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Constructed Wetlands Revisited: Microbial Diversity in the –omics Era

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Abstract Constructed wetlands (CWs) constitute an interesting alternative option to conventional systems for wastewater treatment. This technology is based on the utilization of the concerted activity of microorganisms for the removal of contaminants. Consequently, knowledge on the microbial assemblages dwelling CWs and the different environmental factors which can alter their activities is crucial for understanding their performance. In the last decades, the use of molecular techniques to characterize these communities and more recently, application of –omics tools, have broaden our view of microbial diversity and function in wastewater microbiology. In this manuscript, a review of the current knowledge on microbial diversity in CWs is offered, placing particular emphasis on the different molecular studies carried out in this field. The effect of environmental conditions, such as plant species, hydraulic design, water depth, organic carbon, temperature and substrate type on prokaryotic communities has been carefully revised, and the different studies highlight the importance of these factors in carbon, nitrogen and sulfur cycles. Overall, the novel –omics open a new horizon to study the diversity and ecophysiology of microbial assemblages and their interactions in CWs, particularly for those microorganisms belonging to the rare biosphere not detectable with conventional molecular techniques.

Keywords Constructed wetlands · Microbial diversity · Molecular techniques · –omics · Environmental factors

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Introduction

Constructed wetlands (CWs) are engineered systems formed by lagoons, shallow ponds or channels planted with aquatic vegetation (macrophytes), which constitute a decentralized solution to treat wastewater in small communities and dwellings within a controlled environment. They remove pollutants using natural processes such as volatilization, sedimentation, sorption, photodegradation, plant uptake, transpiration flux, accretion and chemical procedures [35], as well as microbial-mediated transformations [35, 62]. They are extensively used in many countries all over the world, such as Australia, Canada, Korea, Sweden, Taiwan, Thailand, the UK and the USA among others [69] due to their efficiency and low cost in terms of installation, maintenance and operation. Several types of constructed wetlands can be distinguished depending on the criteria like hydrology (free water surface flow and subsurface flow), type of macrophytes (free floating, emerged, submerged) and flow path (vertical or horizontal) [68]. Originally, CWs were used to treat domestic wastewaters, but now their applications comprise industrial and agricultural wastewaters, as well as landfill leachate, mine drainage or stormwater runoff [68]. Besides, the array of pollutants removed by them is large and include not only soluble labile forms of organic carbon but also hydrocarbons like toluene [44, 45, 47], herbicides [20], pesticides [46, 70], antimicrobial agents as triclosan [78] or pharmaceutical residues like ibuprofen [40] among others. Furthermore, removal of pathogenic microorganisms has also been a primary target for CWs [48, 67]. Recently, an emerging technology has arisen combining the use of microbial fuel cells (MFCs) and CWs, since redox conditions necessary for MFCs can be found naturally in CWs [18]; the combination of these two biological systems is aimed to produce electrical power while improving the wastewater treatment capacity. Also, other combined

wetland-bioelectrochemical systems offer the simultaneous production of electrical current and H_2O_2 for disinfection of wastewater [5].

Many studies have highlighted the importance of microorganisms in CWs, since different processes and reactions are based on their concerted activity [1, 23, 28, 65]. For example, they constitute key participants in nitrogen and metal removal, as well as in sulfide oxidation, being dynamic players in carbon, nitrogen and sulfur cycling [23]. Thus, active populations of biofilms can be found associated to plant's roots or to the filter bed material, and they are assumed to be responsible for the degradation performance of CWs [60]. Hence, the rhizosphere of CWs is considered to be the most important compartment where many organic compounds are transformed by microorganisms, due to the release of oxygen and organic exudates by the plant. As a consequence, knowledge of the diversity of the communities involved in these biological processes and their link to certain environmental factors is crucial in order to understand the functioning of these systems.

Several decades ago, before the advent of cultivation-independent techniques, it was arduous to ascertain the role of the key drivers of wastewater treatment processes, but nowadays, the scenario has overturned with the development of molecular methods, which circumvent cultivation and its inherent selectivity. Moreover, the current –omics era has extended our understanding on the diversity of these complex systems. In this manuscript, a review of the most recent findings concerning the diversity of prokaryotic microorganisms in CWs is presented, highlighting their influence in some relevant microbial processes and their correlation with environmental variables.

## Molecular Approaches to Study Diversity in the –omics Era

In the last decades, a great number of studies dealing with molecular characterization of diversity in constructed wetlands have been published. The first culture independent approaches employed fingerprinting techniques such as denaturing gradient gel electrophoresis (DGGE) or terminal fragment length polymorphism (TRFLP) of PCR-amplified 16S ribosomal RNA (rRNA) gene fragments (Fig. 1), which allowed to obtain an overall pattern of the microbial community from different samples [8, 11, 12, 19, 31, 60]. DGGE further allows the assessment of population diversity by subsequent sequencing of bands. These methodologies are still currently used in a number of studies for assessing diversity in CWs [2, 20, 48, 54], although they present a bias associated to PCR amplification and primer sets (Table 1) and, overall, do produce a limited number of reads (bands or peaks) compared to tag sequencing of 16S rRNA, which prevent the former from being the future molecular methods in modern microbial ecology laboratories.

