MICROBIAL COMMUNITY DYNAMICS AND ACTIVITY IN CONSTRUCTED WETLAND TREATING AGRO-INDUSTRIAL AND DOMESTIC WASTEWATER: A REVIEW

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Abstract. This study reviews the microbial techniques and microbial population responsible for the removal of organic matter and nutrients in constructed wetlands (CWs). In addition, it aims to analyze the effect of presence and absence of macrophytes and identify major phenomena that affect microbial community dynamics as well as compares performance efficiency of CW types. Removal of particular pollutants in each CWs type was mainly related with a particular microbial functional group. In CWs the dominant bacterial groups were α, β, and γ Proteobacteria (Firmicutes, Actinobacteria, Bacteroidetes groups). Microbial dynamics in subsurface flow are more diverse than in surface flow. Diverse and distinct bacterial community inhabits each CW type where different gradients create variable niches in which different biochemical processes take place. Vertical flow CWs favor aerobic microbes and have higher removal efficiency for Organic Carbon and ammonium, while HSSF systems favor anoxic and anaerobic microbes. Therefore, use of hybrid CWs, design and operational methodologies that enhance the activity of the targeted group would better optimize performance. CWs with plant species have higher microbial density and activities than unplanted. The interaction between plant roots, microorganisms and substrates, along the operation time, might have contributed to the establishment of diverse assemblages of microbes CW system.
Keywords: *constructed wetlands, microbial community, microbial techniques, biofilm, nutrient removal*

Abbreviations: 16 SrRNA: 16S ribosomal RNA, ARDRA: amplified ribosomal DNA restriction analysis, BOD: biological oxygen demand, CLPP: community level physiological profiles, COD: chemical oxygen demand, CSUP: carbon source utilization pattern, CWs: constructed wetlands, DGGE: denaturing-gradient gel electrophoresis, DNA: deoxy-ribonucleic acid, FWS: flow water surface, HSSF: horizontal sub surface flow, OC: organic carbon, OUT: Operational taxonomic group, PCR: polymeric chain reaction, SOB: sulfur-oxidizing bacteria, SRB: sulfur-reducing bacteria, T-RFLP: terminal restriction fragment length polymorphism, VF: vertical flow, WWT: waste water treatment

Introduction

Constructed wetland systems (CWs) are designed and constructed to mimic natural wetland systems which involve the use of wetland plants, substrate (gravel, soil), and associated microbial consortia (biofilms) to treat almost all kinds of wastewater. They are designed to take the same processes that occur in natural wetlands within a more controlled environment (Brix and Schierup, 1989; Kadlec and Wallace, 2009; Vymazal, 2010; Parde et al., 2020). CW systems are environmentally friendly treatment methods

which can be used for the treatment of industrial, domestic, agricultural wastewater and ground water (Kadlec and Wallace, 2009).

Industrial wastewater with high chemical oxygen demand (COD), high biological oxygen demand (BOD), pesticide, nutrients and high salt content are now possibly treated by CWs (Faulwetter et al., 2009; Calheiros et al., 2010; Lv et al., 2016, 2017). However, a better understanding of CWs designs and a configuration in order to optimize the removal of a specific pollutant is still under investigation (Faulwetter et al., 2009). A variety of removal mechanisms including physical (sedimentation, filtration), chemical (precipitation, adsorption, volatilization), and biological (microbial degradation, microbial nutrient transformation, plant uptake, microbial competition) processes are employed in CWs (Kadlec and Knight, 1996; Faulwetter et al., 2009; Vymazal, 2011).

However, the removal of most pollutants in CWs is mainly due to microbial activity (Kadlec and Knight, 1996; Stottmeister et al., 2003; Faulwetter et al., 2009; Lv et al., 2017). For example, organic matter and the majority of total nitrogen (TN) removal is basically through microbial transformations, while uptake of nutrients by plants is a minor process (Nurk et al., 2005; Kadlec and Knight, 1996). Thus, pollutant removal and microbial activity in CWs are closely tied to the cycling of carbon, nitrogen and sulfur compounds. Plant root morphology and development and substrates in CWs have an effect on wastewater treatment that partially results from their effect on bacterial assemblages (Stottmeister et al., 2003; Vymazal et al., 2001) and through its influencing microbial-plant interaction (Gagnon et al., 2007; Lv et al., 2017).

Early publications assumed the influence of microbial processes in CWs, and were based primarily on measurement of changes in water quality, and microbial identification of OTU (operational taxonomic Unit) but there is a lack of direct evidence of specific microbial groups. Recent advances in qualitative and quantitative microbial techniques make direct evidence for the presence of specific microbial species or functional groups influencing pollutant removal (Sims et al., 2013). This paper was trying to review the microbial community dynamics and their role in pollutant removal efficiencies in each CW type, bacterial populations critical to Sulfur and nitrogen removal and/or transformations, pesticide removal and the factors that influence the microbial community dynamics in CW systems (Sims et al., 2013; Ibekwe et al., 2016; Lv et al., 2017).

Objective

This article was aimed at analyzing and providing a comprehensive literature review on the microbial community dynamics and activity in constructed wetland treating agro-industrial and domestic wastewater. It also discusses its feasibility in pollutant removal efficiency and additional benefit to give an overview about microbial communities for the scientific community.

Methods

This review article was written using search engine on key phrases "microbial community dynamics and activity in constructed wetland treating agro-industrial and domestic wastewater "and "Factors affecting treatment efficiency and "usefulness and constraint of Constructed wetland" in science direct, springer link, library genesis,

jester, and www.nap.org searching web pages. From these searching, peer-reviewed journals and review papers were used. The interpretation of the result of each document was done using tables and bar graphs, in Microsoft Excel. Microbial community dynamics was systematically assessed and summarized based on considering taxonomic group abundance both numerically and in %. Result measurement units of physicochemical parameters investigated by different scholars were reorganized and expressed in similar units for comparison.

