Microbiology in Treatment Wetlands

Methodological advances have allowed for the direct measurement, comparison, and characterization of microbial community structure and function in wetlands, which will now help researchers perform more microbiologically comprehensive studies ultimately moving the field closer to a complete understanding of the quantitative and functional role of microbial communities in treatment wetlands.

Authors: Kela P. Weber and Vincent Gagnon

Abstract

Microbial communities play a central role in treatment wetland systems, contributing to both contaminant removal and hydrological development. Given that both of these parameters are key to the proper operation of a treatment wetland system, consultants and researchers alike have identified microbiological research in the field treatment wetlands as a priority area. Through many studies, ranging from 1988 onward, researchers and design consultants have gained a general understanding of the importance of microbial communities in treatment wetland systems. Earlier studies focused on microbial enumeration or activity quantifications. Recent advances in both functional and structural characterization methods and equipment have provided researchers the opportunity to adapt and develop these methods for treatment wetland systems. The field of treatment wetlands is now at a point where studies can assess both microbial communities and water treatment simultaneously. Future work investigating both spatial and temporal microbial community dynamics in treatment wetland systems is expected to uncover the quantitative role of microbial communities and create connectivity with water treatment performance. It is expected that microbiological research will soon assist in optimizing overall performance and treatment wetland design. Several recent studies have moved the field forward in this fashion; however because of the large number of unique treatment wetland designs operating under a large variety of conditions throughout the world, significant effort uncovering the role and contribution of microbial communities in treatment wetland systems is still required.

Introduction

Microbial communities play an important role in wetlands designed for water pollution control (Kadlec and Wallace, 2009; Truu et al., 2009; Faulwetter et al., 2009; Garcia et al., 2010). Microbial communities 1) directly influence and contribute to contaminant removal, 2) develop biofilms which can affect hydrological development, 3) have a close interaction with plant roots within the rhizospheric region, and 4) can contribute to other beneficial or negative ancillary effects related to treatment wetland operations. Treatment wetlands (TWs) house many different microenvironments within a single system. Each microenvironment can have varying conditions, such as oxygen concentration, redox potential, ionic strength, pH, nutrient availability, or pollutant concentration, to name a

Main outcome of the session:

- Direct evaluations and linkages between the roles of microbial communities and water treatment would assist in our understanding and eventual design improvements for treatment wetlands.
    - Direct measurements evaluating both spatial and temporal variations of microbial community structure, function and activity within systems over several seasons are needed to advance our understanding of the roles of microbial communities in treatment wetland operations.
- Recent methodological developments have given researchers the tools to better characterize and understand both the structure and function of microbial communities in treatment wetland systems.
    - Not all research groups have the equipment or expertise on staff for microbial community investigations but several groups have developed the expertise and attained the instrumentation required. It is expected that collaborative studies between groups will increase over time allowing for more comprehensive studies looking at both water treatment and microbial communities in conjunction.

few. These variations allow for the development of diverse microbial communities within different microenvironments of a treatment wetland. Figure 1 presents a simplified depiction of microbial community interactions with plant roots, and the bed media.

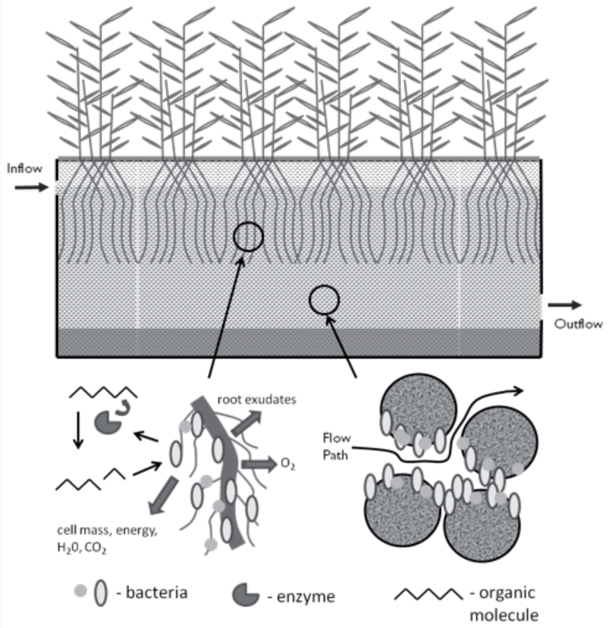

Figure 1: Simplified depiction of microbial community interactions with bed media, plant roots, and organic wastewater components in a horizontal subsurface flow treatment wetland system. (Diagram not to scale)