However, despite the disadvantages resulting from its use, PCR is still utilized to assess the distribution of certain functional genes and shed light on the diversity of main players in certain microbial processes, i.e. the nitrogen cycle [39]. By contrast, fluorescent in situ hybridization (FISH) with specific probes overcomes the problem of PCR bias, although the technique is limited in practice by the number of probes that are presently known (Table 1).

Currently, the rise of the –omics era has changed the present perception of diversity in wastewater treatment systems at an inconceivable scale [24]. The development of high-throughput sequencing (HTS) methods, like Illumina or 454-pyrosequencing among others, has proved increasingly valuable because they are able to produce for the first time millions of sequence reads both easy and inexpensive, while allowing large scale analysis of microbial assemblages and contributing to a better overview of the functioning of populations. Thus, metagenomic characterization of microbial communities (DNA sequencing of all genes) in CWs can provide information about the phylogenetic diversity and the genomic potential of these systems without bias, avoiding isolation of microorganisms and PCR (Table 1). On the other hand, metatranscriptomics (RNA sequencing) allows the analysis of gene expression, while metaproteomics results in the analysis of proteins, the processes

Fig. 1 Molecular techniques used in the last decades for the study of microbial diversity. The recent –omics offer a new perspective for studying diversity and function due to the production of millions of sequence reads at an unprecedented scale
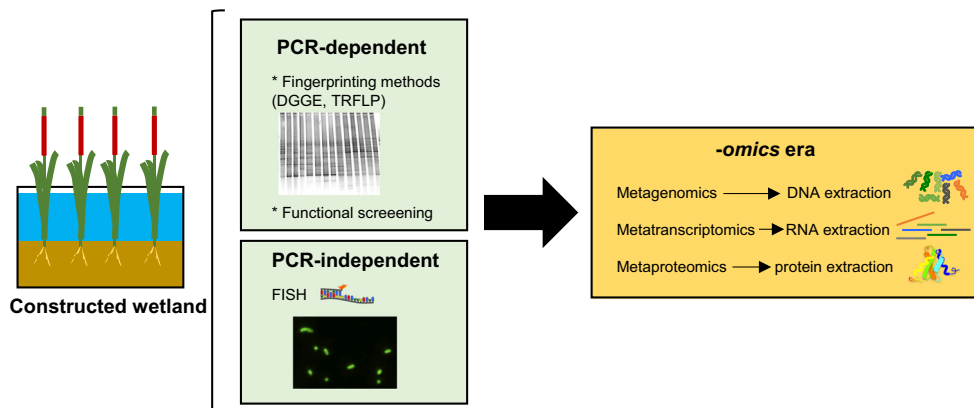
Table 1 Advantages and disadvantages of the most used molecular methods to assess microbial diversity in CWs

Method	Advantages	Disadvantages	Reference
Fingerprinting methods			
DGGE	• Simultaneous analysis of a large number of samples	• PCR bias	[2, 8, 11, 12, 19, 30, 31, 40, 48, 58, 75, 79]
	• Rapid, reliable and reproducible	• Depends on DNA lysis and extraction efficiency	
	• Bands can be excised and sequenced	• Band co-migration	
		• Microheterogeneity and multiple operon presence can lead to band overestimation	
		• Detection of dominant species only (>1 %)	
TRFLP	• Easy and highly reproducible	• PCR bias	[20]
	• Rapid automated analysis of a large number of samples	• Depends on DNA lysis and extraction efficiency	
	• Sensitive to little variations in community structure	• Different sequences may form a single peak	
		• Choice of restriction enzymes can influence community fingerprint	
ARISA	• Highly reproducible analysis of community profiles	• PCR bias	[1]
	• Rapid automated analysis of a large number of samples	• Depends on DNA lysis and extraction efficiency	
		• Different sequences may form a single peak	
FISH	• Non-PCR dependent method	• Limited by the number of available probes	[8]
	• Fast and reliable identification and quantification of microbial groups in environmental samples	• Not all bacterial and archaeal cells can be permeabilised	
		• Hybridization conditions cannot accurately be determined for uncultured microorganisms	
Tag sequencing (16S rRNA gene)	• Production of a large number of sequence reads at low cost	• PCR bias	[1, 4, 20, 27, 28, 32, 40, 41, 44, 71, 73, 74, 78]
	• Allows identification on abundant and rare species	• Depends on DNA lysis and extraction efficiency	
		• Subsequent bioinformatics analyses are needed to remove sequencing errors and process data	
Metagenomics	• Non-PCR dependent method	• Depends on DNA lysis and extraction efficiency	[7, 79]
	• Massive DNA sequencing of all genes present in a microbial community	• Lack of standardization in data processing	
		• Subsequent bioinformatics analyses are needed to remove sequencing errors and process data	
		• Specialized computational facilities and expert bioinformatic researchers are needed	

in which they are involved and the reconstruction of the subsequent metabolic routes (Fig. 1).

