.80%	1.15%
Mycobacteriaceae	1.60%	2.01%	3.69%
Nitrospiraceae	8.31%	5.00%	4.40%
Thermodesulfovibrionaceae	-	1.23%	2.12%

Bradyrhizobiaceae	2.19%	5.05%	3.70%
Hyphomicrobiaceae	6.82%	13.07%	11.36%
Caulobacteraceae	1.55%	2.23%	1.62%
Rhizobiaceae	1.23%	3.17%	3.30%
Rhodospirillaceae	3.51%	7.47%	9.52%
Comamonadaceae	12.52%	12.24%	14.60%
Rhodocyclaceae	23.21%	29.07%	33.09%
Pseudomonadaceae	-	2.66%	1.28%
Xanthomonadaceae	26.10%	10.61%	6.89%
Chromatiaceae	9.12%	2.27%	-
Others	2.71%	2.12%	3.27%

Table S10. Percentage of reads classified as bacteria family > 1% using Greengenes 13_8. Comparison between the three treatment stages (ST, UAF and HFCW) for organic matter degrading families.

	GreenGenes %		
Family > 1%	**ST**	**UAF**	**HFCW**
Bacteroidaceae	6.07%	2.82%	1.96%
Cytophagaceae	-	-	3.05%
Chitinophagaceae	-	-	5.53%
Porphyromonadaceae	9.79%	6.07%	1.82%
Saprospiraceae	-	-	1.11%
Acidaminobacteraceae	-	1.70%	-
Clostridiaceae	16.36%	17.97%	5.20%
Lachnospiraceae	9.12%	2.60%	1.33%
Lactobacillaceae	4.15%	-	-
Mogibacteriaceae	1.36%	2.47%	-
Ruminococcaceae	16.45%	4.95%	1.13%
Acetobacteraceae	-	1.06%	6.66%
Methylocystaceae	1.37%	3.36%	6.98%
Rhodobacteraceae	1.02%	1.21%	2.56%
Shingomonadaceae	-	-	3.18%
Hydrogenophilaceae	-	1.19%	4.62%
Enterobacteriaceae	5.74%	1.62%	-
Methylophilaceae	-	-	2.60%
Methylococcaceae	-	1.19%	5.06%
Moraxellaceae	3.59%	1.20%	-
Sinobacteraceae	1.12%	1.91%	15.82%
Desulfobacteraceae	1.02%	2.68%	2.79%
Desulfobulbaceae	2.55%	2.00%	1.41%
Desulfomicrobiaceae	1.01%	-	-
Desulfovibrionaceae	6.81%	3.46%	-
Geobacteraceae	2.01%	4.42%	2.48%
Syntrophaceae	6.55%	28.82%	17.45%
Syntrophobacteraceae	-	3.88%	2.45%
Others	3.91%	3.44%	4.82%

Table S11. Percentage of reads classified as bacteria family > 1% using Greengenes 13_8 involved in organic degradation at vertical variations within the UAF.

	GreenGenes %	
Family > 1%	**Surface**	**Bottom**
Bacteroidaceae	3.68%	1.27%
Porphyromonadaceae	6.38%	5.51%
Acidaminobacteraceae	2.59%	-
Clostridiaceae	16.37%	20.85%
Lachnospiraceae	1.53%	4.52%
Mogibacteriaceae	1.82%	3.63%
Ruminococcaceae	3.89%	6.86%
Acetobacteraceae	-	1.18%
Methylocystaceae	3.88%	2.42%
Hydrogenophilaceae	1.53%	-
Enterobacteriaceae	1.97%	-
Methylococcaceae	1.36%	-
Moraxellaceae	1.54%	-
Rhodobacteraceae	-	2.50%
Sinobacteraceae	1.34%	2.92%
Desulfobacteraceae	3.71%	-
Desulfobulbaceae	1.80%	2.35%
Desulfovibrionaceae	2.51%	5.16%
Geobacteraceae	5.12%	3.16%
Syntrophaceae	28.83%	28.81%
Syntrophobacteraceae	5.62%	-
Others	4.52%	8.84%

Table S12. Percentage of reads classified as bacteria > 1% using Greengenes 13_8 involved in organic degradation of vertical variations within the HFCW depth.