Microbial communities can exist as free-floating microorganisms within the interstitial spaces of the bed media or as anchored/attached colonies surrounding either the bed media or integrated within the rhizosphere or roots of the plants. It is generally accepted that interstitial microbial communities, although present, play a relatively small role in contaminant removal when compared to rhizospheric or other biofilm-bound microbial communities. Weber and Legge (2013) reported quantitative microbial activity observations supporting this view. Nonetheless, the interstitial water contains free enzymes excreted by fixed bacteria which will assist in the degradation of organics and other contaminants. Depending on the oxygen concentrations and redox potential in a specific region within a TW, different microbial communities will develop and therefore different metabolic pathways will be responsible for the removal of pollutants from the water (Figure 2). For example, microenvironments within the near-root zone (within 1 mm of a root) of horizontal subsurface flow wetlands can be largely aerobic (redox potential +250 to +700 mV), even though the rest of the bed is dominated by anaerobic processes (redox potential +250 to -400 mV, Truu et al., 2009). The potential for localized conditions is one feature of TWs that has allowed for unique and sometimes improved contaminant removal capabilities over more conventional, high-energy input, water treatment technologies.

Microbial communities play a role in organic matter degradation, nitrogen transformations, phosphorus cycling, and other more specific processes such as methanogenisis, sulphate reduction, dehalogenation, iron oxidation/reduction, or the degradation of specific contaminants such as MTBE and BTEX, among others. Significant interest has also been given to the role of microbial communities in the treatment of pathogens and emerging contaminants (pharmaceuticals, personal care products, antibiotics, nanomaterials, synthetic hormones, etc.), in addition to the possible generation of antibiotic resistant microorganisms within wetland systems receiving antibiotics at low concentrations (ng/L).

Besides directly treating, utilizing, mineralizing or transforming pollutants in TWs, microbial communities also play a role in terms of contaminant retention through the creation of biofilms. The attachment or anchorage of microorganisms in TWs depends on the capsule or slime layer surrounding the specific microbial communities developing in the TW, the grain size of the bed media, the availability of roots or root hairs, and the local water velocity in the immediate region. Microbial attachment/detachment occurs readily, with extracellular polymeric substances (EPS) excreted into the slime layer or capsule region assisting attachment, and shear stress working to detach the same microorganisms. These EPS's are made up largely of polysaccharides, as well as proteins that give a sticky exterior. This sticky exterior also allows for the adsorption of contaminants from the interstitial waters. This biofilm adsorption aids the physicochemical removal processes and also provides non-motile microorganisms entrapped within biofilms access to a carbon and energy source. Water

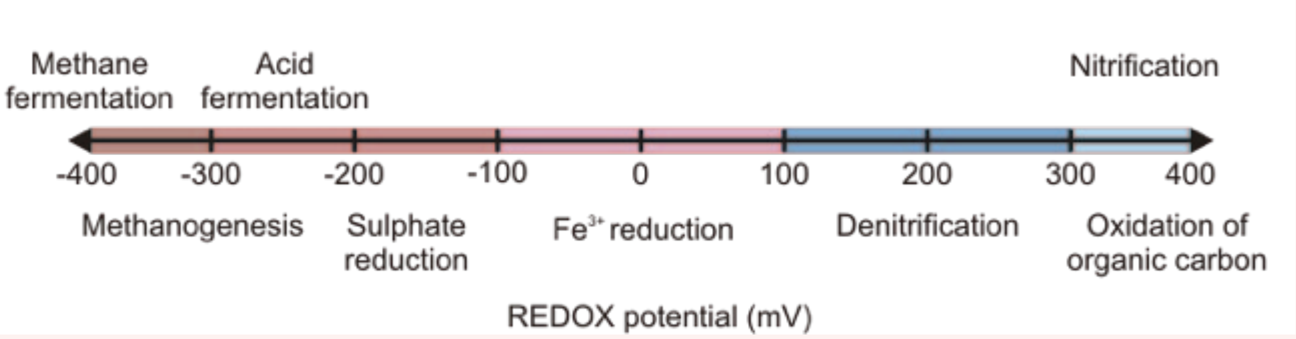

Figure 2: Relationship between microbial process and REDOX potential. Adapted from (Gagnon et al., 2007).

velocity and the associated shear stress will have an effect on microbiological development, which may lead to the selection of specific groups or even microbiological species developing within a system. Biologically based biofilm development has been documented in the literature and can have a significant effect on overall system porosity (Weber and Legge, 2011). Porosity reduction based on microbiological development also affects dispersivity (mixing) characteristics, and can lead to preferential flow paths (short-circuiting), and even eventual clogging given specific conditions (unpublished personal observations discussed during the workshop).