The number of studies involving the different –omics in CWs is still scarce. In 2014, Bai et al. [7] used metagenomic analysis to explore the composition of the microbial community in the rhizosphere soil of a constructed wetland; by means of function annotation of metagenomic data, they were able to identify several biodegradation pathways associated with 14 xenobiotic compounds, as well as different genes involved in the nitrogen cycle and related to nitrogen fixation, nitrification and denitrification, along with genes involved in Fe and Mn transformations. At the same time, they could characterize the most abundant groups in this system, providing new insights in microbial mediated bioremediation of contaminants in CWs. Also, a metagenomic approach using 454-pyrosequencing was provided by Zhong et al. [79], who studied the composition of the bacterial community in horizontal subsurface flow CWs with and without front aeration, and shed light to the genera of microorganisms that could be involved in the nitrogen cycle concerning processes like nitrification, autotrophic and heterotrophic denitrification, or anaerobic ammonium oxidation; they found as well different dominant families of bacteria depending on the CW and related to sulfur cycle.

On the other hand, a metaproteomic approach was used by Lünsmann et al. [45] to investigate the microbial processes in the rhizosphere of a CW treating toluene-contaminated water, observing a stable aerobic toluene turnover by *Burkholderiales* during day and night, and an upregulation of polyhydroxyalkanoate synthesis during the day. Functional assignment of the proteins showed that main functions were cell envelope biogenesis, lipid and amino acid metabolism, as well as proteins belonging to the translation apparatus. In general, their study presented information about the microbial transformations occurring in the rhizosphere of CWs.

However, although the progress of –*omics* becomes unstoppable, culture-dependent methods should not be mistreated. Isolates are a source for laboratory models in physiological studies and are relevant to determine the role of newly discovered genes and functions through the abovementioned molecular methods.

Microbial Diversity in Constructed Wetlands

Fingerprinting methods based on 16S rRNA gene analysis such as DGGE or TRFLP have been used in the last decades to characterize the prokaryotic dynamics and structure in CWs (Table 2). More recently, these methods, which provide a rough estimate of the dominant bacterial composition, have been combined with HTS techniques, so that a more detailed picture of the communities which develop in these systems was obtained (Table 2). On the other hand, other authors have used only HTS to determine diversity through the analysis of the 16S rRNA gene or through a metagenomic approach (Table 3). Tables 2 and 3 include the most remarkable phylogenetic groups found in CWs using these techniques and their relative abundances when available.

In virtually all these studies, independently of the origin of influent waters, there is a permanent dominance of the phylum

Table 2 Some molecular studies using fingerprinting techniques or a combination of fingerprinting and HTS methods, highlighting the most remarkable phylogenetic groups in CWs and their relative abundances in brackets (when available)

Type of CW	Remarkable phylogenetic groups (%)	Method	Main contaminant	Reference
Horizontal subsurface CWs	*Proteobacteria, Firmicutes, Tenericutes*	DGGE	Dairy wastewater	[31]
Horizontal subsurface CWs	*Bacteria, Archaea,* Sulphate-reducing bacteria	DGGE, FISH	Settled sewage from a wastewater treatment plant; filtered beer	[8]
Horizontal and vertical flow CWs, and biofilters	*Proteobacteria, Firmicutes, Bacteroidetes, Actinobacteria*	DGGE	Tannery wastewater	[11]
Two-stage horizontal subsurface CWs	*Proteobacteria, Firmicutes*	DGGE	Tannery wastewater	[12]
Surface flow marsh-pond-marsh CWs	*Proteobacteria, Firmicutes, Bacteroidetes, Planctomycetes*	DGGE	Swine wastewater	[19]
Vertical subsurface flow CWs	*Proteobacteria, Acidobacteria, Bacteroidetes*	DGGE	Pretreated wastewater	[30]
Surface flow CW	*Proteobacteria* (41), *Cyanobacteria* (31), *Bacteroidetes* (12), *Planctomycetes* (7), *Chloroflexi* (3)	ARISA, 454 pyrosequencing (16S rRNA)	Oil	[1]
Horizontal and vertical flow CWs, and biofilters	*Proteobacteria, Bacteroidetes, Firmicutes, Actinobacteria, Chloroflexi, Acidobacteria*	DGGE	Domestic wastewater	[2]
Subsurface-flow CWs (batch and continuous flow)	*Proteobacteria* (inland water 50–90; rhizosphere 50–65)	TRFLP, 454 pyrosequencing (16S rRNA)	S-metochlator	[20]
Horizontal subsurface flow CWs	*Proteobacteria, Bacteroidetes, Firmicutes, Acidobacteria, Sinergistetes, Deferribacteres, Nitrospirae*	DGGE	Urban wastewater	[48]
Free water surface and subsurface flow CWs	*Proteobacteria, Bacteroidetes, Actinobacteria, Firmicutes, Acidobacteria*	DGGE	Primary settled wastewater from a urban wastewater treatment plant	[58]
Horizontal subsurface flow CW with and without front aeration	*Aeration: Proteobacteria* (38), *Nitrospirae* (11), *Acidobacteria* (7); *No aeration: Proteobacteria* (55), *Chloroflexi* (7), *Bacteroidetes* (6)	DGGE, 454-pyrosequencing (metagenomics)	Pretreated wastewater	[79]
Horizontal subsurface flow CW	*Proteobacteria, Acidobacteria, Bacteroidetes, Chlorobi, Chloroflexi, Firmicutes, Spirochaetes*	DGGE, 454-pyrosequencing (16S rRNA)	Ibuprofen	[40]
Horizontal subsurface flow CW	*Proteobacteria, Bacteroidetes*	DGGE	Industrial runoff containing polycyclic aromatic hydrocarbons	[75]

