

Review

Microbial Community Assessment in Wetlands for Water Pollution Control: Past, Present, and Future Outlook

Kela P. Weber

Environmental Sciences Group, Department of Chemistry and Chemical Engineering,
Royal Military College of Canada, Kingston, ON K7K 7B4, Canada; kela.weber@rmc.ca;
Tel.: +1-613-541-6000 (ext. 3633)

Academic Editors: Hans Brix, Carlos A. Arias and Pedro N. Carvalho

Received: 31 August 2016; Accepted: 26 October 2016; Published: 2 November 2016

Abstract: The field of treatment wetlands (TWs) is rapidly expanding and, arguably, is tasked with studying and understanding one of the most complex water treatment systems available. Microbial communities are generally considered to be responsible for the majority of wastewater constituent degradation in TWs. However, they are also known to be spatially heterogeneous, temporally dynamic, as well as structurally and functionally diverse. Presented here is a meta-analysis of all peer reviewed TW journal articles which utilized a microbial community assessment methodology over the period of 1988 to July 2016. A total of 1101 papers were reviewed, 512 from 1988 to 2012, 215 of which included a microbial community assessment aspect and were subsequently classified as representing past research, and 589 from 2013 to July 2016, 196 of which were classified as representing current TW microbial community research. In general, TW microbial community research has increased over time, with a marked surge in the past four years. Microbial community structure is currently the most commonly used methodological type followed by activity, enumeration and function, respectively. Areas of research focus included nitrogen transformations (156), organic degradation (33), and emerging contaminants (32), with general characterization studies also accounting for a significant proportion (243). Microbial communities from a range of TW systems have been investigated over the last four years with meso-scale (10–1000 L) being the most commonly studied system size followed by large-scale (>100,000 L), micro-scale (<10 L), and pilot-scale (1000–100,000 L). Free water surface flow (SF), horizontal subsurface flow (HF), and vertical flow (VF) systems are being studied in approximately equal proportions with the majority of studies focused on gaining fixed media/biofilm samples for analysis (rather than from the rhizosphere or interstitial water). Looking at efforts from a regional perspective shows Asia to be publishing the majority of research with a main focus on VF systems and structural community assessment. European and North American studies are generally more evenly distributed among structure, function, activity, and enumeration with the majority of studies completed on HF systems. South America, Africa, and Oceania published fewer studies but focused on structural community assessment with a selection of HF, SF and VF investigations. Great strides are being made in the field of microbial community assessment in TWs with functional assessment methods being developed, better utilized, and being related directly to water treatment. The use of high-powered metagenomics sequencing such as Illumina HiSeq instrumentation is on the rise, as is the development and utilization of functional assays such as DNA microarrays and community level physiological profiling allowing for more complete community assessment. Used in concert with activity, enumeration and newly implemented stable isotope methodologies, the field of TWs is certainly moving away from the black-box understanding of the past.

Keywords: microbial community; biofilm; function; structure; activity; enumeration; treatment wetland; constructed wetland; wetland; water treatment

1. Introduction

Wetlands for water pollution control have been in use for more than 100 years. Both Kadlec and Wallace [1] and Vymazal [2] give detailed accounts of the major developments in the field over the past century and describe the field as evolving, over time, towards more intensely engineered designs and process train layouts for water treatment optimization purposes. The term “constructed wetland” (CW) is generally used when a wetland for water pollution control has been explicitly built and designed for a water treatment purpose. The term “treatment wetland” (TW) generally encompasses constructed wetlands but is more expansive and includes natural wetlands that are intentionally used as catchment areas for water pollution. Kadlec and Wallace [1] describe several early 1900s North American examples of natural wetlands receiving water pollution, by design, over extended periods of time as a general polishing (tertiary) water treatment step.

Vymazal [2] classifies TW systems based on vegetation (emergent, submerged, floating leaved, and free-floating) and hydrology (free water surface, and subsurface flow), with subsurface systems classified into horizontal and vertical flow, and hybrid systems involving any combination thereof. Free water surface systems are generally more common in North America and Australia [3], where subsurface flow systems are more common in Europe and were recently described as one of the most common extensive water treatment processes in the world [4]. Although many design variations exist, often three main (more commonly implemented) configurations are discussed and compared in the literature being free water surface flow (SF), horizontal subsurface flow (HF), and subsurface vertical flow (VF). SF systems are more often used for tertiary water treatment applications, where both HF and VF are more commonly used for secondary water treatment (although many exceptions do exist). VF systems are also sometimes used in primary treatment applications (termed “French systems”). Engineering augmentation, design, and intensification is currently an active area of research with aerated systems being implemented for full-scale applications in many locations worldwide (see Nivala et al. [5]).

The study of the use and utility of plants in water treatment systems is generally credited to German scientists in the 1950s, the first being Seidel [6] (as described in [1,3]). More recently, the rhizosphere (subsurface zone of interaction between root structures and microbial communities) has been described as a “sunlight driven hotspot” for the degradation of organics [7], and has been shown to degrade, remove, immobilize, and/or transform a range of contaminants including but not limited to nitrogen compounds, organics, pharmaceuticals, petrochemicals, chlorinated solvents, pesticides, explosives, heavy metals, and radionuclides to name a few [8,9]. Removal mechanisms involved in water treatment in TWs include a number of physiochemical, plant related, and microbiologically mediated mechanisms, the host of which are outlined in Kadlec and Wallace [1]. TWs are relatively straightforward in their implementation. However, their internal operation is quite complex. Microbial communities can be found throughout TWs, however, three main areas are commonly identified: attached, within close proximity to, or associated with roots (rhizospheric); within biofilms surrounding the general media; or in the free water (for SF) or interstitial water (for HF or VF). As wastewater passes through a TW the chemical constituents can be considered food for microorganisms. As this food is utilized by microbial communities in specific areas of the TW, they can anchor and create fixed biofilms through secretion of extracellular polymeric substances [8]. As this anabolic action of biofilm and microbial mass creation continues, pore space in HF or VF systems can change (on a local-scale) thus driving water through a slightly different hydrological regime, and therefore overall flow-path in the TW. This action is quite interesting as the nutrient flux to biofilms in areas of reduced pore space is thus reduced, naturally limiting biofilm growth. As this process continues over time a subsurface TW can develop to a point where biofilm is either well distributed or perhaps heterogeneous. Stratification of biomass in subsurface flow systems is well documented [10]. At some point an equilibrium between attachment and detachment (due to local-scale velocity and shear stress) can occur allowing for steady state operation with no bio-clogging [11]. This steady-state operation is however sometimes not possible due either to simple solids build up, or in

part due to the nutrient loading which can drive the microbial community towards anabolic based processes (i.e., creation of biomass/biofilm) which can eventually lead to clogging of pore spaces (see Nivala et al. [12]). Recent modelling exercises have included these dynamics into fundamental TW investigations [11,13–15], and predict either heterogeneous or homogenous clogging depending on the specific model used.

As described, microbial communities are said to facilitate water treatment through metabolic actions resulting in the general degradation of waste constituents. This occurs through using the waste constituent for either cellular mass and reproduction (anabolism), or energy (catabolism). These metabolic actions are facilitated through a different series of enzymatic reactions based on the specific functional requirement/action of the community, or subset of the community. Different areas within TWs house different environments, which help drive and select for certain functional abilities of inherent TW microbial communities [4,8,10,16]. It is through these diverse and iterative sets of different conditions, in which wastewater passes through, that TWs are thought to have exceptional and sometimes surprising water treatment potential [8]. For example, CWs have shown promise for the removal of pesticides [17] and emerging contaminants, including antibiotics, pharmaceuticals and personal care products [18].

Weber and Gagnon [8] describe four classes of microbial community assessment: activity, enumeration, function, and structure. Enumeration methods look to provide quantitative data on the number or amount of microorganisms in a sample. Activity measurements do not directly account for the number of microorganisms in a sample, but rather look to understand how metabolically active they are. This activity is often directed at a specific type of transformation, for example CO₂ evolution implying catabolic respiration and the mineralization of organic material. Functional analysis looks to profile the overall function of a microbial community over a range of metabolic transformations. This in some ways can be thought of as gathering an understanding of many transformation-specific activity measures to build a full picture of the microbial community overall function. Function can also be assessed through piecing together gene pathways available (DNA) or active (mRNA) in a microbial community through molecular techniques. Structural analysis is focused on what microorganisms are present in a sample (often at the individual level, or as operational taxonomic units) and is completed through many techniques ranging from light microscope profiling to metagenomics sequencing. Weber and Gagnon [8] further define structural analyses to include methods where communities are compared based on structural components, even if not all individuals or taxonomic units are expressly identified. Using this premise, community based DNA fingerprinting methodologies are categorized as a type of structural analysis. Truu et al. [10], Faulwetter et al. [16], and Weber and Gagnon [8] provide an account regarding the different microbial community assessment methodologies available and their general utility. However, a solid quantitative survey of what specific microbial community assessment methods are being utilized in the field of TWs, and for what purpose has not been reported.

