Spatial and Temporal Bacterial Community Dynamics in Constructed Wetland Mesocosms

by

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I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

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Abstract

The objective of this work was to understand microbial population density and diversity, both spatially and temporally, in wetland mesocosms to gain a better fundamental understanding for use in the optimization and design of constructed wetlands (CWs).

A standardized community level physiological profiling (CLPP) data analysis protocol was adapted and utilized for CW mesocosms. A new one-dimensional metric was developed to track community divergence using BIOLOG^{TM} ECO plate data. The method proved easy to use, did not require a background in multivariate statistics, and accurately described community divergence in mesocosm systems.

To study mesocosm biofilm-bound bacterial communities an appropriate detachment protocol was required. Various shaking protocols were evaluated for their effectiveness in the detachment of bacteria from mesocosm pea gravel, with a focus on detachment of viable and representative bacterial communities. A protocol based on mechanical shaking with buffer and enzymes was identified as an optimal approach and used further in this study. The bacterial communities associated with the interstitial water, pea gravel media, and rhizospheric regions from both planted and unplanted CW mesocosms were profiled using the CLPP method and compared. Vertical community stratification was observed for all mesocosm systems. Rhizospheric communities were found to be significantly more active than their gravel-associated counterparts, suggesting that although rhizospheric bacteria were less abundant in the mesocosms they may play a more significant role in the removal and fate of water born contaminants.

The start-up dynamics of CW mesocosms was investigated using the CLPP and standard CW characterization methods over an eight month period. All mesocosms showed a steep increase in interstitial community divergence until day 75-100, at which point a steady-state was reached. The interstitial communities were also characterized in terms of similarity based on experimental design treatments (planted/unplanted and origin of seeding inoculum). Four stages were identified during the start-up consisting of an initial stage where mesocosm communities were differentiated based on origin of the inoculum, a period where adjustments and shifts occurred in all mesocosm, a time where all mesocosm communities were quite similar, and a final state where community differentiations were made based plant presence in the mesocosms.

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1.1 Background

1.1.1 Constructed Treatment Wetlands

The ability of wetland ecosystems to improve water quality has been recognized since the 1970s (Knight *et al.*, 1999). The main principle behind a constructed wetland, used for water treatment, is to reproduce a wetland ecosystem with the expectation that contaminated water will be treated as it passes through the wetland system. Constructed wetlands have been used to treat a number of different contaminated waters including organic farm waste (Cronk, 1996), food processing waste (Burgoon *et al.*, 1999), human wastewater (Decamp and Warren, 2000), and acid mine drainage (Mitsch and Wise, 1998; Kadlec and Kinight, 1996).

There are three general types of constructed wetland (CW) systems; free surface water (FSW), horizontal subsurface flow (HSSF), and vertical flow (VF) constructed wetlands. FSW CWs are built by first digging a trench with a slight gradient (1%) from inlet to outlet to allow movement of the water by gravity. This trench is then lined with an impermeable polymer, or low a permeability soil such as densely packed clay. This lined trench is then filled with the desired bed media. Bed media is most often selected based on desired flow patterns, nutrient and mechanical support for the chosen plant types, and as a nutrient source required for some biologically-mediated treatment processes. In some geographical locations the native soil may have a permeability low enough such that no liner is required. In this case the trench can simply be dug and filled with the desired bed media. Common media include peat, gravel, sand, soil and compost. FWS constructed wetlands contain standing water on the surface of the treatment system and can be fed from either below ground or above ground (Figure 1.1).

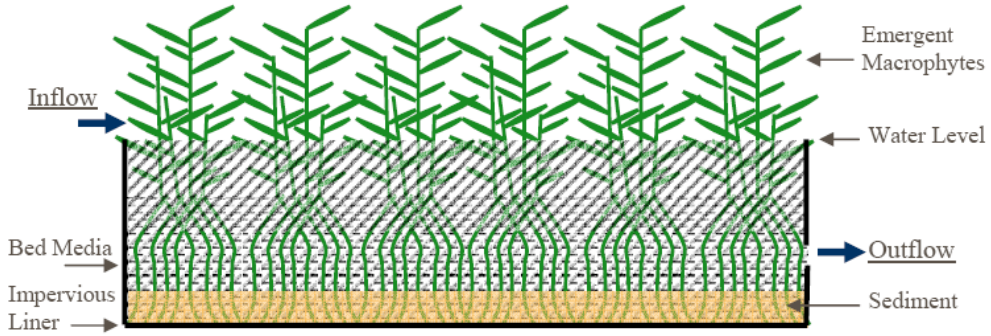

Figure 1.1: Free surface water constructed wetland.