Constructed wetlands

Wetlands are found at the interface of aquatic and terrestrial ecosystems in a biome spanning from the tundra to the tropics. CW comprises of a bed of soil, sand or gravel which together treat wastewater. Root system of plants and media (soil and gravel) act as filters and support biofilms which help in removing contaminates. In addition, plants utilize nutrients and bioaccumulation contaminates such as metals. First experiment using wetlands with macrophytes for wastewater treatment was carried out in Germany during 1950. Various European countries including the UK adopted this technology during the 1980s. An international conference on the use of CWs in water pollution control was organized in Cambridge in 1990. Constructed wetlands are classified based on vegetation type (emergent, submerged, floating leaved, free floating) and hydrology (free water surface and sub-surface flow wetland).

Constructed wetlands, based on its water flow, can be divided in to two basic types; free water surface flow (FWS) and sub-surface flow (SSF) wetland. SSF CWs could be classified into horizontal and vertical according to the flow direction through a permeable medium (typically sand, gravel or crushed rock) (Decamp and Warren, 2001; Vymazal, and Kröpfelová, 2008; Vymazal, 2010; Mina et al., 2011; Parde et al., 2020). Both types utilize wetland/emergent aquatic vegetation and are similar in appearance to a natural wetland (Siti et al., 2011).

In FWS CWs the water surface is exposed to the atmosphere and flows horizontally over the media/soil surface. The mean water depth is usually less than 0.4 m, and thus, FWS CWs are frequently dominated by floating, rooted emergent or submersed vegetation (Vymazal, 2010). In SSF CWs, the water surface is kept below the surface of the substrate, which may support different types of rooted emergent vegetation. In HF systems, the influent enters in the bed subsurface at the beginning of the wetland cell and flows through horizontally using pressure or gravity forces. In vertical flow (VF) wetland system the wastewater is fed from the top and then percolated down through the filter media (substrate) and gradually collected by collecting drainage system at the bottom of the wetland (Kadlec and Wallace, 2009; Parde et al., 2020). SSF system allows for filtration, biodegradation with microbial and plant uptake of contaminants (*Fig. 1*). The benefits of SSF over FWS wetland are odor minimization, control of insect vector and greater surface area for pollutant treatment (*Table 1*) (Parde et al., 2020).

Wetland types microbial processes influencing performance of CWS

The performance of constructed wetlands is based on the combined action between microbes, plants, filtering media (Kadlec and Knight, 1996; Faulwetter, 2009; Vymazal, 2011), substrate and nutrient availability and loading rates (Truu et al., 2009). The mineralization of organic matter is mainly carried out by microbes both in aerobic and anaerobic conditions. Removal or transformation of a particular pollutant such as

organic carbon (OC), total nitrogen (TN) and other nutrients in CWs is typically associated with a specific microbial group, therefore employment of design and operational methodologies that enhance the specific activity of that group will improve performance (Kadlec and Knight, 1996; Faulwetter et al., 2009; Chang et al., 2015). Adsorption and Sulfate reduction is also recognized as an important mechanism for metals removal in CW (Dvorak et al., 1992), but the latter may also play an important part in organic carbon (OC) removal. Thus, pollutant removal and microbial activity in CWs are closely tied, especially to the cycling of carbon, nitrogen and sulfur compounds (Fu et al., 2011).