The objective of this study is to provide a comprehensive and precise overview of exactly what microbial community assessment methodologies are being used and developed for use in the field of TWs, and for what purposes. To this end a meta-analysis was completed in order to quantitatively and concisely summarize these aspects throughout the history of TWs. This study is not meant to comment on or recap the findings of all microbial community assessment studies completed in the field of TWs, rather to gain an historical perspective on microbial community assessment in TWs, understand what tools are currently available in the field of TWs, and to provide perspective and comment on current and future developments.

2. Literature Review

Literature was gathered and reviewed in two separate phases. The first phase was aimed at understanding past practices and developments with respect to microbial community analysis in the field of TWs. The second phase was aimed at understanding current practices and state of the art in microbial community assessment in TWs.

2.1. Phase One—1880 to 2012

For phase one all potentially relevant peer reviewed journal publications were gathered by searching through the years of 1880 to 2012 using the databases of Compendex (engineering focused), and Web of Science (more generally captures fundamental science journals). Keywords used for this search were: wetland, constructed wetland, treatment wetland, which were then individually combined with the word microbiology, microbiological, or microbial, giving a total of nine searches. The key word “wetland” was designated as useful over this time period as many early publications did not identify systems as “treatment wetlands” or “constructed wetlands” yet they certainly involved the study of a wetland used for water pollution control. These nine individual searches were then combined and duplicates removed leaving a total of 512 papers. Conference proceedings and non-TW related papers were then removed (206 total) which included a large number of studies investigating natural wetlands where no water pollution control was involved. After this, an additional 91 papers were removed, as they did not include the direct study of a microbial community. The latter removal step was found to be a common occurrence as many publications included the word “microbiology”, “microbiological”, or “microbial” within the paper in order to help discuss and contextualize water treatment results, but the authors did not actually directly investigate the inherent TW microbial community using any methodologies in that specific publication. Pathogen removal focused papers also fell into this category as many looked at the removal of microorganisms from influent water, but did not investigate the inherent TW microbial community. Following this process, the remaining 215 publications were then reviewed, with specific content tracked for further analysis. An excel spreadsheet was used to track the publication title, year, focus area of the study, and specific microbial community assessment methodologies used. Methodologies were then classified into the following categories: activity, enumeration, function, and structure (a full listing of methodologies used and their classifications will be presented later). Focus areas were identified based on the type of water contaminant(s) or research area being investigated and included: nitrogen, organics, phosphorus, methane, sulphur, agricultural, chlorinated volatile organic compounds (cVOCs), petrochemicals, BTEX (benzene, toluene, ethylbenzene and xylene), pesticides, emerging contaminants (ECs), metals, and microbial fuel cells (MFCs).

2.2. Phase Two—2013 to 2016

Phase two was conducted in a similar fashion to phase one, but was augmented in several key ways in order to gather additional info to fully understand the current state of the art and general research efforts currently being expended in the field of TWs. Compendex and Web of Science were again used however only across the years of 2013–2016 (which included up until the month of July in 2016), and the searches did not use the keyword “wetland”. It was deemed that the field of TWs was well established by 2013 and in order to gain insight into TW specific state of the art, catching all wetland related studies (i.e., natural wetland studies without water pollution control involved) was not desirable. A total of 1445 publications were gathered. Table 1 summarizes the specific search match results. After removing duplicates, a total of 589 publications remained. In reviewing these 589 publications, 420 were later removed, as they did not utilize a specific microbial community assessment methodology, leaving 169. An interesting note is that phase two gathered a large number of review articles. In addition, a large number of articles were found which reviewed or described alternative water treatment methodologies that then also referred to TWs in some fashion. This was interpreted as evidence that the TW field certainly has matured to a point where it is thought to be a mainstream and useful comparison reference for other novel and developing technologies.

Table 1. Summary of phase two literature search matches—2013 to 2016 (July).

Constructed Wetland			Treatment Wetland		
Compendex	microbiology	81	Compendex	microbiology	94
	microbiological	81		microbiological	94
	microbial	179		microbial	196
Web of Science	microbiology	7	Web of Science	microbiology	5
	microbiological	25		microbiological	22
	microbial	353		microbial	308
Total		726	Total		719

The remaining 169 publications were then reviewed for content and information tallied in an excel spreadsheet. In addition to the same suite of information gathered from publications in phase one, phase two also gathered information on the region where the study was undertaken, the system configuration/design, and the system size. Region was captured as the country from which the corresponding author resided. This was the only way to gather consistent information throughout the entire process, however it is identified that small biases may exist. For example, the corresponding author of Button et al. [19] is from North America, even though the actual systems studied are in Europe. System configuration was gathered through the authors own system configuration identification, or from provided schematics and other descriptions. In some cases, informed estimations were required. For example, a large number of publications which did not identify the system configuration surrounded the investigation of large-scale systems (often more than 10 ha in area), in those instances the author chose to identify the systems as surface flow, as it was deemed unlikely that systems of such size would be subsurface vertical or horizontal flow. The system size was quantified in terms of total system volume in litres (L). The system sizes were then further categorized into four size ranges: microcosm (0.1–10 L), mesocosm (10–1000 L), pilot-scale (1000–100,000 L), and large-scale (>100,000 L). In many cases, system depth was not given. In those instances, the author chose to use a standard assumed depth of 1 m for all system types (SF, HF, or VF). In the majority of cases, only publications focused on pilot-scale or large-scale systems chose to not report a system depth, and therefore the majority of systems where an assumed depth of 1 m was used happened to be surface flow (which admittedly means 1 m may be an overestimation in some cases). There were a small number of cases where the authors chose to not report size dimensions of any sort. In those instances, an implied system size identification was sought, such as the authors describing their system as “pilot-scale”. In order to estimate a size from an implied size description, the author chose a value one order of magnitude above the lowest value for that size category: 1 L for microcosms, 100 L for mesocosms, 10,000 L for pilot-scale, and 1,000,000 L for large-scale.

3. Results and Discussion

Phase one and phase two literature reviews returned a large amount of information. Much of the information spans across the entire time period surveyed, however in the interest of the objectives, information has been packaged to generally describe and comment on past practices and development, and current state of the art.

3.1. Past

3.1.1. Historical Perspective

The field of CWs owes homage to many scientists from other research fields who have paved the way for microbial community assessment. Although it is not possible to mention all, some notable historical mentions are made here.

The first identification of a microorganism was completed using a light microscope in 1665 by Hooke and Leeuwenhoek (as described by Gest [20]). Since then, microbial community assessment methods have been steadily developed, albeit more slowly until the 1970s. The first microbial community structural assessment in TWs could be said to be directly connected to the work of Hooke and Leeuwenhoek. Vymazal et al. [21] used a light microscope to spatially assess and compare microbial community structural differences in a HF system. The foundation for another relatively simple method for the identification of microbial community structural shifts was first suggested by White et al. [22] (as described in Morris et al. [23]) where they introduced the concept of extracting and profiling phospholipids from microbial community cellular membranes, which lead to the further refinement of FAME (fatty acid methyl ester profile) and PLFA (phospholipid-derived fatty acid profile) methodologies. More recently, specialized metagenomics sequencing equipment has been developed and is available in benchtop formats.

The inception of the idea of bacterial culturing/plating and enumeration first began in 1824 through Bartolome Bizio's observation of red colony bacteria on foodstuffs (as described by Merlino [24]). This observation and the concept of bacterial colonies later became the cornerstone for colony enumeration using agar-based media and was later developed and refined by scientists such as Robert Koch and Julius Richard Petri (to name only a few). Through the meta-analysis performed here, Hatano et al. [25] was found to be the first to enumerate inherent TW bacteria and fungi in a SF systems treating pulp mill wastewater.

The ideas behind measuring microbial community activity could be argued to originate from many areas. In the opinion of the author, one of the principal activity based breakthrough concepts comes from the Royal Commission on Sewage Disposal [26] when they recommended the use of the BOD5 method to test water pollution. The BOD5 method, at its origin, is an oxygen uptake measure. This concept could be said to later develop into standardized substrate uptake and gas evolution activity measures. Arguably, microbial activity had been studied and measured much earlier in time than this though (although origins and examples are difficult to pin down). The first mentioning found by the author was that of MacDougall [27] who generally mentions that the idea of soil activity credited to microorganisms was not known until 1877 (two decades previous to his own paper) where he says previously they understood soil to be made of non-living forms of geologic origin. It should also be noted that the concept of plant respiration, which can be said to lead to the concepts of soil bacteria respiration, was studied as early as the 1770s in England by Joseph Priestly who is credited with discovering oxygen gas.