Current background / status

The field of microbial community characterization has been through an immense growth period within the last 30 years. Figure 3 summarizes the main categories of microbial community characterization in a simplified fashion.

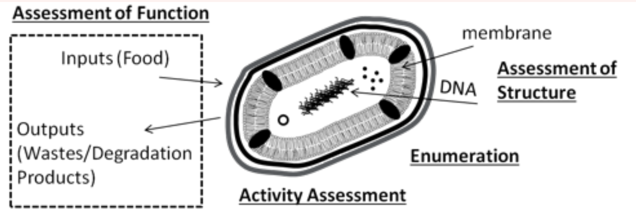

Figure 3: Microbial community characterization techniques. (Cellular components not to scale)

Enumeration Techniques

Enumeration was one of the first characterization techniques utilized in TWs. Originally this involved plate cultures and the subsequent counting of colony forming units, filtering and dry weight measurements of total organic matter, and direct counting and/or identification under a microscope (e.g. Petroff-Hauser counting). Later developments included microbial staining techniques, flow cytometry, and eventually real-time polymerase chain reaction (RT-PCR) (also known as quantitative PCR – qPCR).

Quantification of Microbial Activity

Microbial activity methods were also developed and utilized very early in the field of TWs (although not always expressly described as microbial activity). For example measurements of soil respiration have been used and described as far back as the 1980s. Respiration rates have generally been measured in aerobic systems or using samples from aerobic regimes and have most often tracked either O2 utilization rates, or CO2 production rates. Other activity measurements include the direct or indirect quantification of adenosine triphosphate (ATP - the main coenzyme used in cellular metabolism) or nicotinamide adenine dinucleotide (NADH - coenzyme involved in cellular metabolism), and the quantification of extracellular enzyme activities (eg. fluoresceine diacetate method).

Structural Characterization of Microbial Communities

Some of the first methods available for microbial community structure comparisons were fatty acid methyl ester (FAME), and phospholipid-derived fatty acid (PLFA) analysis. Although not used for direct identification of microorganisms they give the ability to compare or differentiate complex microbial communities based on the specific make-up of the plasma membrane of prokaryote cells.

A number of methods have been developed based on the characterization of PCR amplified DNA segments from a mixed microbial community. Most methods utilize primers that amplify a highly conserved region of DNA encoding for the 16s ribosomal unit to gain an understanding of all prokaryotes in a sample; however other regions or specific genes can be targeted to gain more specific information. Some of these methods include denaturing gradient gel electrophoresis (DGGE), temperature gradient gel electrophoresis (TGGE), and single-strand conformation polymorphism (SSCP), each of which yield patterns of bands embedded within a gel which can then be excised and sequenced. To gain a full understanding of microbial community structure, sequencing is required; however useful information regarding structural diversity can also be gained without sequencing.

Other methods that allow for community comparisons include terminal restriction fragment length polymorphism (TRFLP), amplified rDNA (Ribosomal DNA) restriction analysis, ribosomal intergenic spacer analysis (RISA), length heterogeneity PCR (LH-PCR), and random amplification of polymorphic DNA (RAPD). Although all methods mentioned can give useful information, perhaps the most powerful methods to be developed are the high-throughput sequencing methods. Several different methods/platforms have been developed by various companies and research groups throughout the world including but not limited to, pyrosequencing, ion torrent semiconductor sequencing, sequencing by ligation, and reversible dye-terminator sequencing. These high throughput sequencing methods allow for the simultaneous relative quantification and sequencing of all targeted genes within a sample. These methods hold great potential as they give a complete snapshot of a sample's microbial community structure in one simple method, but they are currently the most costly microbial community characterization methods available, which can be prohibitive.