*Table 1. Major properties of the different constructed wetland types*

Characteristics	Surface flow	Subsurface flow	Vertical flow
Bed	• Long, narrow channels with an impermeable liner to prevent seepage	• Trench or bed with impermeable liner to prevent seepage	
CW plant type	• With emergent vegetation	• With emergent vegetation	• With emergent vegetation
Water flow	• Wastewater flows at a shallow water depth and in CW media	• Wastewater flows latterly through the medium	• Wastewater flows vertically through the medium
Substrates (media)	• Usually soil, sand and gravel	• Sand and gravel	• Sand and gravel
Treatment	• Purified by microorganisms attached to plant stalks, litter and on media surface	• Purified by microorganisms attached on the surfaces of the root zone of the vegetation and medium surface	• Purified by microorganisms attached on the surfaces of the root zone of the vegetation and medium • Support aerobic system
Advantage	• Provide "green space" in a community • BOD, TSS, COD, metals, and organic material removal in a reasonable detention time • N and P removal in a significantly longer detention time • Minimization of mechanical equipment, energy, and skilled operator requirements	• Long flowing distances possible; nutrient gradient can establish • Nitrification and denitrification possible • Formation of humic acids for N and P removal • Longer life cycle	• Smaller area demand • Good oxygen supply - good nitrification • Simple hydraulics • High purification performance from the beginning
Disadvantage	• Higher area demand • Anoxic environment—poor nitrification • Mosquito production	• Higher area demand • Careful calculation of hydraulics necessary for optimal O2 supply • Equal wastewater supply is complicated	• Short flow distances • Poor denitrification • Higher technical demands • Loss of performance esp. in P-removal (saturation)
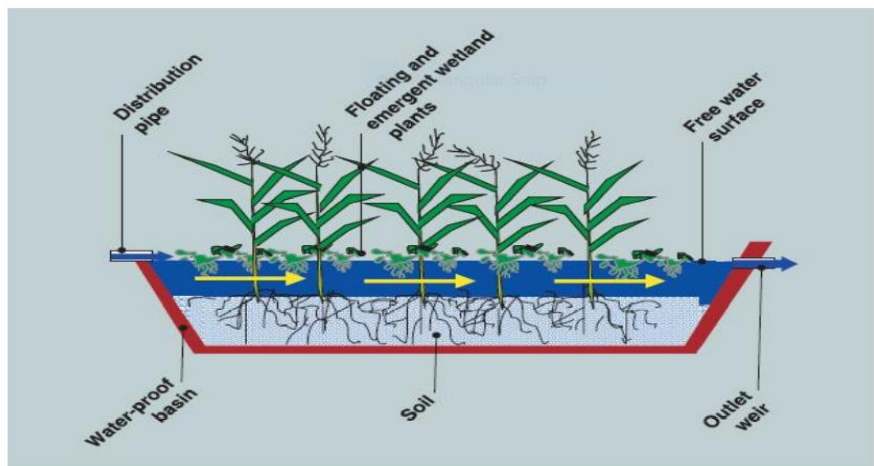
(a) Free surface flow wetland (FSW)
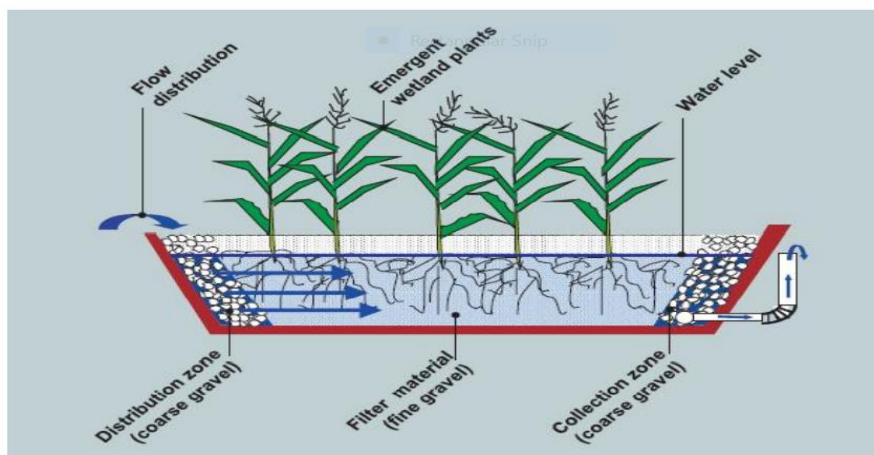
(b) Horizontal subsurface flow (HSSF) wetlands
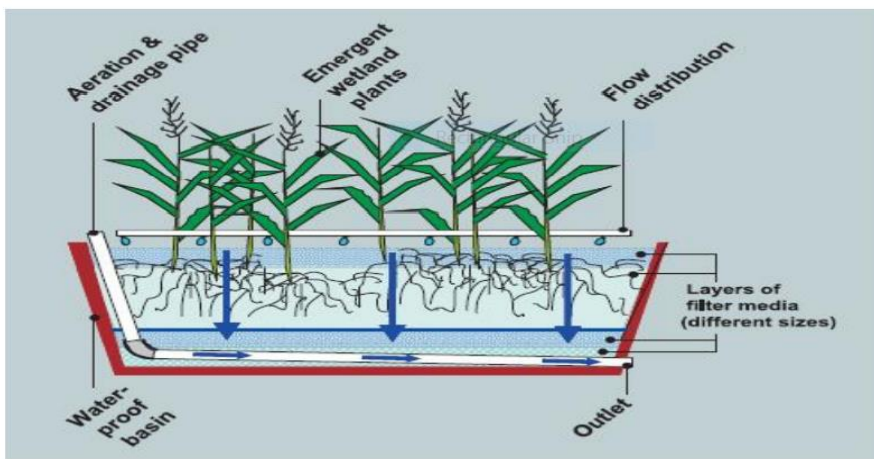
(c) *Vertical subsurface flow (VSF) wetlands*

Figure 1. Free surface flow wetland (FSW) and subsurface flow wetlands (SSF) (source: WSP 2008)

Constructed wetland is also used to treat pesticides. Budd et al. (2009) reported that organophosphate insecticides removal in CWs ranged from 52-94%. Vymazal and Březinová (2015) also reported that the highest average removals (97%) were achieved

for pesticides from organochlorine group (endosulfan, pentachlorophenol) followed by organophosphate pesticide (94%) and Urea based pesticide (50%). On the other hand, the lowest removals were achieved for triazinone pesticide (24%). Rose et al. (2006) also reported that CWs designed for pesticide removal (retention) should comprise of both open water and vegetable zones, to increase the potential for complementary chemical, microbial, photolytic and plant mediated pesticide break-down. Maillard and Imfeld (2014) pointed out Wetland vegetation enhanced the pesticide removal.

The treatment efficiency VF CWs (except TN and TP) is relatively more efficient (73-90%) than FWS and SSF CWs (*Fig. 2; Table 2*). VF CWs allow unsaturated conditions and excellent oxygen transfer, which results in high redox potentials that favor aerobic microbial processes (Houda et al., 2014). Similar studies showed that BOD removal and nitrification were significantly higher in VF compared to FWS and SSF CW (*Table 2*) (Li et al., 2008; Vymazal, 2007, 2010).

Table 2. *Treatment efficiency (Eff, in %) of various types of constructed wetlands (CWs) for organics, Total suspended solids (TSS), total nitrogen (TN), ammonia nitrogen (NH4 -N) and total phosphorus (TP); HLR = hydraulic loading rate (cm/d)*

CW types	BOD5		TSS		TN		NH4-N		TP		References
	HLR	Eff.	HLR	Eff	HLR	Eff	HLR	Eff	HLR	Eff.	
FSF	4.1	74	4.8	77	4.9	45	5.4	48	5.4	34	Vymazal, and Kröpfelová, 2008
	3.3	72	3.1	68	3.2	58	3.1	53	3.5	50	Bulc, 2006
HSSF	11.8	75	15.4	75	10.6	43	14.1	39	11.4	50	Tadesse et al., 2012
F		61		65		61		43		68	Wu et al., 2013
	8.2	90	9.7	89	9.1	43	8.4	73	8.2	56	Tadesse et al., 2012