Microbial community function is perhaps the end goal of microbial community assessment in TWs. TWs are designed for water treatment, and understanding the mechanistic contribution of microbial communities is generally understood as a cornerstone in advancing and optimizing TWs. That being said, there are relatively few microbial community function methodologies available for TWs. Hench et al. [28] first used community level physiological profiling, borrowing from soil scientists and the development of micro-titre plates for soil bacteria differentiation [29,30]. It was, however, not until recently (for example see Button et al. [19]) that the carbon sources on the plate were truly considered as measures for organic degradation and used in data interpretation and TW optimization recommendations. Molecular based techniques have been more recently been used and will be discussed later.

3.1.2. Microbial Community Assessment in Treatment Wetlands from 1988 to 2013

Weber and Gagnon [8] performed a literature survey where publications from 1988 to 2012 were gathered and classified based on the type of microbial community assessment methodology utilized. Here, the phase one analysis was expanded to list the specific methodologies utilized and the focus area of the study, and to additionally gather region, system configuration, system size information as performed in phase two. Figure 1 summarizes all journal publications in the field of TWs wetlands where a microbial community assessment technique was used over the period of 1988 to July 2016. The

field of microbial community assessment and microbiology (in general) goes back much farther than 1988, however, as far as the author was able to find (using the methods employed) Brodrick et al. [31] is the first available peer reviewed journal article where a constructed wetland microbial community is directly assessed. The article was published in *Water Research* and describes the study of a 3.3 ha natural wetland in southeast Australia that had been used for tertiary water treatment for more than a decade. The article stated that it was aimed at moving forward from the “black-box” understanding of the systems operation at the time. Nitrogen removal in the system had met expectations for many years, however the study aimed to estimate denitrification potential in different vertical cross sections using an ex-situ benchtop nitrogen transformation activity methodology. Samples taken downstream of the water inlet were compared to background samples from the same geographical wetland area upstream of the wastewater influent point. Findings showed the top layers of the wetland and the wetland samples taken downstream of the influent point to be most active. Recommendations were made to spread the wastewater in a more diffuse fashion across the top of the wetland area to better make use of the more active top layers for denitrification. For the present meta-analysis, Brodrick et al. [31] was classified as being focused on nitrogen removal, and utilized a method of activity measurement, specifically a nitrogen transformation methodology of activity assessment. Brodrick et al. [31] is a good example of using microbial community data to better understand a system’s operation, and further use this information to recommend a design augmentation to potentially increase the water treatment effectiveness of the investigated TW (or TWs in general).

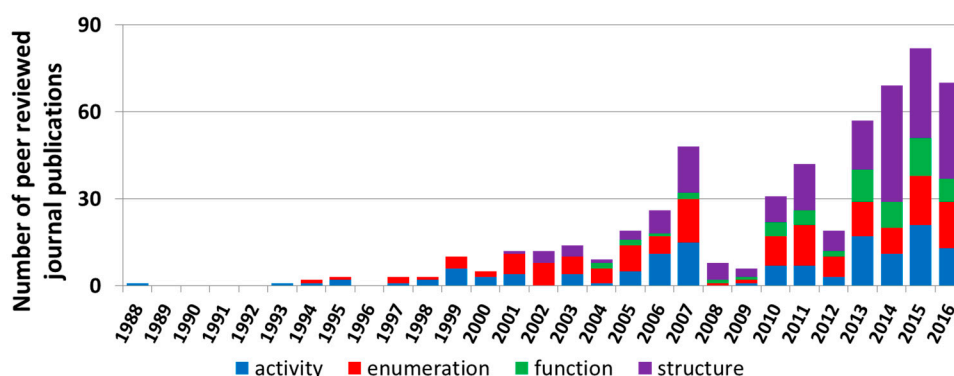


Figure 1. Number of journal publications per year where a microbial community assessment method was directly applied to a treatment wetland for investigative purposes.

Following this first known activity assessment, the first study utilizing enumeration was found in 1994, the first study utilizing a structural analysis was found in 2001, and the first microbial community function study in 2004. Many of the methodologies found in the early studies borrowed from soil microbial community analysis techniques. Up until 2007 a steady increase in publication rate can be observed with the main areas of interest being activity and enumeration. Starting in 2008 (a somewhat thin year for publications), structural assessments started to proportionally become more popular, with functional studies really only becoming proportionally noticeable in 2010. This trend is not unexpected as it generally follows the trends of methodology development.

3.2. Present

Phase two results showed a large increase in microbial community methodology use over the past three and a half years (2013 to July 2016). Figure 1 shows all four types of methods are being utilized in large numbers, with sequencing perhaps becoming the most popular, especially in 2014–2016. It can also be seen that the use of activity measures, and functional assessment has generally increased from the years previous to 2013. Use of enumeration methods has not changed to any great degree since 2001; enumeration sees a consistent use of approximately 10 studies per year, save the generally sparse

years of 2008 and 2009. Perhaps the most important observation is the overall increase in TW studies directly investigating microbial communities over the last four years. Given this exceptional trend and overall research activity, the author suggests that the field of TWs is working to move beyond the black-box understanding which has been referenced as recently as 2016 (see Lünsmann et al. [32]).

3.2.1. Study Focus Areas

In order to fully understand how and why microbial community assessment techniques are being utilized each instance of methodology use was mapped against the specific target area of the research article. The majority of TW research is aimed at better understanding water treatment mechanisms, or ways in which to optimize for water treatment. There are of course many other areas of research that can either be concurrently studied, or individually studied which contribute to our better understanding of other TW aspects (e.g., hydrology, translocation of materials in plants, and methane release). All can be important to better understand and optimize water treatment, or for the improved implementation and long-term management of TWs. Table 2 quantitatively summarizes the usage of microbial community assessment methods over the past 28 years. A large number of methodological implementations focused on a general characterization of the microbial community from TWs (243 total). This could have included single samples, a spatial survey, or in some cases tracking temporal trends. They generally did not directly correlate microbial community results with water treatment, or have a specific water treatment application focus area contextually identified. There were however a large number of studies where microbial community assessment was completed in the context of nitrogen removal (156 total). Generally, where a characterization was the goal, all methodology categories were evenly used.

Nitrogen is a species where we generally understand the different mechanisms involved and even know of several groups of bacteria which are able to perform specific nitrogen transformations (see Parades et al. [33]). Therefore, it is not surprising that this was the second most popular area of focus with respect to microbial community assessment. There was a total of 35 instances where microbial community assessment was completed to better understand the treatment of organics (third most popular), again not surprising given that the degradation of organics is generally well understood from a mechanistic standpoint. Although it could be said that some of the earlier papers which studied nitrogen removal also studied organics removal in some way as well, the first purely organics focused study was found to be Baptista et al. [34] where a suite of microbial assessment methods were utilized and a dedicated effort was made to relate microbial community parameters to observed treatment of organics (in terms of overall removal).

Interestingly, emerging contaminants (ECs) was the fourth most heavily studied area. Here, mechanistic degradation of ECs is not well understood, however TWs do show an exceptional capacity for removing ECs from wastewater. Activity was less utilized than the other categories for ECs. Many studies were looking to understand the impacts of compounds such as antibiotics on the inherent microbial community and any potential long term impacts to TW health and water treatment capabilities. Methane, petrochemicals (which included BTEX), sulphur, and metals were all also studied to a reasonable degree with the categories of assessment generally evenly distributed. A large number of MFC papers were reviewed as part of the meta-analysis (approximately 20). Five of those MFC studies directly assessed the microbial community, and one of them, Corbella et al. [35], was multiphasic in using both a functional analysis and structural analysis in the same study. There the authors used qPCR (quantitative real-time polymerase chain reaction) to detect methyl-coenzyme M reductase (*mcrA*) genes, which are generally considered to be a functional marker of methanogenesis as it catalyzes the last step in methanogenesis and is present in all methanogens [36]. The same study also performed 454 pyrosequencing for the V1–3 region of the 16S rRNA gene for eubacteria, and then separately targeting the V3–4 for archaeobacteria.

Table 2. Usage of microbial community assessment methods mapped against specific research areas. Includes the period of 1988 to July 2016.