Table 3 Some recent molecular HTS studies with the most remarkable bacterial phylogenetic groups in CWs and their relative abundances in brackets

Type of CW	Remarkable phylogenetic groups (%)	Method	Main contaminant	Reference
Hyerarchical Mosaic of Artificial Ecosystems (HMAE)	*Proteobacteria* (50–61), *Verrucomicrobia* (5–26), *Chloroflexi* (0.4-22)	454 pyrosequencing (16S rRNA)	Municipal wastewater	[4]
Surface water CW	*Proteobacteria* (50–65), *Firmicutes* (1–13)	Illumina (metagenomics)	Urban water	[7]
Freshwater wetland	*Proteobacteria* (25–55), *Acidobacteria* (6–24), *Bacteroidetes* (6–9), *Actinobacteria* (3–21), *Verrucomicrobia* (3–5)	Illumina (16S rRNA)	River water	[41]
Vertical flow CWs with different substrate materials (sand, zeolite, gravel)	*Proteobacteria* (29–54), *Chloroflexi* (4–19), *Bacteroidetes* (6–9), *Acidobacteria* (3–21), *Cyanobacteria* (3–5)	Illumina (16S rRNA)	Polluted river water	[27]
Surface flow CSs (batch) with different plants	*Proteobacteria* (40–74), *Bacteroidetes* (13–25), *Actinobacteria* (2–17), *Cyanobacteria* (3–12)	454 pyrosequencing (16S rRNA)	Triclosan	[78]
Vertical flow CW	*Proteobacteria* (28–63), *Chloroflexi* (7–27), *Firmicutes* (3–36), *Acidobacteria* (3–12), *Bacteroidetes* (2–18), *Nitrospirae* (2–8)	Illumina (16S rRNA)	Pond water with agricultural and rural pollutants	[28]
Continuous flow CW	*Proteobacteria* (35), *Bacteroidetes* (22), *Firmicutes* (10), *Cyanobacteria* (6), *Actinobacteria* (3)	454 pyrosequencing (16S rRNA)	Swine waste	[32]
Planted fixed bed reactor	*Burkholderiales* (19), *Rhizobiales* (24), *Acidobacteria* (19)	qPCR, Illumina (16S rRNA), protein-SIP	Toluene	[44]
Subsurface flow CW	*Proteobacteria* (31–48), *Actinobacteria* (2–16), *Bacteroidetes* (5–15), *Cyanobacteria* (7–30), *Firmicutes* (2–5), TM7 (1–15), *Acidobacteria* (1–4)	qPCR, 454-pyrosequencing (16S rRNA)	Domestic wastewater	[71]
Combination of vertical subsurface flow CW, free water surface CW and horizontal subsurface flow CW in series	*Proteobacteria* (21–40), *Acidobacteria* (5–13), *Bacteroidetes* (5–10), *Verrucomicrobia* (2–12)	Illumina (16S rRNA)	Sanitary and industrial wastewater	[73]
Combination of vertical subsurface flow and horizontal subsurface flow CW	*Proteobacteria* (36–64), *Bacteroidetes* (15–22), *Actinobacteria* (5–13)	Real-time PCR, Illumina (16S rRNA)	Secondary effluent of an industrial park wastewater treatment plant	[74]