	GreenGenes %	
Family > 1%	**10 cm**	**50 cm**
Bacteroidaceae	1.00%	2.73%
Cytophagaceae	6.14%	-
Prophyromonadaceae	-	2.67%
Chitinophagaceae	10.44%	-
Saprospiraceae	1.25%	1.00%
Clostridiaceae	5.50%	4.95%
Lachnospiraceae	-	1.65%
Ruminococcaceae	-	1.38%
Acetobacteraceae	9.42%	4.40%
Methylocystaceae	9.03%	5.30%
Rhodobacteraceae	3.27%	1.97%
Sphingomonadaceae	5.33%	1.41%
Xanthobacteraceae	1.42%	-
Hydrogenophilaceae	2.52%	6.33%
Methylophilaceae	2.10%	3.00%
Enterobacteriaceae	1.21%	-

Methylococcaceae	6.84%	3.61%
Sinobacteraceae	15.98%	15.69%
Desulfobacteraceae	1.11%	4.17%
Desulfobulbaceae	-	2.07%
Geobacteraceae	2.75%	2.26%
Syntrophaceae	6.96%	26.04%
Syntrophobacteraceae	2.52%	2.40%
Others	5.20%	5.43%

Table S13. Percentage of reads classified as bacteria family > 1% using Greengenes 13_8. Longitudinal variations of organic matter degrading families within the HFCW (Inlet, Middle and Outlet).

	GreenGenes %		
Family > 1%	**Inlet**	**Middle**	**Outlet**
Bacteroidaceae	4.16%	-	-
Cytophagaceae	1.47%	3.26%	4.69%
Chitinophagaceae	6.38%	5.44%	4.62%
Porphyromonadaceae	3.08%	-	1.23%
Saprospiraceae	-	1.87%	1.03%
Acidaminobacteraceae	-	-	1.12%
Clostridiaceae	4.08%	4.26%	7.47%
Lachnospiraceae	-	1.28%	2.10%
Mogibacteriaceae	-	-	1.27%
Ruminococcaceae	4.08%	-	1.52%
Acetobacteraceae	1.12%	8.17%	4.55%
Methylocystaceae	7.15%	7.49%	6.81%
Rhodobacteraceae	6.69%	2.37%	3.71%
Sphingomonadaceae	1.73%	3.11%	4.93%
Xanthobacteraceae	-	-	1.48%
Hydrogenophilaceae	7.08%	4.13%	2.20%
Methylophilaceae	-	3.22%	3.92%
Enterobacteriaceae	-	1.05%	1.25%
Methylococcaceae	8.21%	3.84%	2.60%
Sinobacteraceae	13.70%	17.43%	16.69%
Desulfobacteraceae	2.51%	2.53%	3.39%
Desulfobulbaceae	-	1.41%	2.37%
Desulfovibrionaceae	-	-	1.32%
Geobacteraceae	2.66%	3.30%	1.44%
Syntrophaceae	21.07%	16.88%	13.77%
Syntrophobacteraceae	1.77%	3.35%	2.36%
Others	5.41%	5.58%	2.18%

Table S14. PerMANOVA and ANOSIM analyses for the vertical (UAF and HFCW) and longitudinal variations (HFCW) for nitrogen and organic matter degrading families.

Bacteria	**ANOSIM R**	**Significance**
	Unit:0.5486	Unit: 0.001*

Nitrogen related bacteria	UAF Depth: 0.4889 HFCW Longitudinal variations: 0.2549 HFCW Depth: 0.4933	UAF Depth: 0.007* HFCW Longitudinal variations: 0.001* HFCW Depth: 0.001*
Organic matter related bacteria	Unit: 0.6311 UAF Depth: 0.3056 HFCW Longitudinal variations: 0.1996 HFCW Depth: 0.4575	Unit: 0.001* UAF Depth: 0.003* HFCW Longitudinal variations: 0.008* HFCW Depth: 0.001*