Classification	Method	Crtz	N	C	ECs	CH4	Petr	S	M	MFC
Activity	Adenosine Triphosphate	6								
	Amino Acid Assimilation	3								
	O ₂ utilization	1		1						
	CO ₂ production	10	6	4		3				
	CH ₄ evolution		1			1				
	Enzyme Activity	24	11	2	2		1		1	
	Stable Isotope Probes	6	4	2			3	1		
	Ex-situ N Transformations	2	28	2		4				
	Other Transformations		3	1	1	4	1		1	
Function	CLPP	19	3	1	2		1			
	qPCR (DNA-specific gene)		1	1	4	2	2	1		1
	qPCR (DNA-N gene)	1	21	1		2		1		
	FISH (specific gene)	1	4		1	1		1		
	qPCR (mRNA)						1			
	Microarray						1			
	Proteomics			1			1			
Enumeration	Plate counting	21	6	5	2		2	3	2	
	Most Probable Number	5	5	1				1		
	Staining	7	1		1					
	Flow Cytometry	2								
	qPCR (16S rRNA only)	11	5	1	3	3	1	1		
	Grid Counting	8							1	
	FISH	9	5			1				1
	Organic Mass/Fumigation	8	2							1
	EPS Components	5								
Structure	Stable Isotope Probes	1	1	1		1		1		
	FAME/PLFA	9	1		1		1			
	DGGE/TGGE/SSCP	30	16	4	6	1	3	2		
	TRFLP/ARDRA	8	7	1		1	1		1	
	RISA	1	1		1		1		1	
	DGGE Band Sequencing	21	10	2	1	2		1		
	454 Pyrosequencing	9	5		2	1	1		1	2
	ABI PRISM sequencing	4	2				1		1	
	Sanger sequencing	2								
	Illumina Miseq	4	3	1	1		2			
	Illumina Hiseq		2							
	Unknown Sequencing	4	1		2					1
	Microscopy	1	1	1	2					

Notes: Research area abbreviations: Crtz, characterization; N, nitrogen; C, carbon/organics; ECs, emerging contaminants; CH₄, methane; Petr, petrochemicals; S, sulphur; M, metals; MFC, microbial fuel cell. Methodological abbreviations: CLPP, community level physiological profiling; qPCR, quantitative real-time polymerase chain reaction; FISH, fluorescence in-situ hybridization; EPS, extracellular polymeric substances; FAME, fatty acid methyl esters; PLFA, phospholipid-derived fatty acids; DGGE, denaturing gradient gel electrophoresis; TGGE, temperature gradient gel electrophoresis; SSCP, single-strand conformation polymorphism; TRFLP, terminal restriction fragment length polymorphism; ARDRA, amplified ribosomal DNA restriction analysis; RISA, ribosomal intergenic spacer analysis.

Functional Assessment

The definition of functional assessment can be debated. A functional assessment implies that one is gaining an understanding of what the microbial community is able to accomplish (i.e., what they are doing), where structural assessment is aimed at understanding what specific species or phylogenetic groups are present. With respect to TWs, water treatment capacity is the main consideration for improvement and end application. When it comes to microbial community mediated water treatment, the majority of functional activity/capacity is due to chemical transformations facilitated by enzymes either intracellularly or extracellularly. These transformations can be related to a number of important water treatment aspects such as organic matter degradation, nitrogen cycling, or methane production (to name only a few). The range of enzymes expressed by the microbial community constitutes the

overall water treatment capability. For an enzyme to be expressed and involved in water treatment in some way, the inherent microbial DNA first requires the gene encoding this enzyme. This gene can be found within the genomic DNA, or housed within a plasmid, which is carried in the cellular matrix separately from genomic DNA. Plasmids are quite interesting as they can be transferred between cells (called horizontal gene transfer) allowing for exceptional possibilities in microbial communities as a whole. Plasmids are also commonly engineered, augmented, and utilized in microbial community assessment laboratory methods. Whether a gene is found on genomic DNA or in a plasmid, the next step towards end function is transcription, where the DNA is transcribed into mRNA. This mRNA is then translated into an actual protein or group of proteins which can then mediate chemical reactions. Collectively known as enzymes, these proteins/enzymes physically interact with chemical compounds in wastewater to degrade or transform them into other molecules.

If looking to identify and quantify genes relevant to water treatment from a microbial community, qPCR targeting the gene of interest (i.e., not the 16S region, but a region/gene encoding for a different protein/enzyme) in DNA is commonly used. This methodology does not look for mRNA or the specific enzyme of interest, rather it looks to see if the genetic code to potentially create the required mRNA and enzyme is available. The greater research community sometimes generally accepts this to be a measure of implied function, even though it is more accurately a measure of potential function. This can be contentious as interpretation can be mixed. For example, facultative microbial communities are able to switch between aerobic and anaerobic metabolism. In order to survive under either environmental regime, the community is required to express different enzymes depending on the regime they are currently experiencing. This, for example, would be a common situation in tidal flow, or intermittently aerated TWs. Microbial communities will certainly carry DNA for enzymatic transformations in either regime for extended periods while experiencing either environmental condition. In a practical sense this means that a microbial community can carry genetic material for waste transformations that they are currently unable to express, or perhaps are simply not expressing because there is a more energetically favourable metabolic process available. The presence of a gene in DNA does not directly indicate active function at the time of sampling. This type of debate over interpretation was well communicated among Zhi et al. [37], Chen and Vymazal [38], and Zhi et al. [39]. Assessing DNA to better understand functional potential is a common practice, however researchers are cautioned to rigorously consider findings in the context of how they were collected and over the spatial and temporal scales which may be relevant. In this meta-analysis, a FISH (fluorescence in situ hybridization) or qPCR analysis where a specific functional gene was investigated was considered a functional analysis. However, as stated, it is more specifically a measure of potential function, not active function.

With respect to assessing function, two specific studies require mention. Martinez-Lavanchy et al. [40] documented the first TW peer reviewed study utilizing a microarray methodology. Microarrays are powerful tools and able to semi-quantitatively detect 10,000+ genes in a single sample, allowing one to piece together potential enzymatic pathways. In this particular study, toluene removal was of interest, and the authors were able to find evidence suggesting the presence of the ring monooxygenation pathway for toluene degradation. The microarray was based on DNA, and therefore potential function was assessed, however the authors followed up with stable isotope analysis to provide a second line of evidence pointing to the ring monooxygenation pathway for toluene degradation in their systems. The second study requiring mention is that of Lünsmann et al. [32], which involved a functional analysis of microbial communities in lab-scale TWs also treating toluene contaminated water. Lünsmann and coauthors describe a rigorous assessment of toluene degrading function through mRNA extraction, cDNA construction and subsequent qPCR. This is the first TW specific instance of mRNA extraction and analysis found through this meta-analysis exercise. The authors, in the same study, went on to perform proteomic analysis looking for specific proteins/enzymes involved in toluene degradation. This was also the first study documented to use proteomics. Proteins corresponding to quantified mRNA were not found, however the proteomic survey was a general success. A follow up study by Lünsmann et al. [41] utilized proteomics, stable isotope analysis and metagenomics to further elucidate

a specific bacterial family (*Burkholderiaceae*) and functional pathways mainly responsible for toluene degradation in lab-scale TWs.

mRNA is not often used for the assessment of microbial community function of environmental samples due to its general instability, and difficulty in its proper extraction [42]. The study of Lünsmann et al. [32] was completed in lab-scale, well controlled TWs. Perhaps this assisted in creating a matrix where mRNA extraction could be successful. In a similar fashion proteomics has been described as challenging for environmental samples, and an area of research where lab-scale systems are very useful, if not a requirement, given current methodologies [43]. Small-scale systems do have the advantage of being well controlled and replicable, however they are generally thought to not perfectly replicate large-scale systems, especially the inherent heterogeneity of large-scale systems, which is generally thought to be an advantage in water treatment.

Stable isotope studies are also challenging to perform, as they require specialized expertise and equipment, however the technology has been used in the field of TWs for quite some time. The first stable isotope study found was that of Pollard et al. [44] where isotopes were used to directly and confidently track the growth of bacteria in biofilms on plant roots. This was an example where stable isotope analysis was used for an enumeration type purpose. Stable isotope analysis was also sometimes classified as a type of activity measure. For example, Braeckevelt et al. [45] used stable isotope analysis to study the degradation of monochlorobenzene, and Lünsmann et al. [41] for the study of toluene degradation. It was however admittedly difficult to properly categorize these studies (and other isotope studies) within the four-category construct (activity, enumeration, function, and structure) of this meta-analysis. For example, Lünsmann et al. [41] used isotope analysis for identifying specific bacteria involved in toluene degradation which could perhaps be said to be aimed at a more structural analysis objective. Another example was that of Martinez-Lavanchy et al. [40] who used isotope analysis as a second line of evidence to verify functional pathways, which could have been classified as a functional assessment measure rather than an activity based measure. There may be some bias in how isotope studies were captured in this meta-analysis however it is clear that stable isotope analysis can be used for many purposes and has been successful in several TW studies.

Structural Assessment

When assessing structure via sequencing, the 16S rRNA region of DNA is most often investigated. This is a region that is well conserved between generations and encodes for the specific proteins required for the process of translation. Different species have slightly different sequences encoding for the 16S rRNA gene. From this type of analysis one generally receives an indication of phylogenetic diversity, and if data is mined further, known species can be identified. This can be useful in assessing shifts in microbial communities or generally understanding diversity over space or time. It however does not directly assist with understanding the exact microbial community mediated mechanisms of water treatment available to that community. If the identified species are well characterized and their known enzymatic/functional capabilities are considered, one could generate an idea of potential function. This is however challenging and likely the reason the author found this type of extended analysis only present in a select few of the 200 structural analyses reviewed.