(a)   (b)
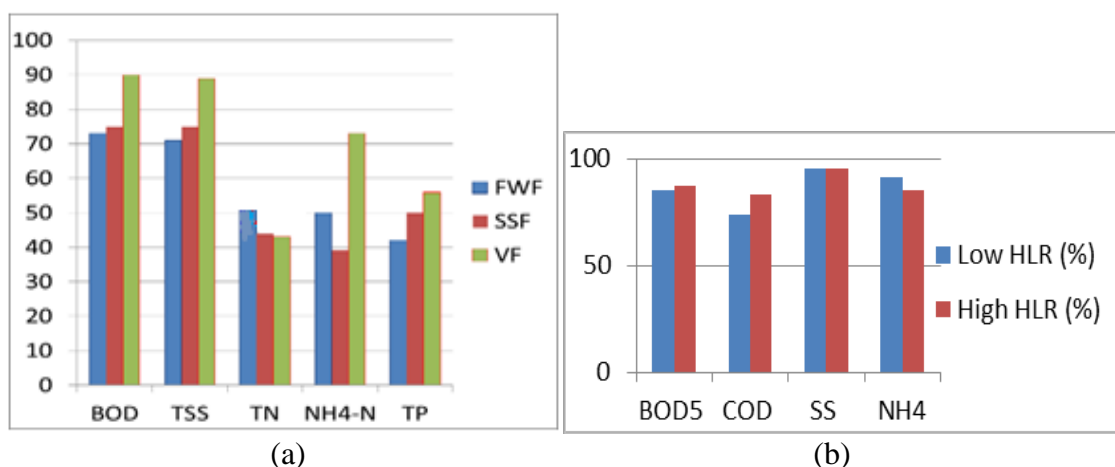
Figure 2. *Comparison of removal efficiency (a) between CW types (b) Low and high hydraulic loading rates (VSSF CW) (Sources Melian et al., 2010; Vymazal; Kröpfelová, 2008; Bulc, 2006 and Tadesse and Seyoum, 2015)*

Total phosphorus removal in all types of constructed wetlands is low (34-56%) due to very low capacity of substrates for sorption and precipitation of phosphorus (Vymazal and Kröpfelová, 2008; Vymazal, 2007, 2010). Similarly, removal of TN in all

types of CW systems is also usually low (43-61%) due to low nitrification in water-saturated SSF constructed wetlands and very low denitrification in FWS and VF CWs, respectively (Vymazal, 2007; Kadlec and Wallace, 2008; Vymazal and Kröpfelová, 2008; Wu et al., 2013) (*Table 2*).

In the free water surface flow constructed wetland nitrogen is removed via two major processes; nitrification and denitrification in aerobic and anaerobic (anoxic) litter layers of upper and bottom bed of the CWs, respectively. At VF CWS, very high nitrification proceeds, but, because of entirely aerobic conditions in the vertical bed, no denitrification takes place (Vymazal, 2010). To achieve maximum nitrogen removal vertical flow CWs should be combined with horizontal Flow CWs.

Melián et al. (2010) reported that the effects HLR (hydraulic loading rate) on the removal of pollutant in hybrid CW (HSSF followed by VSSF). At the first period of application, HLR was 37 mm/d and in the second period, HLR was 79 mm/d. Average removal efficiency of hybrid CW mainly for $NH4^+$ was higher than in any other reports. This is may be due to the presence of both aerobic and anaerobic conditions that supports both nitrification and denitrification. TN and TP removal in all types of CW is very low (*Fig. 2*). COD removal was higher in high HLR mode, but BOD and NH_3 removal was approximately greater than in high and low HLR mode (*Fig. 2*) (Melian et al., 2010; Parde et al., 2020).

Microbial assessment techniques

To generate information regarding the dynamics and properties of a given microbial 'fingerprint' or 'profile' in a CW at a given time, there are a number of both classical and novel methods available for characterizing microbial communities in the environment (Gonzalez et al., 2012). The methods are generally grouped into two; culture-dependent (plate count method) or molecular-based (culture independent) (Kirk et al., 2004; Bernardes et al., 2019).

Culture-dependent techniques

In this method certain living cells are able to grow and replicate on suppling biochemical substrates in specific physicochemical environments to evaluate bacterial abundance by plate counting techniques. Due to their cost effectiveness, and lower level of expertise needed, and the availability of media, the method remains among the most popular when measuring fecal contamination and the presence of pathogens (Morgan et al., 2008) (*Table 3*). However, the method may be unable to detect viable, but-not-culturable bacteria, which is less than1-15%, in environmental samples (Oliver, 2005; Signoretto and Canepari, 2008). For this reason, there is a need to use molecular methods for the assessment of pathogenic environmental organism (Bernardes et al., 2019; Fu et al., 2016).

Molecular (culture independent) methods/techniques

The three most often molecular applied methods are FISH, PCR-DGGE and ribosome gene cloning. Phylogenetic information from DGGE is more limited and the method is the most suitable for monitoring community succession or spatial distribution within a particular system while FISH and ribosome gene cloning allow the determination of the presence and abundance of certain microbial populations at

different resolution levels. PCR DGGE and 16 S rRNA gene cloning require extracted DNA. DNA from environmental samples, was extracted using Ultra Clean soil and Water DNA kits (MO BIO, Inc., Solana Beach, CA, USA) (Ibekwe et al., 2006) or Fast DNA spin kit for DNA extraction (BIO101, La Jolla, CA) (Henry et al., 2006; Malik et al., 2008; Cristae et al., 2014).