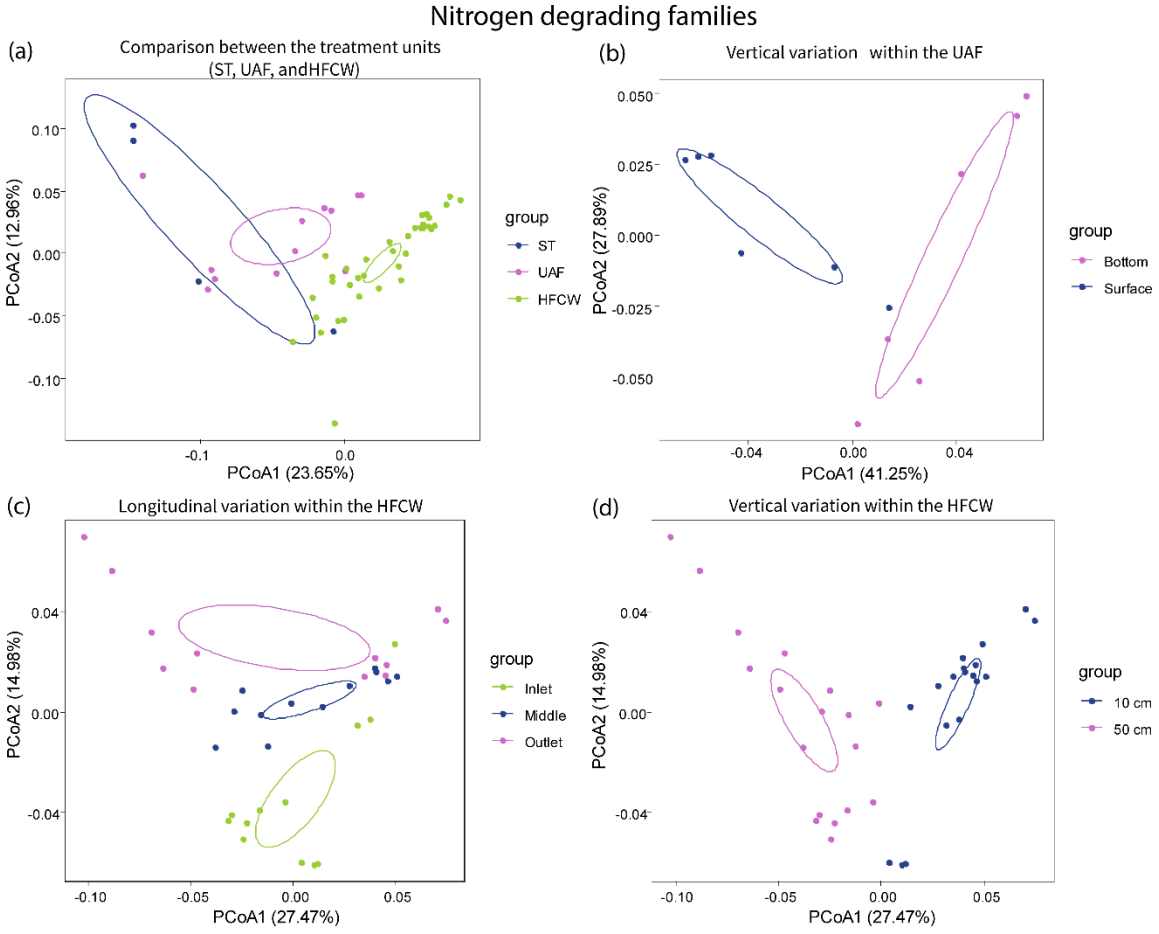

Figure S2. PCoA for nitrogen removal related bacterial families. **(a)** Comparison between the treatment units (ST, UAF, and HFCW), **(b)** Vertical variations within the UAF. **(c)** Longitudinal variations within the HFCW **(d)** Vertical variation within the HFCW.