Meta-genomic sequencing is a powerful tool which as stated can yield large amounts of information. Different levels of information are attained depending on the sequencing method used (base pair length sequenced, and error rate being two important parameters). Sequencing in TWs first started (and continues) with Sanger, ABI PRISM and 454 pyrosequencing, but has also moved on to more powerful platforms such as Illumina[®] Miseq and Hiseq platforms. Ligi et al. [46] were the first to use an Illumina[®] Hiseq 2000 to spatially investigate a constructed riverine complex in Ohio, USA. Unfortunately, a fair number of studies surveyed (8 in total) did not give any information regarding sequencing methods utilized, therefore it was difficult to ascertain the level of confidence in results communicated.

3.2.2. System Sizes

Figure 2 summarizes the size of systems surveyed in this meta-analysis over the period of 2013 to July 2016. It can be seen that a broad range of system sizes have been consistently studied each year. The most frequently investigated category is the meso-scale, with the pilot-scale being studied to the smallest degree. These observations likely indicate the difficulty and cost in maintaining pilot-scale systems (especially when maintained for research specific purposes, and not as a required water treatment service to a population). For research purposes, micro-scale systems (<10 L) can certainly serve an important purpose, however in general larger systems seem to be preferred for research, which is perhaps the reason meso-scale systems were the most common category. Large-scale systems are also being investigated to a reasonable degree, however it should be noted that the majority of these were SF, with only two VF and two HF.

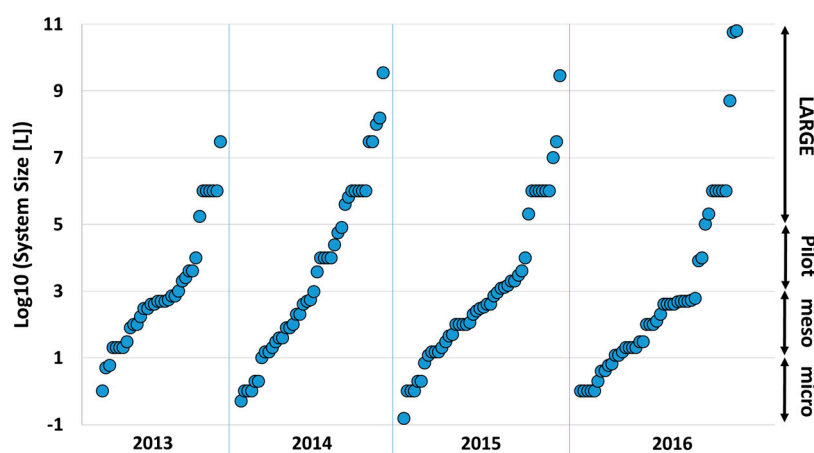


Figure 2. Size of TWs where a microbial community assessment method was applied for the period of 2013 to July 2016.

3.2.3. Internal Sampling Location

There were three categories surveyed for internal sampling location: water, media, and rhizospheric. For classification purposes water was either free water from a surface flow system, or interstitial water from a horizontal flow or vertical flow. Interstitial water was classified generally as water found within the pore space, and sampled via vertical wells or horizontal/slanted sampling ports in either HF or VF systems. The term interstitial water was deemed appropriate (rather than pore water) due to the basal definitions found in two different fields of research. Geologists refer to interstitial water as “water that occurs naturally within the pore space”, whereas in the medical sciences, it is generally used to describe “fluid that surrounds cells”. In the case of TWs, both descriptions are quite well suited (fluid can be said to be within pore spaces, but also surrounding biofilm found on the media), likely the reason the terminology has been kept and used by microbial community based TW researchers up until present day. Media was classified as sediment in a SF system, or as soil, gravel, sand or other material that was used to create the base porous media in HF or VF systems. The media itself is of course not what is actually being sampled in the case of microbial community assessment, rather it is the biofilm found on the surface (and perhaps within depending on the media used). Therefore, when researchers used wells (as more common for interstitial samples) but made a concerted effort to rigorously detach and sample biofilm, that sample was also classified as a media sample. More commonly, though, media samples were gathered through destructive system sampling. Rhizosphere samples constituted actual root samples where microbial communities were sampled/detached or directly detected/assessed, or in some cases if the researchers were taking mixed root/media samples from the rhizosphere and directly comparing them to lower non-rhizosphere media regions, the root/media sample would

have been classified as rhizospheric. The purpose here was not to split hairs regarding what a true rhizosphere sample is, but rather catalogue what areas the papers reviewed were investigating.

Figure 3 summarizes the sample types of largest focus through the period of 2013 to July 2016. The first observation is perhaps that the different system types are all well studied, and in an even fashion. The second observation is the heavier focus given to media samples. This is not surprising as the media generally covers the largest volume of subsurface flow systems, and is generally thought to be an active area in surface flow systems. In general, one can be safe in assuming that a media sample represents a microbial community responsible for some aspect of water treatment, however the rhizosphere, which was more sparsely studied, is also considered a region of high activity and has been proven so in direct comparisons. For example, Weber and Legge [47] found rhizospheric communities in VF systems to be at least ten times more active when degrading organics than corresponding microbial communities found in general media samples.

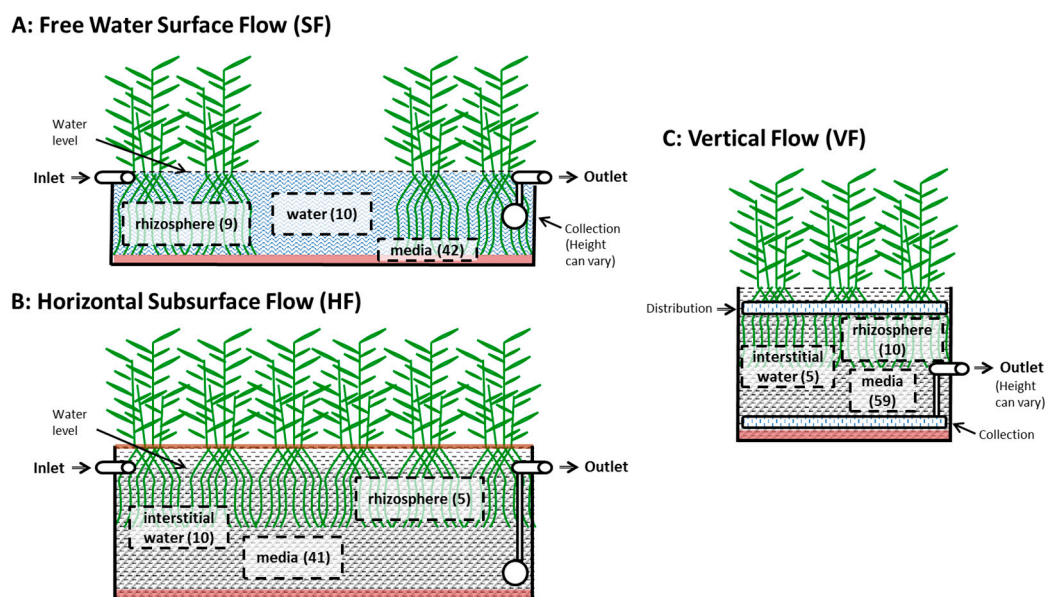


Figure 3. System sampling locations for microbial community assessment over the period of 2013 to July 2016 in free water surface flow (A); horizontal subsurface flow (B); and vertical flow (C) treatment wetlands.

Microbial communities can be described as in flux between the biofilm and interstitial water via attachment and detachment throughout time and in response to stressors and other influences [8,14]. The use of interstitial water samples can be convenient, however may also not fully represent the overall function of the microbial community responsible for water treatment in large-scale systems. Weber and Legge [47] showed some evidence that for meso-scale saturated VF systems run in recirculated batch mode interstitial water samples could in some ways represent the media-biofilm. However, it was also found that it was not a perfect representation. This could be a situation where recirculating batch mode keeps and houses detached biofilm over an extended period allowing for a somewhat reasonable representation of active biofilm populations, however from a theoretical stand-point flow-through systems would not benefit from this longer hold time and potentially equilibrated interstitial water/biofilm interaction seen in batch mode recirculated systems. It is suggested that this relationship between biofilm samples and interstitial water could be of interest in future studies.

Water samples were taken from SF systems on a number of occasions and interpreted to represent the inherent system microbial community. The author would suggest that free water samples may not represent the inherent microbial community responsible for the majority of water treatment in SF systems, and therefore in the future additional media and rhizospheric samples be taken for

comparison in the same study. It is not fully understood where the majority of water treatment occurs for the different waste constituents in SF systems (and to a smaller degree in HF and VF systems), therefore sample type comparison studies investigated in a spatial or temporal fashion could be quite informing.