Table 3. Advantages and limitations of microbial methods (cultural and molecular) used to characterize constructed wetland microbial community

	Methods	Advantage	Limitation	References
	Culture-based methods	• Assessing living (culturable) microbes • Able to recognize viable cell in a sample • Cost effective, and lower level of expertise needed, and media availability	• Risk of Contamination • High skill level is necessary for optimal result • Time and resource intensive • Less specific	Morgan et al., 2008; Figdor and Gulabivala, 2011; Bernardes et al., 2019; Fu et al., 2016
Molecular methods	DGGE/TGGE	• Sensitive to variation in DNA sequences • Band can be exited, cloned and sequenced for identification	• Time consuming • Used only for short fragments • Multiple bands for a single species- hence complex for community identification • Difficult to reproduce (gel to gel variation)	Kirk et al., 2004; Ibekwe et al., 2006; Sanz and Kochling, 2007; Cristae et al., 2014
	ARDRA	• Highly useful for detection of structural changes in simple microbial communities • No single equipment required	• More applicable to environments with low complexity • Several restrictions are needed for adequate resolution • Labor and time intensive • Different band ca belongs to the same species	Nocker et al., 2007
	T-RFLP	• Enable analysis of wide array of microbes • Highly reproducible • Convenient way to store data and compare between complex samples	• False peaks may appear • Distinct sequence sharing a restriction site will result in one peak • Unable to retrieve sequences	Okubo and Sugiyama, 2009; Chikere, 2013
	FISH	• Allows detection and special distribution of more than one sample at the same time • Highly specific identification of different microbial species	• Auto-florescence of microorganisms • Staining only bacteria with intact membrane • Accuracy and reliability is highly dependent on specificity of probe(s)	Moter and Gobel, 2004; Faulwetter et al., 2009; Caltereiros et al., 2010
	16S rRNA (next generation sequencing)	• Rapid method to assess biodiversity and abundance of many species (OTU) simultaneously and at the considerable depth compared to the methods that have been available so far	• Relatively expensive • Replication and statistical analysis are essential • Challenge in terms of data analysis	

Metagenomics (next generation sequencing)	• Biodiversity can be studied in more detail • Reveals the presence of thousands of microbial genomes • Provide information about the functions of microbial communities in a given environment	• High cost • Data analysis is challenging and time consuming • Difficult to use low abundance communities • Current sequencing method	Manichanh et al., 2007;

The PCR product amplified from environmental DNA is separated by:

Denaturing-gradient gel electrophoresis (DGGE)

Electrophoresis is the biochemical technique used for separating compounds such as DNA, RNA, proteins in an electrical gradient based on variation in molecular or physical structure and chemical properties (e.g. size, shape and natural charges). The differing mobility generates band patterns that directly reflect the genetic biodiversity of the sample. The number of bands corresponds to the number of dominant species (Sanz and Kochling, 2007).

Amplified ribosomal DNA restriction analysis (ARDRA)

ARDRA also is used to digest the PCR product amplified from environmental DNA using tetra-cutter restriction endonucleases, and restricted fragments are resolved on agarose or polyacrylamide gels.

In addition, ARDRA is implemented for estimating OTUs and identifying the unique clone in environmental clone libraries based on the principle of restriction of profile clones (Smit et al., 1997). One of the major limitations of ARDRA is that restriction profiles generated from complex microbial communities are sometimes too difficult to resolve by agarose (Bernardes et al., 2019)

Terminal-restriction fragment length polymorphism (T-RFLP)

T-RFLP are markers randomly distributed throughout the genome of an organism. Restriction enzymes are used to digest DNA; followed by electrophoresis. The PCR primers used in T-RFLP analysis are fluorescently labelled at the 5′-terminus and the resulting PCR products are visualised and quantified. T-RFLP depends on differences in the sites of restriction position among sequences the lengths of fluorescently labelled fragments of terminal restriction gel electrophoresis were determined by high resolution gel electrophoresis on an automated DNA sequence (Chikere, 2013).

Staining techniques

Quantification of bacterial cells or communities, can be performed by using staining techniques such as staining with 4',6-diamido-2-phenylindole (DAPI), viability staining, fluorescent antibodies, green fluorescent protein or fluorescence *in situ* hybridization (FISH). The DAPI and FISH staining techniques were widely used for publication.

Fluorescence in situ hybridization (FISH)

FISH is a phylogenetic staining technique which makes use of fluorescent oligonucleotide probes (DNA or RNA oligonucleotide attached to a fluorescent dye) complementary in base sequence to determine bacterial genome (Amann et al., 1995; Faulwetter et al., 2009). These phylogenetic probes can penetrate and hybridize with bacterial ribosomal RNA through different procedures, and the targeted bacterial cells fluoresce according to the dye used. By using fluorescence or confocal microscopy, the presence of the targeted microorganisms can be shown and enumeration of cells can be performed (Schmidt et al., 2002; Leta et al., 2004; Caltereiros et al., 2010). The most common microbial techniques used to assess microbial community in CWs and their advantage and disadvantage inherent to each microbial method is summarized in *Table 3*.

Microbial activity detection

Microbial activity refers to a measure of the microbial driven biological processes occurring in a CW. Microbial activity can be measured by situ, mostly by measuring a specific gas production (e.g. CO_2, N_2, CH_4) enzyme production, metabolic capacity (Faulwetter et al., 2009). An estimation of the production or consumption of enzymes used in various biological processes important for wastewater treatment can shed light on those processes. Targeting specific enzymatic activities can help to better understand degradation mechanisms of a variety of pollutants and specific pathways within the carbon, nitrogen, phosphorus and sulfur transformations in CWs (Wu et al., 2011; Fu et al., 2016). Community-Level Physiological Profiles (CLPP) method also allows a relatively quick and cost-effective analysis of the metabolic capacity, or the carbon source utilization pattern (CSUPs), of the microbial community (Cristea et al., 2014).