Proteobacteria, including members of the classes *Alpha-*, *Beta-*, *Gamma-*, *Delta-* or *Epsilonproteobacteria*, which are in different proportions depending on the conditions. This phylum is present in different types of samples from CWs, such as soil or sediment [4, 41, 78], rhizosphere [7, 44], manure influent [32], lagoon water [20, 32], inlet and outlet water [1] or biofilms from substrate particles [28, 79]. Within this group, the proteobacterial ammonia oxidizers (AOB), which include the *Betaproteobacteria Nitrosomonas* and *Nitrosospira*, and the *Gammaproteobacteria Nitrosococcus* (except *Nitrosococcus mobilis*, a betaproteobacterium), can carry out aerobic or anaerobic ammonia oxidation [57], while nitrite oxidizing bacteria (NOB) perform the conversion of nitrite to nitrate and include members of the genera *Nitrobacter* (*Alphaproteobacteria*) and *Nitrococcus* (*Gammaproteobacteria*) among others. Ammonium removal by nitrification is a well-documented process in different types of CWs [23], where sequence DGGE analyses have revealed the presence of two dominant AOB lineages corresponding in fact to *Nitrosomonas europaea*/*Nitrosococcus mobilis* and *Nitrosospira* [31, 63, 64]. Recent metagenomic analyses confirmed the presence of a complete set of nitrification genes (*amo*, *hao*) in both water and soil samples of a CW treating urban water associated to *Nitrosomonas eutropha* [7], suggesting that this bacterium might be responsible for nitrification activity in the rhizosphere soil. Some genera of Proteobacteria are also responsible for denitrification in CWs, a process spread in diverse phylogenetic groups, which involves the transformation of nitrate or nitrite into gaseous products (N_2 and N_2O). They are mainly facultative anaerobic chemoheterotrophic bacteria such as *Pseudomonas*, *Aeromonas* or *Vibrio* among others, although some AOB like *Nitrosomonas* sp. can also denitrify when grown under oxygen limitation [9].

Moreover, some pathogenic bacteria belong to the phylum *Proteobacteria*, and actually, proteobacterial indicator microorganisms (*Enterobacteriaceae*) are routinely used to investigate microbial removal in CWs [66]. In fact, CWs offer a combination of mechanisms suitable to remove pathogenic microorganisms [48]. Proteobacteria are also involved in sulfur cycling, an important process in CWs due to the considerable amounts of sulfur present in wastewater. The Deltaproteobacteria sulfate reducing bacteria (SRB) can oxidize organic substrates anaerobically and utilize sulfate as a terminal electron acceptor in CWs, although some works have showed that they can persist in oxic conditions [10, 15, 29, 59]. Different genera, such as *Desulfobacter*, *Desulfovibrio*, *Desulfobulbus* and *Desulfobacterium* have been detected in CWs by means of culture dependent and independent techniques [36, 43, 56]. Interestingly, SRB metabolism is closely related to precipitation of metal sulfides and they are considered of great interest in the bioremediation of metal-polluted environments [37]. In fact, different microbial processes can affect the mobility, toxicity and bioavailability of metals, particularly activities like biosorption, precipitation, redox tranformation, methylation, or microbe-plant interactions [37].

Another group usually abundant in CWs is the phylum *Bacteroidetes*, formed by Gram-negative chemoheterotrophic bacteria widely distributed in different environments, such as soil, sediments, seawater, aside from the guts and skin of animals. It is known by its ability to degrade complex organic matter, and it is suggested to be strongly involved in the degradation of aromatic compounds used in the post-tanning process [17], or in denitrification processes [2] from different CWs. A list of some genera of *Bacteroidetes* found in CWs is shown in Table 4.

Other prokaryotes, including *Firmicutes*, *Actinobacteria*, *Acidobacteria*, *Planctomycetes*, *Chloroflexi*, *Sinergistetes*, *Deferribacteres*, *Nitrospirae*, *Cyanobacteria*, *Verrumicrobia* or *Archaea*, can be found in different types of CWs (Tables 2 and 3), but usually their contribution is low, in most cases being part of the rare biosphere, that is, those species that are represented by a low number of individuals [52, 53]. Also, in these surveys, a great proportion of sequences can be hardly affiliated to a specific group, and remain as "uncultured" in the databases, highlighting the fact that further efforts to characterize these microorganisms are essential in order to explore the diversity and functional abilities of microbial communities in CWs.