3.2.4. Regional Analysis

A regional analysis was completed to better understand what types of systems, what types of microbial community analyses, and what focus areas are being studied throughout the world. Figure 4 summarizes the regional analyses and separately presents different continental/spatial regions for the period of 2013 to July 2016. Asia is most certainly publishing the majority of studies with respect to microbial community analysis, followed by Europe, and North America, respectively. The majority of Asian studies originated from China, where it can be seen that structural analyses are preferred (which accounted for almost half of all analyses), followed by activity, function, and enumeration respectively. Both Europe and North America had a more balanced approach (although less studies in total) with structure, function, and activity being the top three types of analyses and those being more even in their usage. The southern hemisphere was in general less active in microbial community analysis but, similar to Asia, preferred to assess for structure.

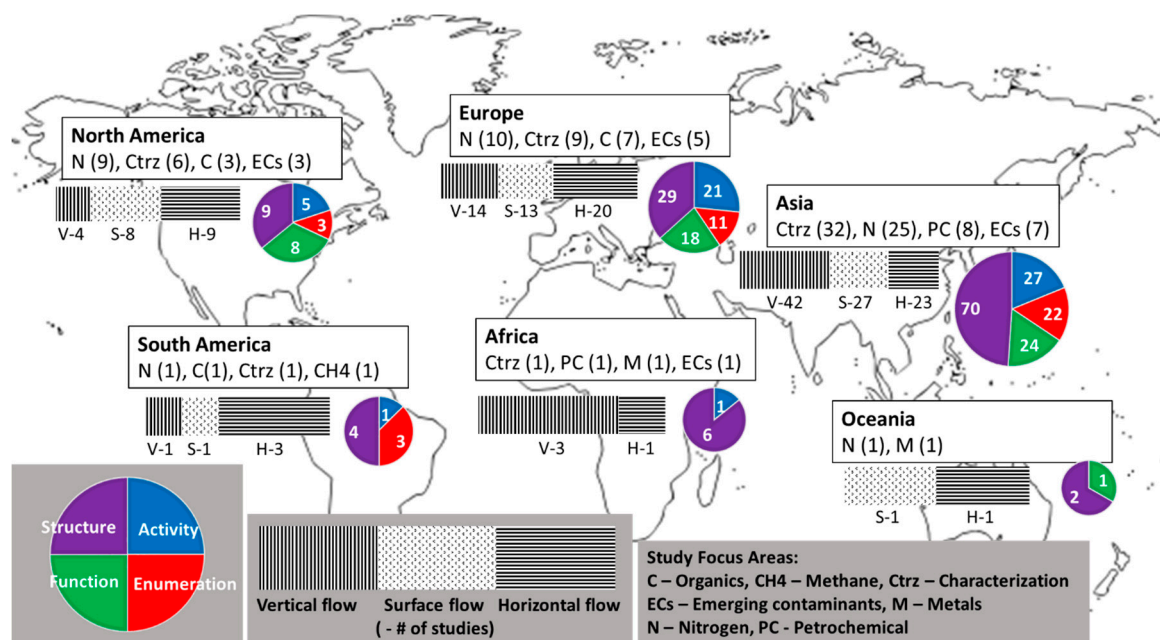


Figure 4. Summary of regional focus for microbial community assessment for the period of 2013 to July 2016.

In terms of types of systems studied there is a reasonable spread of system types with Asia focusing on VF followed by SF systems, Europe focusing on HF followed by VF systems, and North America focusing on HF followed by SF systems. Again in the Southern hemisphere there were less studies total but South America focused on HF systems, Africa on VF systems, and Oceania on a split between SF and HF system types. A number of the VF system studies also looked at aeration or some other type of intensification or flow regime (such as intermittent, or tidal). In general, there is significant effort being expended for the study of all three types of TW configuration.

With respect to focus area, there was a wide variety of study types, and it was not always easy to classify any single study. However, the author believes that Figure 4 gives a reasonable account for the areas of focus since 2013. The majority of studies consisted of a survey or general characterization of a TW system. On occasion these studies included a spatial or temporal aspect to

the characterization. There were a significant number of characterization studies looking at structure, activity and enumeration with far less utilizing a functional technique; Figure 4 covers the recent time period of 2013 to July 2016, however the same trend can be seen from Table 2, which covers the entire range of meta-analysis (1988 to July 2016). Nitrogen was a well-covered area of study. Here, authors looked to glean specific microbial community information and either correlated or directly compared/related results to nitrogen treatment. For nitrogen, in general, there were a large number of activity and structural assessments, with function closely following. As this meta-analysis included qPCR of functional nitrogen transformation genes as a form of functional assessment, it is not surprising that function was well covered in this area of focus. Many fields of research have contributed to pushing the boundaries of understanding and characterizing nitrogen cycling genes, and the field of TWs is keeping pace in borrowing and adapting methodologies. Worldwide the third largest focus area was ECs. These studies generally looked at removal and effects of ECs in TWs. Some examples of first known investigations are Helt et al. [48] who looked at general enumeration based antimicrobial resistance in response to an antimicrobial, Weber et al. [49] who looked at temporal population responses in response to an antimicrobial, Nolvak et al. [50] who investigated the presence of antimicrobial functional genes in response to an antimicrobial agent, and Button et al. [51] who investigated the fate and effects of silver nanoparticles in wetland microcosms. Other study areas included organics, petrochemicals, and metals. In general, there is a large number of studies covering a wide range of focus areas. The field of TWs seems to be keeping pace with the growing need for water treatment professionals to understand new and poorly understood wastewater constituents, generally considered here as ECs (pharmaceuticals, personal care products, antibiotics).

4. Conclusions and Future Horizons

TW research takes many forms. Where in past decades one could attend the WETPOL (Wetland Pollution Dynamics and Control) and the IWA: Wetlands for Water Pollution Control conferences and generally be up to date on all research progress occurring, the current amount of research activity in the field of TWs is beyond the point where this is possible. TWs are becoming truly internationalized, and this means the range of applications will most certainly increase with new situations and challenges yet to be discovered. This meta-analysis showed that TW research involving the assessment of microbial communities is on the rise, especially since 2013. SF, HF, and VF system types are all being investigated in approximately equal proportions worldwide.

High powered metagenomics sequencing methods are being applied, microarrays have been used to map functional pathways, and stable isotope analysis has been used to assist in functional analysis. The field of TWs truly is at the edge of microbial community analysis technological implementation. mRNA has also been extracted from lab-scale TW systems. This is quite a step. mRNA can be exceptionally difficult to extract from environmental samples [42]. If mRNA extraction for TWs is possible on a wider scale, micro-array and qPCR analysis could begin to return active function information. Although exciting, this type of extraction and methodology will require significant development and expertise to be applied on a wider scale.

Detachment of representative microbial community samples is a basic question that has not received sufficient attention in the field of TWs. When assessing biofilm originating from gravel, roots, sediment, or otherwise, one wants to gain a representative sample for analysis, yet not bias the community in the process. This can be challenging. Two example studies are Weber and Legge [52], which investigated the effects of different detachment methodologies on culturable bacteria from CW gravel, and Button et al. [53], which evaluated the effects of sample preparation on CLPP specifically. Neither Weber et al. [52] or Button et al. [53] were able to find absolutely perfect methodologies, but did provide an evaluation of the suitability of commonly utilized methods/techniques and were able to provide general recommendations. Similar evaluation studies have not been completed for DNA extraction in TWs specifically. Methodological advancements are expected and therefore studies similar to Weber et al. [52] and Button et al. [53] are anticipated and recommended for the future. Such

studies would help researchers standardized their sampling/extraction methodologies allowing for more appropriate and accurate comparisons between studies.

In relation to challenges surrounding the extraction/detachment of representative microbial community samples (especially from biofilm matrices), a similar challenge exists when deciding when and where to take a microbial community sample from a system. In the majority of investigations, the researcher is interested in gaining samples which are representative of the microbial communities contributing to the water treatment capabilities of a TW, however communities from different regions in a TW could contribute in different ways at different times. Since 2013, 33 spatial studies and 20 temporal studies have been published, marking good progress. However, with the vast array of system modifications, intensifications, and hydrological regimes being now employed, in order to gain a solid understanding of microbial community dynamics in all situations, additional effort is required. Understanding both spatial and temporal microbial community dynamics is central to comparing different studies to each other, and in being confident in the results of any single study.

Studies are increasingly becoming multiphasic, meaning more than one class of microbial community assessment is implemented in the same study (63 total from 2013 to July 2016). Multiphasic analysis is enhancing the impact of research by offering multiple lines of evidence. Structural and metagenomics analysis has developed rapidly and is currently the most popular microbial assessment tool in TWs. It is suggested that structural analysis be a part of multiphasic studies or extended to include interpretation of the functional potential of species identified to better assist in connecting the microbial community observations to TW water treatment.