There is a little need for isolation, amplification processing or enrichment of dilution, re-suspension and centrifugation (Calbrix et al., 2005). Creation of a CLPP is done through the use of BIOLOG™ ECOplates™ (by Biolog Inc.) which contain 96 wells, three blanks along with 31 carbon sources in triplicate (Weber and Legge, 2010; Ramírez-Vargas et al., 2020). This method measures the ability or rate of a given microbial community to metabolize carbon. CSUPs or metabolism difference is expressed in terms of richness, evenness and diversity (Gonzalez et al., 2012). The major limitation of CLPP is that it is not able to provide a reliable picture of the community structure; because it is unknown whether or not carbon utilization is due to a single species or is a result of cooperation among microbes (Gonzalez et al., 2012).

Microbial community dynamics in constructed wetlands

CWs are a mysterious assemblage of microorganisms which includes bacteria, viruses, protozoa, fungi, algae and other microscopic organisms. However, the use of the term 'microbial community' dynamics refers to the consortia of various bacterial populations in CWs. Microbial community composition, type, size and dynamics are the key factors for efficient wastewater treatment in CW systems (Faulwetter et al., 2009). The influent wastewater, with its native microbial species, is flowing through the wetland matrix (gravel, root, soil). A portion of these microbial populations are attached to this matrix and assemble in a biofilm. Biofilms varies in terms of

biological and chemical compositions depending on the type of treated wastewater, CW types and plant species (Truu et al., 2009; Qiaohong et al., 2009). For this reason, the microbial community composition of different biofilms is relatively unknown and usually considered a black box (Samaso and Garica, 2013). Analysis of influent and effluent chemical parameters and microbial dynamics in CW treatment indicates that there are consortia microbial activities involved in the treatment processes. The presence of plants in CW also enhances microbial diversity and its activity (Ibekwe et al., 2007). As a result, the plant species, development, oxygen uptake and root morphology seem to be a key factor influencing microbial-plant interactions (Gagnon et al., 2007) and biofilm population and its density (Vymazal et al., 2001; Fu et al., 2016).

Microbial communities are active in aerobic, anoxic, and anaerobic zones of CWs. In the sediments, gravel or soil, as the oxido-reduction potential decreases with depth, the succession of microbial community with increasing depth is: denitrificating, iron reducing, sulfate reducing, methanogenic microbes (Kadlec et al., 2002; Caltheiros CSC et al., 2009). Since wetlands are producing methane (CH_4) to the atmosphere, methanogens play an important role in BOD reduction in wastewater effluents. In CW systems a variety of bacteria originating from different phyla were found (Ahn et al., 2007; Yan et al., 2017). The microbial community of the wetland sediment was dominated by α-proteobacteria (48–60% of the clones) and second in abundance bacteria were related to *Actinobacteria* and *Firmicutes* (Ahn et al., 2007). A direct microbial measurement study has shown that the microbial density and activity were maximized in the first 5–10 cm of the vertical flow filter (Tietz et al., 2007). The ample oxygen supply and high nutrient contents in the upper zone of vertical flow CWs causes the higher abundance of microorganism (Wu et al., 2006; Tietz et al., 2007; Bernardes et al., 2019).

Most functional biological WWT systems depend on naturally occurring microorganisms that are responsible for the organic carbon degradation and nutrient cycling (Daims et al., 2006). Microbial biofilms attached to the CW matrix (plant root, gravel and solid particles), are responsible for most of the biological transformations and decompositions of contaminants in the wastewater (Wuetz, 2003; Faulwetter et al., 2009). According to Ibekwe et al. (2007) the majority of obtained sequence from sediment and rhizosphere samples of SF CW belongs to unclassified taxa, while the second dominant group consists of *proteobacteria* members (*Table 2*). The very dynamic and variable nature of a wetland system is a result of different gradient of redox conditions, substrate availability, and environmental conditions such as oxygen, pH and temperature (Milenkovski, 2009). The different gradient creates variable niches through the vertical and longitudinal section of the wetlands, in which different biochemical processes take place (Scholz and Lee, 2005). A study by Truu et al. (2005) also showed that wetland depth affected the microbial community structure of the biofilm with respect to communities of bacteria (ammonia oxidizing bacteria and Archaea bacteria).

Studies on the microbial diversity from CW treating high salinity tannery WW also reported the presence of bacterial isolates phylogenetically related to *Firmicutes, Actinobacteria, Bacteroidetes, α, β, and γ Proteobacteria* (Calheiros et al., 2009). Similarly, a study by Calheiros et al. (2009) and Lefebvre et al. (2006) on the microbial diversity from CW treating tannery wastewater reported the presence of bacterial isolates belonging to the *α - and γ -Proteobacteria, Firmicutes,*

Actinobacteria and Bacteroidetes groups. Aguilar et al. (2008) performed a study on the characterization of Sulphur oxidizing bacteria in wetland treating tannery wastewater and reported bacterial isolates with similarities to *α, β, and γ - Proteobacteria* subgroups and affiliated with *Actinobacter* spp (*Table 4*). Sequence analysis of bands excised from DGGE derived from bacterial 16S rRNA extracted from wetland sediment, rhizo-sphere plants, and surface water (*Table 4*) showed that the different niches of CWs have its own microbial population. In CW sediments δ-proto bacteria are dominant (14.3%). However, the dominant bacterial groups in rhizosphere and water phase are *γ–Proteobacteria* (45%) and *Cyanobacteria* (16%) respectively (*Table 4*) (Ibekwe et al., 2007). Microbial biomass C/N ratio is higher in horizontal flow systems compared to vertical flow systems, indicating the structural differences in microbial communities between those two constructed wetland types (Ibekwe et al., 2007; Fu et al., 2016).

Modeling results on spatial distribution of microbes in CWs also showed that there are dominant microbial populations in each vertical and longitudinal section (Samso and Garcia, 2013). A fermentation bacterium occupies the inlet position, Heterotrophic and nitrifying bacteria on the top and methanogens and SRB occupies the bottom sediments (*Fig. 3*).