Correlating Microbial Communities With Environmental Parameters in CWs

There is a large set of environmental conditions that can affect microbial physiology and community structure in CWs, and different publications have dealt with this issue in the last years. Microbial communities can be altered with different

Table 4 Genera affiliated to the phylum Bacteroidetes found in different CWs

Genus	Type of CW	Reference
Algoriphagus sp.	Combination of vertical subsurface flow and horizontal subsurface flow CW	[74]
Arenibacter sp.	Combination of vertical subsurface flow and horizontal subsurface flow CW	[74]
Bacteroides sp.	Surface flow CW	[32]
Chitinophaga sp.	Vertical flow CW	[27]
Chryseobacterium sp.	Horizontal and vertical subsurface flow CWs, and biofilters	[2, 28, 40, 58]
Dysgonomonas sp.	Vertical flow CW	[27, 28]
Flavobacterium sp.	Free water surface, horizontal and vertical subsurface flow CWs, biofilters	[2, 27, 58, 74, 79]
Haliscomenobacter sp.	Combination of vertical subsurface flow and horizontal subsurface flow CW	[74]
Hallella sp.	Surface flow CW	[32]
Lewinella sp.	Surface flow CW	[1]
Myroides sp.	Surface water CW	[7]
Ohtaekwangia sp.	Combination of vertical subsurface flow and horizontal subsurface flow CW	[74]
Paludibacter sp.	Vertical flow CW	[28]
Pedobacter sp.	Horizontal and vertical subsurface flow CWs, biofilters	[2]
Porphyrimonas sp.	Vertical flow CW	[28]
Prevotella sp.	Surface flow CW	[32]
Sphingobacterium sp.	Surface flow CW, horizontal and vertical subsurface flow CWs, biofilters	[1, 2]

environmental factors, such as plant species, hydraulic design, availability of organic matter, temperature, dissolved oxygen (DO) or substrate type among others [6, 27, 58, 66, 73]. As a consequence, investigation of fluctuations in microbial populations can shed light to the response of a CW to variations in operational and environmental settings.

Several studies have highlighted the importance of vegetation in shaping microbial communities in CWs both directly through the comparison of populations in planted and unplanted CWs by molecular methods [6, 58, 60, 66] or indirectly by reporting loss of performance in nitrogen processing for unplanted wetlands [33]. The wetland rhizosphere seems to play an important role in the establishment of oxic-anoxic interfaces where aerobic and anaerobic microorganisms can operate in close vicinity, enhancing elemental cycling and therefore stimulating microbial activity [25, 50]. Plants can provide carbon compounds through the excretion of exudates and can release oxygen via the aerenchyma system; oxygen is consumed during the night and redox diurnal fluctuations are established, allowing the coexistence of oxic and anoxic conditions and probably contributing to niche differentiation in the rhizosphere [50]. Other effects of vegetation in CWs include the role of plants in the control of hydraulic efficiency due to modification of internal flow patterns, the influence on water temperature and energy balance, the decrease of reaeration due to wind blocking and shading or the provision of surfaces (litter and stems) to which microbes attach [33].

Gagnon et al. [25] showed that microbial density and activity increased when plants were present, and these values presented differences within plant species, being higher values associated with *Phalaris arundinacea* compared with *Typha angustifolia* and *Phragmites australis*. The effect of plant species in microbial profiles was further investigated by Sidrach-Cardona et al. [58], who studied how the presence of *T. angustifolia* or *P. australis* could influence the microbial composition of the rhizoplane, gravel biofilm and interstitial water in CWs; they concluded that plants exerted an effect on all the microbial communities examined within the mesocosms, confirming their influence in interstitial water and gravel-associated bacteria far beyond their roots. In the case of *Phragmites*, differences between environments were higher than in *Typha*. Faulwetter et al. [22], by means of quantitative PCR and DGGE, examined also effluent and biofilms associated to gravel and roots and observed a clear influence of plants on abundance and diversity of SRB and AOB populations. On the other hand, Arroyo et al. [6] obtained evidence of a most effective metal removal of *P. australis* compared to *Typha latifolia*, highlighting that those CWs planted with *P. australis* presented the highest diversity and richness, measured with PCR-amplified 16S rRNA clone libraries. Concerning the nitrogen cycle, Kadlec [33] reported a reduced ability of unvegetated CWs to process nitrogen compared to planted ones; nitrification and denitrification rates decreased

probably due to a reduction in the surface area for microbe attachment. Differences between planted and unplanted CWs by means of quantitative PCR and HTS were also observed by Wang et al. [71], who concluded that both microbial community structure and abundance were positively affected by plants, being bacteria more abundant in the rhizoplane than in sand.

However, other works showed the lack of a strong vegetation effect on the structure of microbial communities [3, 8, 30], and the study of specific groups of organisms like metanotrophic bacteria by quantitative PCR revealed no significant differences in the quantities of Type I or Type II metanotrophic communities between planted and unplanted wetlands [16]. Sidrach-Cardona et al. [58] suggested that inadequate redox conditions for root development could have conducted to the absence of plant effects on microbial communities, a fact that is in accordance with Ravit et al.'s [55] proposals, which point out to less evident effects of plants on microbial communities in anthropogenically disturbed wetlands. In fact, Ahn et al. [3] indicated that the absence of results could be explained by the low growth of plants and sampling anomalies in their CWs. In any case, it is not clear the effect of plants in these systems, making evident that little is known about the mechanisms performing the shape of microbial communities and their functioning in different compartments of CWs.