Rather than recommend any specific microbial community assessment methods the author would like to emphasize the concept of “fit for purpose”. The most important question to ask before choosing a method is, “What information do I require, and how will that information help in meeting the overall objectives of the study”. With the understanding that resources are often limited for any single research endeavour, method selection can become challenging. TWs are one of the most complex water treatment systems in existence, and it is for this reason that truly rigorous studies are so difficult to complete because there are so many interdependent aspects at play. In the context of gaining an understanding of the microbial community, using an exceptionally new and expensive method by default can sometimes be a detriment and limit researchers to investigating only one aspect of the microbial community. Those same resources could often be better spent in performing a multiphasic study looking at perhaps all four aspects in some way (enumeration, activity, function, and structure), rather than a single aspect alone. Converging lines of evidence is something all researchers strive to achieve when publishing findings. Striving for multiphasic experimental designs is perhaps the single most important recommendation to be made here.

The field of TWs is truly multidisciplinary. For example, the study of Lünsmann et al. [41] required collaboration among departments of Biotechnology, Proteomics, Environmental Biotechnology, Isotope Biogeochemistry, Metabolomics, and a Microbial Interactions and Processes Research Group. It is expected that additional collaborations will be developed in the coming years and that TW microbial community research will be multiphasic reaching beyond the current horizons and starting new paradigms.

Acknowledgments: The author would like to thank Sonja Bissegger for abundant and fruitful discussion, which allowed for the synthesis of concepts, and also for her exceptional editing efforts. Thank you to Maëlle Luana Weber for the motivation to complete the meta-analysis in a compressed time frame. The author would also like to thank the reviewers (identities unknown) for their exceptional feedback and ideas, which helped improve the clarity and utility of this review.

Conflicts of Interest: The author declares no conflict of interest.

References

1. Kadlec, R.; Wallace, S. *Treatment Wetlands*, 2nd ed.; CRC Press: Boca Raton, FL, USA, 2009.
2. Vymazal, J. Constructed Wetlands for Wastewater Treatment. *Water* **2010**, *2*, 530–549. [[CrossRef](#)]

3. Vymazal, J. Constructed Wetlands for Wastewater Treatment: Five Decades of Experience. *Environ. Sci. Technol.* **2011**, *45*, 61–69. [[CrossRef](#)] [[PubMed](#)]
4. Garcia, J.; Rousseau, D.P.L.; Morato, J.; Lesage, E.; Matamoros, V.; Bayona, J.M. Contaminant Removal Processes in Subsurface-Flow Constructed Wetlands: A Review. *Crit. Rev. Environ. Sci. Technol.* **2010**, *40*, 561–661. [[CrossRef](#)]
5. Nivala, J.; Murphy, C.; Troesch, S.; Wallace, S.; Esser, D. Intensified and Modified Wetland Designs. *Sustain. Sanit. Pract.* **2014**, *18*, 15–20.
6. Seidel, K. Pflanzungen zwischen Gewässern und Land. *Mitteilungen Max-Planck Gesellschaft* **1953**, *8*, 17–20. (In German)
7. Fester, T.; Giebler, J.; Wick, L.Y.; Schlosser, D.; Kastner, M. Plant–microbe interactions as drivers of ecosystem functions relevant for the biodegradation of organic contaminants. *Curr. Opin. Biotechnol.* **2014**, *27*, 168–175. [[CrossRef](#)] [[PubMed](#)]
8. Weber, K.P.; Gagnon, V. Microbiology in Treatment Wetlands. *Sustain. Sanit. Pract.* **2014**, *18*, 25–30.
9. Truu, J.; Truu, M.; Espenberg, M.; Nolvak, H.; Juhanson, J. Phytoremediation and Plant-Assisted Bioremediation in Soil and Treatment Wetlands: A Review. *Open Biotechnol. J.* **2015**, *9*, 85–92. [[CrossRef](#)]
10. Truu, M.; Juhanson, J.; Truu, J. Microbial biomass, activity and community composition in constructed wetlands. *Sci. Total Environ.* **2009**, *407*, 3958–3971. [[CrossRef](#)] [[PubMed](#)]
11. Samsó, R.; Garcia, J. BIO_PORE, a mathematical model to simulate biofilm growth and water quality improvement in porous media: Application and calibration for constructed wetlands. *Ecol. Eng.* **2013**, *54*, 116–127. [[CrossRef](#)]
12. Nivala, J.; Knowles, P.; Dotro, G.; Garcia, J.; Wallace, S. Clogging in subsurface flow treatment wetlands: Measurement, modeling and management. *Water Res.* **2012**, *46*, 1625–1640. [[CrossRef](#)] [[PubMed](#)]
13. Hua, G.F.; Li, L.; Zhao, Y.Q.; Zhu, W.; Shen, J.Q. An integrated model of substrate clogging in vertical flow constructed wetlands. *J. Environ. Manag.* **2013**, *119*, 67–75. [[CrossRef](#)] [[PubMed](#)]
14. Rajabzadeh, A.R.; Legge, R.; Weber, K.P. Multiphysics modelling of flow dynamics, biofilm development and wastewater treatment in a subsurface vertical flow constructed wetland mesocosm. *Ecol. Eng.* **2015**, *74*, 107–116. [[CrossRef](#)]
15. Meyer, D.; Chazarenc, F.; Claveau-Mallet, D.; Dittmer, U.; Forquet, N.; Molle, P.; Morvannou, A.; Pálffy, T.; Petitjean, A.; Anacleto, R.; et al. Modelling constructed wetlands: Scopes and aims—A comparative review. *Ecol. Eng.* **2015**, *80*, 205–213. [[CrossRef](#)]
16. Faulwetter, J.L.; Gagnon, V.; Sundberg, C.; Chazarenc, F.; Burr, M.D.; Brisson, J.; Campera, A.K.; Stein, O. Microbial processes influencing performance of treatment wetlands: A review. *Ecol. Eng.* **2009**, *35*, 987–1004. [[CrossRef](#)]
17. Stehle, S.; Elsaesser, D.; Gregoire, C.; Imfeld, G.; Niehaus, E.; Passeport, E.; Payraudeau, S.; Schäfer, R.B.; Tournebize, J.; Schulz, R. Pesticide risk mitigation by vegetated treatment systems: A meta-analysis. *J. Environ. Qual.* **2011**, *40*, 1068–1080. [[CrossRef](#)] [[PubMed](#)]
18. Garcia-Rodriguez, A.; Matamoros, V.; Fontas, C.; Salvado, V. The ability of biologically based wastewater treatment systems to remove emerging organic contaminants—A review. *Environ. Sci. Pollut. Res.* **2014**, *21*, 11708–11728. [[CrossRef](#)] [[PubMed](#)]
19. Button, M.; Nivala, J.; Weber, K.P.; Aubron, T.; Mueller, R.A. Microbial community metabolic function in subsurface flow constructed wetlands of different designs. *Ecol. Eng.* **2015**, *80*, 162–171. [[CrossRef](#)]
20. Gest, H. The discovery of microorganisms by Robert Hooke and Antoni Van Leeuwenhoek, fellows of the Royal Society. *Notes Rec. R. Soc. Lond.* **2004**, *58*, 187–201. [[CrossRef](#)] [[PubMed](#)]
21. Vymazal, J.; Sladeczek, V.; Stach, J. Biota participating in wastewater treatment in a horizontal flow constructed wetland. *Water Sci. Technol.* **2001**, *44*, 211–214. [[PubMed](#)]
22. White, D.C.; Davis, W.M.; Nickels, J.S.; King, J.D.; Bobbie, R.J. Determination of the sedimentary microbial biomass by extractable lipid phosphate. *Oecologia* **1979**, *40*, 51–62. [[CrossRef](#)]
23. Morris, B.E.L.; Crable, B.R.; Suflita, J.M. On the contributions of David Cleaveland White, MD, PhD to microbial ecology: Celebrating the life of a pioneer. *ISME J.* **2008**, *2*, 797–804. [[CrossRef](#)] [[PubMed](#)]
24. Merlino, C.P. Bartolomeo Bizio's Letter to the most Eminent Priest, Angelo Bellani, Concerning the Phenomenon of the Red Colored Polenta. *J. Bacteriol.* **1924**, *9*, 527–543. [[PubMed](#)]
25. Hatano, K.; Frederick, D.J.; Moore, J.A. Microbial Ecology of Constructed Wetlands Used for Treating Pulp Mill Wastewater. *Water Sci. Technol.* **1994**, *29*, 233–239.