***Table 4.** Microbial dynamics in CW treating agro-industrial and domestic wastewater treatment systems*

Waste water type	Wetland types	Taxonomic group	Abundance (%)	Sample site	Method	References
Agro-industrial WW	HSSF	α, & γ -Proteobacteria	40%	Root and substrate	DGGE 16S rRNA	Calheiros et al., 2009
		Firmicutes	18%			
		Actinobacteria	10%			
		Bacteroidetes	8%			
	HSSF	α-proteobacteria	1st (48-60%)	Sediment	DGGE 16S rRNA	Ahn et al., 2007
		Actinobacteria and Firmicutes	2nd			
	HSSF	α - and γ -Proteobacteria, Firmicutes, Actinobacteria and Bacteroidetes	NG	NG	DGGE 16S rRNA	Calheiros et al., 2009 and Lefebvre et al., 2006
	HSSF	α, β, and γ -Proteobacteria	NG	CW sediment	DGGE and sequence	Aguilar et al., 2008
	HSSF	α, β, and γ, Proteobacteria	1st, 2nd and 3rd (5-6%) of total eubacteria	CW biofilm	Enzyme gene sequence	Braker et al., 2000
	HSSF	Proteobacteria	Grater than50%	NG	DGGE and DNA Seq.	Imfeld et al., 2010
	HSSF	Proteobacteria	34.94%	Influent, effluent and storage pond	16S rRNA gene	Ibekwe et al., 2016
		Bacteroidetes	22.04%			
		Firmicutes	9.86%			
		Cyanobacteria chloroplast	6.22%			

Domestic/municipal WW	HSSF	β-Proteobacteria	1st	Gravel particles and plant-free microcosms	PCR-DGGE and sequencing of 16S rRNA	Iasur-Kruh et al., 2010
		Bacteroidetes	2nd			
		α- Proteobacteria	3rd			
		δ - Proteobacteria				
		Acidobacteria, Nitrospira				
		Bacillariophyta, lanctomycetacia				
	FWS	Unclassified taxa	1st	CWs sediment	DGGE	Ibekwe et al., 2007
		γ, α, β, and–proteobacteria	2nd			
		Acidobacteria				
		Firmicutes, Bacteroides				
	VF and HSSF	α-Proteobacteria	NG	plant roots	16S rRNA gene sequencing	Houda et al., 2014
		β-Proteobacteria				
		γ-Proteobacteria				
		Actinobacteria, Firmicutes				
	VF	β, α, & δ –proteobacteria	1st	Gravel	DGGE and sequencing	Cristae et al., 2014
		Bacteroides	2nd			
		Acidobacter	3rd			
	HSSF	δ –proteobacteria	37%	Gravel	16S rRNA gene sequencing	Bernardes et al., 2019
		Delta protobacteria	26.5%			
		Synergistia	15.1%			
		α – protebacteria	9.4%			

NB: Community level physiological profiling (CLPP), NG (not given in the article)

1. *Fermentation Bacteria*

2. *Heterotrophic bacteria*

3. *Sulphate reducing bacteria (SRB)*

4. *Metanogenic and SRB*

5. *Nitrifying bacteria*

6. *Metanogenic and SRB*
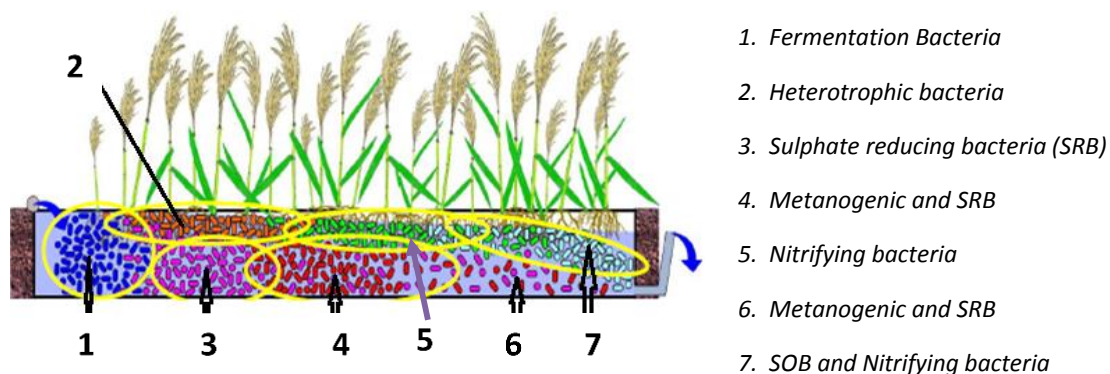
7. *SOB and Nitrifying bacteria*

Figure 3. *Vertical and horizontal microbial profile in CWs (Samso and Garcia, 2013)*

Factors affecting microbial community (biofilm development)

Microbial biomass and activities of different wetland systems were influenced by organic matter, surface property, depth (Tietz et al., 2007) and vegetation. Lv et al. (2017) conclude that season, the presence of plants and species of wetland plants were the main drivers for defining microbial community in saturated CWs system. The presence of plants defined the carbon source utilization pattern of the microbial

community. Lv et al. (2017) also showed that there are clear seasonal shifts in the carbon sources utilization patterns probably because of environmentally induced changes in plant as well as microbial activity.

The physical and chemical properties of the wetland systems may also influence biofilm biomass, its assembly and function. The type of substrate and the presence of plants seemed to have a major effect on the dynamics and diversity of the bacterial community (Gagnon et al., 2007; Vymazal et al., 2001). The root zone (rhizosphere) of CWs is the active reaction zone where the biological and physicochemical processes take places, induced by the soil, interaction of plant pollutants and microorganisms. According to Gagnon et al. (2007) CWs with plant species always had a higher microbial density and activities than unplanted controls. Microbial activities were ten times higher on root surfaces compared with sands (Wang et al., 2014). The differences in root and shoot morphology between plant species are key factors influencing microbial density and activity. Studies showed that oxygen release rates by plants are strongly correlated to the above ground biomass. According to Gagnon et al. (2007) Phalaris had the highest above ground biomass, the greatest number of stems per microcosm and the highest root surface. The high microbial density in Phalaris is due to root oxygen release and high aerobic respiration rates. Higher microbial density and activity associated with Phalaris was may be due to its rapid growth rate, passing from 4 to 88 stems on average per microcosm during the first growing season (Gagnon et al., 2007; Bernardes et al., 2019).