Functional Characterization of Microbial Communities

Microbial community function looks to gain an understanding of exactly what types and in what quantities the microbial community is utilizing and excreting different compounds. It is through these basic functions that microbial communities interact with different trophic levels, participate in different nutrient cycles in the environment, and offer pollutant removal capabilities in TWs. Rather than quantifying and identifying DNA fragments within a sample, primers and probes can be developed for mRNA segments. Although mRNA is more difficult to work with, it gives an actual indication of gene expression and therefore an indication of a specific active function, rather than the potential for a specific function when assessing DNA. qPCR and fluorescence in-situ hybridization have been used to this end.

Community level physiological profiling (CLPP) is another functional characterization method where the metabolic activity of a community sample is measured with relation to 31 to 95 different carbon sources on a microtitre plate. With this method, both a relative activity and total metabolic potential for degrading a range of carbon sources is obtained.

The last functional approach is the use of microarrays, such as the Geochip 3.0, to assess the presence of anywhere from 20,000 to 60,000 genes via RNA (or DNA) segments using specified probes on a small microscope slide. Although in its infancy this methodology also holds great potential. With the expression of so many genes being assessed in a single sample, full enzymatic pathways can begin to be assembled and assessed, giving a more thorough (although not directly measured) indication of overall function. Microarrays can also be costly, which can be a prohibitive factor in its common usage.

Historical View of Microbial Community Characterization in Treatment Wetlands

We completed a literature review to offer an understanding of the efforts invested into microbiological studies for wetland systems to date (Figure 4). Studies included here were for the most part not solely focused on microbiological processes or characterizing the communities, but rather had a characterization or microbiological component to complement other concurrent investigations (see Figure 4 for keywords). The search was also not restricted to treatment wetlands alone, but included natural wetland systems as well. Of the 564 papers identified, 235 were removed as they were not related to wetlands, with another 97 removed as there were no microbiological methods used, leaving a total of 232 papers. Through a review of the remaining papers, a comprehensive understanding of the history of microbiological characterization in wetland systems was gained.

The first studies in the late 1980s used general enumeration or activity techniques. Use of both enumeration and activity measurements continue to date and each year accounts for the majority of publications. In the early 2000's TRFLP, DGGE and more directed genetic sequencing was introduced into several studies along with functional characterization including CLPP. From 2006 onwards structural studies have become more frequent, most likely because of the greater accessibility of the instrumentation and lower cost of materials. In 2010 through 2012 studies have begun to be multiphasic in nature, with combinations of activity, enumeration, structure and function methodologies applied to the same systems. Of the 232 papers reviewed, 49 investigated nitrogen transformations, 40 focused on pathogen removal and/or characterization, and the remaining papers investigated other microbiological processes or focussed on specific microbial community characterization techniques.

In 2013 (up to June when this review was completed) 11 studies that included a microbiological assessment component were published. Three studies used qPCR, four studies used some type of enzymatic activity measurement, two studies were completed using pyrosequencing, one study utilized CLPP, and one study (conference proceeding) used the GeoChip 3.0 microarray.

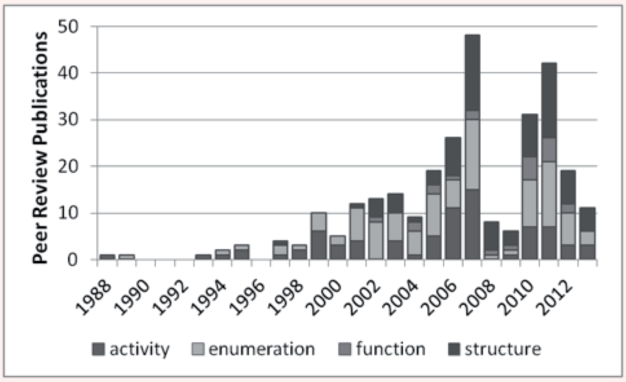

Figure 4: Summary of microbial community characterization publications in the field of treatment wetlands. Keywords: wetland, constructed wetland, treatment wetland, microbiology, microbial, microbiological (with all combinations). Databases: Compendex, Referex, Inspec, GEOBASE, GeoRef, Scifinder, Web of Science.