A second important attribute in CWs is hydraulic design, since different operation and configuration strategies can lead to variations in performance due to changes in redox conditions. Conventional CWs are categorized according to water flow in free water (FW) surface and subsurface flow (SSF) wetlands. FW systems have a better exchange of oxygen than SSF CWs and therefore, have a higher redox and a higher advantage in nitrate removal, while SSF wetlands might provide a more anoxic environment and might have more surface area for microbial attachment and for growth of denitrifying bacteria [42]. Other innovative designs have emerged in the last years, such as circular-flow corridor CWs, towery hybrid CWs, baffled subsurface-flow CWs, or microbial fuel cells CWs [72], which try to intensify performance for wastewater treatment, for instance enhancing nitrogen removal.

Several works have examined the influence of flow on community composition. Thus, Lin et al. [42] studied nitrate removal in CWs with the same size but diverse flow patterns (FW surface vs SSF) and concluded that, in general, there was no significant difference between both types of CWs. In contrast, Arroyo et al. [6] observed that, besides plant presence, the type of flow seemed to be the main design parameter to increase efficiency to remove zinc and arsenic, being the *Proteobacteria* phylum, characterized by 16S rRNA gene amplification and cloning, the most abundant group under all conditions. Zinc and arsenic removal was better in FW flow CWs, although in the case of arsenic, those CWs planted with

P. australis offered higher rates; in the case of zinc, no interaction between vegetation and flow was observed. Sidrach-Cardona et al. [58] also demonstrated that hydraulic configuration, together with the type of vegetation, was important in shaping microbial communities in FW and SSF CWs. However, the choice of the type of treatment wetland is not clear, and apart from the nature of contaminant to be removed, aspects like size, cost, operability, health issues and ancillary benefits should be considered [34].

In addition to flow, another key design factor for CWs is water depth, which in SSF systems has been normally set at 0.60 m to maximize growth and plant effects [14]. Water depth influences the oxygen transfer coefficient from atmosphere to water, and governs the water volume in contact with macrophytes, leading to a compartmentalization of metabolic processes within the wetland, but although it is an important feature to consider, it has not been included in performance models as a variable. Truu et al. [65] concluded that depth gradient was the most important spatial pattern to determine microbial community structure in a horizontal SSF CW treating domestic wastewater. Besides, in the study from Morató et al. [48], it was shown that shallower horizontal SSF CWs (0.27 m) had higher redox values and consequently more oxidized conditions; accordingly, microbial communities were more related to aerobic microorganisms. Also, shallow wetlands combined with fine gravel granulometry were more effective when removing pathogens such as total coliforms and *Escherichia coli*, as well as *Clostridium* spores, a result partially explained by the fact that a larger volume of water is contacting macrophyte roots in this type of CWs. On the other hand, cluster analysis of the DGGE patterns separated samples according to water depth, showing that it was an important feature to consider for microbial community structure.

Bacterial populations can also be affected by environmental variables like organic carbon availability. The carbon source usually comes from wastewater, soil, or products excreted by plant roots. It has been considered that organic carbon can promote denitrification, but in those waters with high nitrate or carbon deficient content, such as waters from agricultural drainage or from secondary treated nitrified effluents, denitrification can be greatly sustained by root exudates [73, 77]. The alternative of adding external organic sources to enhance denitrification revealed that planted wetlands were more efficient in nitrate removal than unplanted ones after organic matter supply, although the loss of the added carbon by other microbial processes increased costs and limited its utilization in CWs [73]. Chang et al. [13] demonstrated that organic matter especially affected the index of fatty acids in CWs, used as biomarkers to characterize microbial community structure. On the other hand, Wu et al. [73] also reported that total organic carbon had a high impact on some denitrifiers. Nevertheless, other environmental factors, such as

dissolved oxygen, can be crucial in microbial processes like nitrogen transformation, since nitrifying bacteria need oxygen, and it is recognized that nitrification is the first limiting step for nitrogen removal. Thus, artificial aeration can be an alternative to enhance nitrogen removal by rising AOB and NOB, while increasing the wetland performance [21]. Furthermore, other factors, such as phosphorus loading, appear to be critical in the treatment efficiency of CWs; high phosphorus loading seems to have a recognizable impact by decreasing and altering the microbial diversity associated to soil [3], and consequently, microbial communities may be used as potential indicators of phosphorus dynamics in CWs.