However, the variations introduced in the systems in terms of hydraulic loading rates did not result in substantial changes in the diversity of the microbial communities along the systems operation (Stottmeister et al., 2003). The substrate is an important wetland component since it supports plant growth, establishment of microbial biofilms and influences the hydraulic processes (Stottmeister et al., 2003). The other important spatial pattern in microbial community structure within the CW was related to the depth gradient. There is a significant difference in bacterial community structure between the upper and deeper layers; where the diversity of the bacterial community was higher in the upper layer than in the deeper horizon (Mina et al., 2011). In terms of CFU/ml there is a significant difference between the wastewater inlet and the outlet of CW (Calheiros et al., 2009).

Comparison of constructed wetland C/N ratio microbial biomass, showed that horizontal flow systems have higher microbial biomass compared to Vertical flow system (Ibekwe et al., 2007; Yan et al., 2017). Many factors can affect microbial sulfur cycling in CWS including carbon availability, the presence of more energetically favorable elements and redox conditions (Faulwetter et al., 2009; Fu et al., 2016). Sulfate reducing bacteria (SRB) are among the most ubiquitous organisms on the planet (Faulwetter et al., 2009) and most abundant of all groups (40%) in CW systems. These organisms utilize sulfate as a terminal electron acceptor in the anaerobic oxidation of organic substrates (Liamleam, and Annachhatre, 2007; Bernardes et al., 2019). The relative concentration of sulfate to other electron acceptor compounds will determine which microbial processes are occurring in anaerobic conditions.

For instance, denitrification is energetically more suitable with sulfate reduction presences after the entire nitrate has been avoided (Whitmire and Hamilton, 2005) and methanogens compete with sulfate reducing bacteria at similar redox level for available carbon (Omil et al., 1998). It has been shown that oxygen released from the roots may be used to re-oxidize reduced metabolites formed in the sulfur and iron cycles (Brune et

al., 2000; Qing yan et al., 2017). Methanogens and sulfate-reducing bacteria may compete for the same electron donors, acetate and H2 at the bottom sediments and anaerobic region (Liamleam, and Annachhatre, 2007).

The report of research on the growth of kinetics of Methanogens and sulfate-reducing bacteria shows that sulfate-reducing bacteria have a higher affinity (Ks 1/4 9.5 mg/l) than methanogens (Ks 1/4 9.5 mg/l) for acetate substrate (Hansen, 1994). This indicates that sulfate-reducing bacteria can out-compete methanogens under low acetate concentrations. This competitive inhibition results in the shunting of electrons from methane generation to sulfate reduction (McFarland and Jewell, 1990; Fu et al., 2016). Damgaard et al. (2001) used a 30 mm diameter methane-microsensor, and reported that the presence of 2 mmole/L sulfate, and the use of H (hydrogen) as an electron donor, inhibited Methanogenesis activity. Methanogenes and sulfate reducers are very competitive at the 1.7 to 2.7 COD/SO4 ratios. An increase of this ratio is favorable to methanogens, whereas a decrease in the ratio is favorable to sulfate reducers (Choi and Rim, 1991).

Conclusion

Recent CW treatment research results generally confirmed the existence of the appropriate microbial functional groups, e.g. nitrifiers, denitrifiers, methanogens, SRB and SOB, which are responsible for the removal of specific pollutants. There is a diverse and distinct bacterial community inhabits in each CW type where the different gradient creates variable niches in which different biochemical processes take place. VF systems favor aerobic microbial populations while HSSF systems favor anoxic and anaerobic microbes. Uses of hybrid CWs enhance higher removal efficiency due to the integration of all redox conditions which can support various microbial functional groups. The types of plant, substrate, redox conditions, organic matter and substrates have an effect on the composition of bacterial communities in CWs. CW with vegetation has higher microbial density and activities than unplanted ones. However, the different hydraulic loading rates did not result in significant changes in the microbial communities and removal efficiencies. The interaction between plant roots, microorganisms and substrate, along operation time, might have contributed to the establishment of diverse assemblages. Over time, in CW systems, anaerobic bacteria dominated over aerobic bacteria in terms of total biomass. Sulfate reducing bacteria were the most abundant of all groups. Based on reviewing the recent CW treatment research findings, information on functional groups of microbes in CW is lacking.

The way forward

The reviewed results provide a snapshot of the composition and structure of microbial community dynamics in the constructed wetland for the treatment of agro-industrial and domestic wastewater. In order to have conclusive information on the microbial community population dynamics playing key roles in the removal of these pollutants, the following key points were recommending:

- It is important to perform a longitudinal investigation of microbes in each component of the treatment system as part of a routine measurement of biotic and abiotic factors over time.

- In addition to the assessment of temporal dynamics of microbial community structure and activity in constructed wetlands there is a need for high resolution sampling strategies for estimating spatial variation of microbial parameters
- It is impossible to make any overall conclusions about the wetlands microbial community structure dynamics or in its operational parameters and relation to removal process. Future research is vital to link microbial community composition, action, and function, combining genotyping systems with activity measurements, in order to reliably estimate the many of bacterial species, associate bacterial communities within and among constructed wetlands, and relate community structure to environmental parameters.

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Engida et al.: Microbial community dynamics and activity in constructed wetland treating agro-industrial and domestic wastewater: a review

- 2687 -

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