Challenges / opportunities

Spatial Microbial Community Dynamics

Spatial variances in microbiological density, activity, and structural or function diversity are not completely understood. There have been a limited number of studies evaluating spatial dynamics. In the majority of cases significant variations have been observed. Understanding these spatial dynamics and why they occur is key to further understanding how design and environmental factors can affect the role of microbial communities in TWs.

Most comprehensive studies have been done at the meso or micro scale, but in moving forward an understanding of spatial dynamics at the pilot or full scale is needed. In doing this, microenvironments within close proximity (for example rhizosphere-region samples taken directly from the root, within 1 mm from the root, and then progressively outwards) would be of use in truly understanding the spatial variances found within what is assumed to be a highly diverse microbiological region. To this end, it is also important that a greater understanding of anaerobic microbial community functions are evaluated as these processes are of importance in TW systems.

Also noted at the UFZ workshop is the need to accurately evaluate the microbial communities contributing to water treatment during microbiological characterization studies. Many mesoscale studies have been able to compare and in some cases make use of interstitial water for characterization due to the specific operational design and configuration. However in larger scale operations characterization of the microbial communities in outlet water samples may not be (and most likely, in the opinion of the authors, is not) a microbial community sample representative of the communities contributing to water treatment in TW systems. Further study comparing biofilm, interstitial, and outlet water samples for microbial community characterization for several TW system scales should be completed to better understand this aspect.

Temporal Microbial Community Dynamics

Much like the spatial studies previously mentioned, temporal variations in microbial community density, activity, structure and function have been recorded. Even fewer temporal studies (compared to spatial) have been completed to date, likely because of project/funding timelines and costs associated with such studies. Temporal variations have been noted with either season or differing input water parameters affecting the microbial community in some way. Again, understanding temporal variations and possibly how external factors can be modified to enhance the microbial community function with TWs could lead to improved water treatment performance.

Additional fundamental research is also required investigating temporal variations in microbial activity and how this can affect results and interpretation. In treatment wetland operations it is suggested that the temporal dynamics of microbial activity in a set spatial area could change based on differing non-continuous wastewater inlet parameters such as organic content, nutrient ratios, and loading. Past research has been completed through the use of a consistent time point for activity measurements throughout studies (eg. a specified time following a bulk wastewater inlet loading), however it is rarely understood if a chosen reference time point is the most appropriate for the study being conducted.

Connectivity between Microbial Community Characterization and Treatment Wetland Operations

Creating connectivity between microbial characterization studies and treatment wetland operations is the end goal for all researchers, but this connectivity was identified as a weakness at the UFZ workshop. This is most likely due to the fact that many recent studies focused on method verification or first time trials using specific microbiological methods in TW systems. Microbiological methodologies are not easily transferred between system types (soil to sediment for example) and require significant effort to adapt and optimize for any one system type. The field of TWs will always be working to adapt new methodologies to TW systems, although the field as a whole is at a point where a large breadth of methods have been adapted and are ready to be applied in new research studies.

As noted previously, both temporal and spatial studies are important stepping stones in moving forward. Nevertheless, there are many other basic questions still requiring further research. Many opportunities were discussed at the UFZ workshop including but not limited to the quantitative role of ANAMMOX pathways in nitrogen transformations; heterotrophic nitrification and aerobic denitrification; the role of microbial communities in industrial effluent treatment (both the identification and possible isolation of specific species or groups of bacteria); the quantitative split between catabolism and anabolism for microbial communities in TWs; the effect of COD:N:P ratios on the structure and function of microbial communities in TWs; the generation of antibiotic resistant bacteria in different system types of varying sizes; the identification of microbial species directly transforming emerging contaminants in TWs; identification of both protozoa and bacteria involved in pathogen removal; the effect of biofilm on hydrological parameters; and verifying several microbiological parameters for use in TW numerical models.

Summary

For many decades, researchers and industry leaders alike have made gains in understanding, while also posing many questions regarding the role of microbial communities in wetland systems. Future research frontiers include both spatial and temporal analyses. At present there are many tools available for microbial community characterization and the future holds many great discoveries.

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Name: Kela P. Weber
Organisation: Environmental Sciences Group, Royal Military College of Canada
Town, Country: Kingston, Canada
eMail: kela.weber@rmc.ca

Name: Vincent Gagnon
Organisation: L'UNAM University, École des Mines de Nantes
Town, Country: Nantes, France
eMail: vincent.gagnon@mines-nantes.fr