CW performance and their associated microbial communities are also sensitive to climate and seasonal changes. During winter, low temperatures have a negative impact in plant activity and microbial growth. Wu et al. [73] made an interesting revision about the different wetland configurations reported to intensify pollutant removal under low temperature conditions. Other works, however, highlighted the seasonal effect on microbial composition. Thus, Morató et al. [48] observed a reduction of *E. coli* in summer, while heterotrophic bacteria decreased in winter, and Paranychianakis et al. [51] described a seasonal shift in the composition of denitrifiers. Beyond the plant influence, Faulwetter et al. [22] described as well a seasonal effect on abundance and richness of SRB and AOB, while Wang et al. [71] concluded in their results that removal efficiencies of contaminants and microbial community structure differed in winter. DGGE analysis of samples from different lab-scale horizontal SSF CWs revealed also that seasonal variations had an effect on the diversity and composition of microbial populations [76]. Other studies reported seasonal changes in microbial communities with indirect methods, such as Song et al. [61], who used BIOLOG-GN plates to describe the metabolic features of airborne microbial communities in CWs and found different degrees of carbon compound utilization between seasons.

Substrate type can influence, too, the formation of microbial biofilms and the microbial assemblage structure in wetlands, as well as its system performance. In this case, molecular methods have also proved to be useful for the investigation of substrate effect on bacterial community composition. Thus, Vacca et al. [66], by using fingerprinting analyses, reported differences on rhizospheral microbial communities depending on filter material (expanded clay and sand). Likewise, Calheiros et al. [12], by numerical analysis of DGGE profiles, indicated that bacterial richness and community structure were affected by the type of substrate (different types of expanded clay aggregates and fine gravel) and the presence of *T. latifolia*. On the other hand, Guan et al. [27] utilized more recently a HTS technique (Illumina MiSeq) to investigate the influence of substrate type (sand, zeolite and gravel) on microbial communities, demonstrating a clear effect of soil material on the different bacterial groups detected. Some authors

have used methodologies other than molecular techniques to investigate the link between microbial community structure and filter media, such as Li et al. [38], who compared the microbial community assemblages of eight types of substrate (zeolites, anthracite, shale, vermiculite, gravel, ceramic filter media, bio-ceramic and steel lag) by measuring the phospholipid fatty acid (PLFA) composition, concluding that PLFA profiles exhibited significant differences among the diverse materials. However, Gorra et al. [26] did not observe a clear effect of substrate (gravel, ceramic wastes, magnetite, zeolite and soil with marble sand) on AOB populations.

Future Directions

Introduction of –omics technologies in the study of CWs microbiology has certainly broadened our knowledge of the diversity in these systems at an unprecedented scale. However, the great amount of information obtained from these studies has not solved some questions about their functioning that still remain to be elucidated. For instance, which are the key species becoming crucial for the performance of a CW? A few metagenomic studies have provided insights about detailed species composition and potential functions in these systems (see Tables 2 and 3 and included references), but information is still scattered and scarce and does not usually provide knowledge of the core microbiome present in CWs. The core microbiome refers to the suite of microorganisms shared within similar environments, and determining its key components helps to understand how that particular system functions across complex microbial populations.

Although usually many authors try to link microbial composition and function, these two issues are not necessarily related, since in some cases, closely related microorganisms may have diferent metabolic features, while on the other hand, several physiological characteristics like the denitrification capacity are spread in different phylogenetic lineages. The different –omics can solve these obstacles by providing, through the combination of metagenomics and metatranscriptomics, the knowledge of microbial identification and gene expression under different conditions and time scales. Consequently, the future challenge to obtain a deeper understanding of CWs will be the integration of molecular tools with ecological theory, enhancing the development of mathematical models able to predict population structure and population functioning in variable environments, including their interactions and activities. In this sense, the use of model CWs can constitute a useful framework for formulating and testing hypotheses about optimization and design of wastewater treatment systems. As a remarkable example, in a context different from CWs but related to wastewater treatment, Nielsen et al. [49] developed a conceptual ecosystem model for describing microbial communities in enhanced biological phosphorus removal plants through the presentation of a core microbiome;

the model offers knowledge of the dominant microorganisms present in these systems, with emphasis on key ecophysiological factors controlling their presence and activity and interspecies interactions. This kind of information lacks for CWs, and accordingly, more effort should be invested in this direction for the future management and design of these systems; some aspects commented above, such as the influence of plant presence, hydraulic design or seasonal variations on microbial communities could be contextualized and modelled to predict detailed metabolic patterns for relevant core species.

However, even though the different –omics offer an extraordinary potential to investigate structure-function relationships, they are not free of some limitations concerning mostly the interpretation of metagenomics and metatranscriptomics data, particularly due to the amazing number of sequences encoding hypothetical proteins generated using these tools, so that further efforts should be directed to improve their output.

Another question from ecological interest concerns all those microorganisms that are present in low numbers and were invisible until the development of HTS techniques. Are those microorganisms involved in the rare biosphere particularly active when favourable conditions appear and thus important for the system functioning? There are evidences from other environments that show that some members of the rare biosphere may actually become active under the appropriate conditions, although this is not always the case [53]. However, the rare biosphere is an unexplored field that has not been investigated in CWs, and the new sequencing technologies open the window to allow the experimental study of these rare bacteria.

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