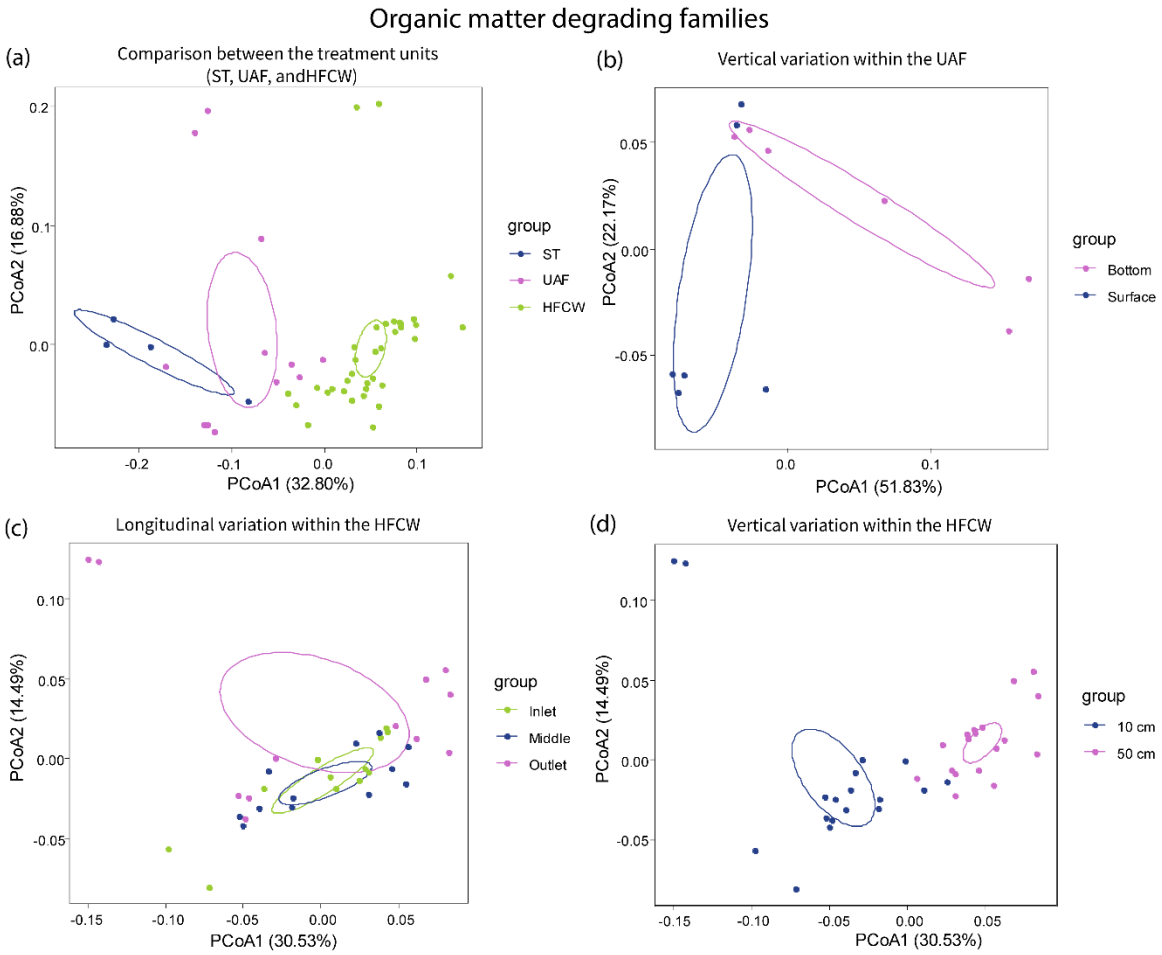

Figure S3. PCoA for organic matter removal degrading families. **(a)** Comparison between the treatment units (ST, UAF, and HFCW), **(b)** Vertical variations within the UAF. **(c)** Longitudinal variations within the HFCW **(d)** Vertical variation within the HFCW.

Table S15. RDA for nitrogen degrading families (first four significant redundancy components).

Variable	RDA1	RDA2	RDA3	RDA4
pH	-0.3575	-0.4277	0.5001	-0.6627
Temperature	-0.7966	0.2480	-0.4519	0.3156
DO	-0.2291	0.4402	0.3653	-0.7876
EC	-0.6288	0.0047	0.5972	0.4980
BOD_5	-0.6750	0.1776	-0.1297	-0.0452
COD	-0.6842	0.2126	-0.0877	-0.0178

TSS	-0.7106	0.0591	0.2101	-0.1142
TNK	-0.6066	0.4802	0.0281	0.1454
NH_3-N	-0.5763	0.5560	0.0238	0.2618
ON	-0.6118	0.4157	0.0303	0.0616
NO_2	-0.7105	0.0453	0.2438	-0.1911

Table S16. RDA for organic matter degrading families (first four significant redundancy components).

Variable	RDA1	RDA2	RDA3	RDA4
pH	-0.09092	-0.5845	-0.5187	-0.6173
Temperature	-0.92198	0.1128	0.3310	0.1663
DO	-0.21471	0.2501	-0.7993	-0.5025
EC	-0.51209	-0.5360	-0.3538	0.5703
BOD_5	0.8045	-0.0810	0.0813	-0.0156
COD	0.7913	-0.0327	0.0795	-0.0063
TSS	0.7276	0.1657	-0.0306	0.0782
TNK	0.6965	0.3853	0.3248	-0.1739
NH3-N	0.6432	0.4078	0.3468	-0.1765
ON	0.7152	0.3599	0.3012	-0.1676
NO_2	0.6855	0.1480	-0.0546	0.2050

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