26. Great Britain Royal Commission on Sewage Disposal. *Final Report of the Commissioners Appointed to Inquire and Report What Methods of Treating and Disposing of Sewage (Including Any Liquid from Any Factory or Manufacturing Process) May Properly Be Adopted. General Summary of Conclusions and Recommendations*; P.S. King and Son Ltd.: Orchard House, Westminster, London, UK, 1915.
27. MacDougall, R.S. The Bacteria of the Soil, with Special Reference to Soil Inoculation. *Trans. Bot. Soc. Edinb.* **1897**, *21*, 25–40. [[CrossRef](#)]
28. Hench, K.R.; Sexstone, A.J.; Bissonnette, G.K. Heterotrophic community-level physiological profiles of domestic wastewater following treatment by small constructed subsurface flow wetlands. *Water Environ. Res.* **2004**, *76*, 468–473. [[CrossRef](#)] [[PubMed](#)]
29. Garland, J.L.; Mills, A.L. Classification and characterization of heterotrophic microbial communities on the basis of patterns of community-level sole-carbon-source utilization. *Appl. Environ. Microbiol.* **1991**, *57*, 2351–2359. [[PubMed](#)]
30. Insam, H. A new set of substrates proposed for community characterization in environmental samples. In *Microbial Communities: Functional Versus Structural Approaches*; Insam, H., Rangger, A., Eds.; Springer: Berlin, Germany, 1997.
31. Brodrick, S.J.; Cullen, P.; Maher, W. Denitrification in a natural wetland receiving secondary treated effluent. *Water Res.* **1988**, *22*, 431–439. [[CrossRef](#)]
32. Lünsmann, V.; Kappelmeyer, U.; Taubert, A.; Nijenhuis, I.; von Bergen, M.; Heipieper, H.J.; Müller, J.A.; Jehmlich, N. Aerobic toluene degraders in the rhizosphere of a constructed wetland model show diurnal polyhydroxyalkanoate metabolism. *Appl. Environ. Microbiol.* **2016**, *82*, 4126–4132. [[CrossRef](#)] [[PubMed](#)]
33. Paredes, D.; Kuschik, P.; Mbawette, T.S.A.; Strange, F.; Muller, R.A.; Koser, H. New Aspects of Microbial Nitrogen Transformations in the Context of Wastewater Treatment—A Review. *Eng. Life Sci.* **2007**, *7*, 13–25. [[CrossRef](#)]
34. Baptista, J.D.C.; Donnelly, T.; Rayne, D.; Davenport, R.J. Microbial mechanisms of carbon removal in subsurface flow wetlands. *Water Sci. Technol.* **2003**, *48*, 127–134. [[PubMed](#)]
35. Corbella, C.; Guivernau, M.; Vinas, M.; Puigagut, J. Operational, design and microbial aspects related to power production with microbial fuel cells implemented in constructed wetlands. *Water Res.* **2015**, *84*, 232–242. [[CrossRef](#)] [[PubMed](#)]
36. Friedrich, M.W. Methyl-coenzyme M reductase genes: Unique functional markers for methanogenic and anaerobic methane-oxidizing Archaea. *Methods Enzymol.* **2005**, *397*, 428–442. [[PubMed](#)]
37. Zhi, W.; Yuan, L.; Ji, G.; He, C. Enhanced Long-Term Nitrogen Removal and Its Quantitative Molecular Mechanism in Tidal Flow Constructed Wetlands. *Environ. Sci. Technol.* **2015**, *49*, 4575–4583. [[CrossRef](#)] [[PubMed](#)]
38. Chen, Y.; Vymazal, J. Comment on “Enhanced Long-Term Nitrogen Removal and Its Quantitative Molecular Mechanism in Tidal Flow Constructed Wetlands”. *Environ. Sci. Technol.* **2015**, *49*, 11241–11242. [[CrossRef](#)] [[PubMed](#)]
39. Zhi, W.; Yuan, L.; Ji, G.; He, C. Response to Comment on “Enhanced Long-Term Nitrogen Removal and Its Quantitative Molecular Mechanism in Tidal Flow Constructed Wetlands”. *Environ. Sci. Technol.* **2015**, *49*, 11243–11244. [[CrossRef](#)] [[PubMed](#)]
40. Martínez-Lavanchy, P.M.; Chen, Z.; Lünsmann, V.; Marin-Cevada, V.; Vilchez-Vargas, R.; Pieper, D.H.; Reiche, N.; Kappelmeyer, U.; Imparato, V.; Junca, H.; et al. Microbial toluene removal in hypoxic model constructed wetlands occurs predominantly via the ring monooxygenation pathway. *Appl. Environ. Microbiol.* **2015**, *81*, 6241–6252. [[CrossRef](#)] [[PubMed](#)]
41. Lünsmann, V.; Kappelmeyer, U.; Benndorf, R.; Martinez-Lavanchy, P.M.; Taubert, A.; Adrian, L.; Duarte, M.; Pieper, D.H.; von Bergen, M.; Müller, J.A.; et al. In situ protein-SIP highlights Burkholderiaceae as key players degrading toluene by para ring hydroxylation in a constructed wetland model. *Environ. Microbiol.* **2016**, *18*, 1176–1186. [[CrossRef](#)] [[PubMed](#)]
42. Wang, Y.; Hayatsu, M.; Fujii, T. Extraction of Bacterial RNA from Soil: Challenges and Solutions. *Microbes Environ.* **2012**, *27*, 111–121. [[CrossRef](#)] [[PubMed](#)]
43. Herbst, F.-A.; Lünsmann, V.; Kjeldal, H.; Jehmlich, N.; Tholey, A.; von Bergen, M.; Nielsen, J.L.; Hettich, R.L.; Seifert, J.; Nielsen, P.H. Enhancing metaproteomics—The value of models and defined environmental microbial systems. *Proteomics* **2016**, *16*, 783–798. [[CrossRef](#)] [[PubMed](#)]

44. Pollard, P.; Flood, J.A.; Ashbolt, N.J. The direct measurement of bacterial growth in biofilms of emergent plants (*Schoenoplectus*) of an artificial wetland. *Water Sci. Technol.* **1995**, *32*, 251–256. [[CrossRef](#)]
45. Braeckevelt, M.; Rokadia, H.; Imfeld, G.; Stelzer, N.; Paschke, H.; Kusch, P.; Kastner, M.; Richnow, H.-H.; Weber, S. Assessment of in situ biodegradation of monochlorobenzene in contaminated groundwater treated in a constructed wetland. *Environ. Pollut.* **2007**, *148*, 428–437. [[CrossRef](#)] [[PubMed](#)]
46. Ligi, T.; Oopkaupa, K.; Truu, M.; Preema, J.-K.; Nolvaka, H.; Mitsch, W.J.; Mander, U.; Truu, J. Characterization of bacterial communities in soil and sediment of a created riverine wetland complex using high-throughput 16S rRNA amplicon sequencing. *Ecol. Eng.* **2014**, *72*, 56–66. [[CrossRef](#)]
47. Weber, K.P.; Legge, R.L. Comparison of the catabolic activity and catabolic profiles of rhizospheric, gravel-associated and interstitial microbial communities in treatment wetlands. *Water Sci. Technol.* **2013**, *67*, 886–893. [[CrossRef](#)] [[PubMed](#)]
48. Helt, C.D.; Weber, K.P.; Legge, R.L.; Slawson, R.M. Antibiotic resistance profiles of wetland bacteria and fecal indicators following ciprofloxacin exposure in lab-scale constructed mesocosms. *Ecol. Eng.* **2012**, *39*, 113–122. [[CrossRef](#)]
49. Weber, K.P.; Mitzel, M.R.; Slawson, R.M.; Legge, R.L. Effect of ciprofloxacin on microbiological development in wetland mesocosms. *Water Res.* **2011**, *45*, 3185–3196. [[CrossRef](#)] [[PubMed](#)]
50. Nolvak, H.; Truu, M.; Tiirik, K.; Oopkaup, K.; Sildvee, T.; Kaasik, A.; Mander, U.; Truu, J. Dynamics of antibiotic resistance genes and their relationships with system treatment efficiency in a horizontal subsurface flow constructed wetland. *Sci. Total Environ.* **2013**, *461–462*, 636–644. [[CrossRef](#)] [[PubMed](#)]
51. Button, M.; Auvinen, H.; Van Koetsem, F.; Hosseinkhani, B.; Rousseau, D.; Weber, K.P.; Du Laing, G. Susceptibility of constructed wetland microbial communities to silver nanoparticles: A microcosm study. *Ecol. Eng.* **2016**, *97*, 476–485. [[CrossRef](#)]
52. Weber, K.P.; Legge, R.L. Method for the detachment of culturable bacteria from wetland gravel. *J. Microbiol. Methods* **2010**, *80*, 242–250. [[CrossRef](#)] [[PubMed](#)]
53. Button, M.; Weber, K.P.; Nivala, J.; Aubron, T.; Muller, R.A. Community-level physiological profiling of constructed wetland microbial communities: Effects of sample preparation. *Appl. Biochem. Biotechnol.* **2015**, *178*, 960–973. [[CrossRef](#)] [[PubMed](#)]



© 2016 by the author; licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC-BY) license (<http://creativecommons.org/licenses/by/4.0/>).