HSSF CWs are similar in construction to FSW CWs, but are typically fed from below ground and are designed so the maximum flow rate will not allow surface water formation on top of the system (Figure 1.2).

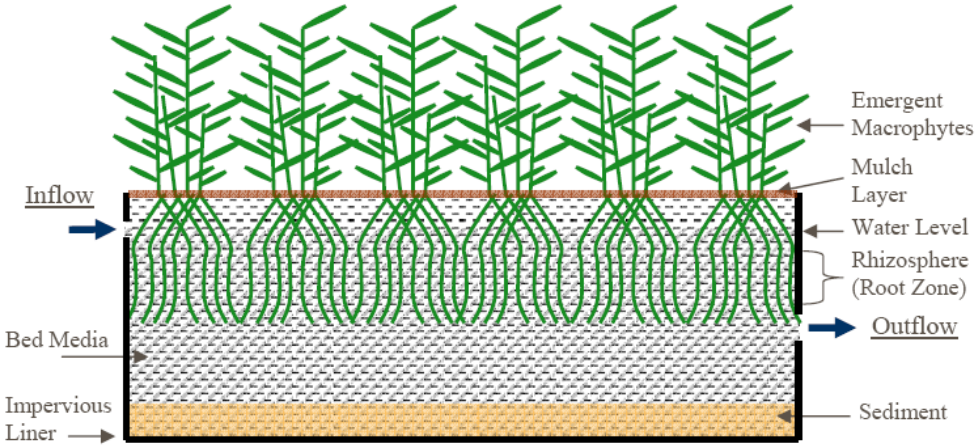

Figure 1.2: Horizontal subsurface flow constructed wetland.

VF CWs have a number of construction elements similar to those of HSSF CWs, however are not built with a gradient as the water is fed into the top of the wetland system. A large impermeable enclosure is built and filled with a substrate, water is then fed into the top of the VF CW. Water is usually fed into VF wetlands intermittently allowing for bed aeration and to

deter surface water formation (Figure 1.3). All three types of CW systems may contain different emerging macrophytes (large aquatic plants rooted to the bed media). In some cases, usually for research purposes, macrophytes are not included in the CW design. The two most commonly utilized macrophytes in CW designs are *Phragmites australis* (common reed) and *Typha* species (cattail or bulrush). FSW wetlands can also contain floating plants rather than rooted macrophytes; these types of systems do not require substrate and are popular in tropical regions.

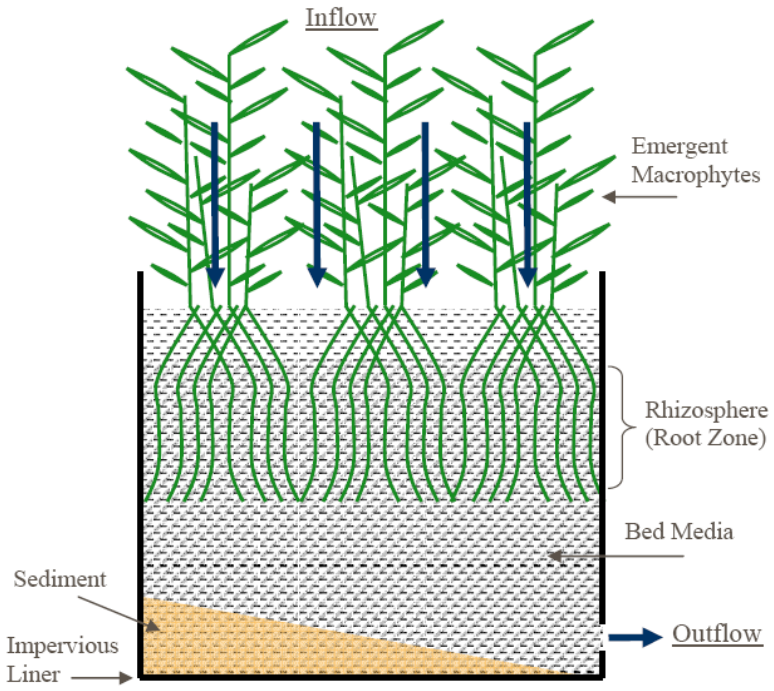

Figure 1.3: Vertical flow constructed wetland.

Currently CW design is based mainly on configuration, size, and flow records from previously employed systems. Wetlands are land (area) intensive technologies making implementation, for the most part, infeasible in densely populated areas. Although CWs have proven to be an effective treatment technology for a variety of contaminants, CW systems will on occasion fail due to temperature changes, hydraulic issues (clogging/short circuiting), and other

undetermined reasons. Diemont (2006) reported more than 650 full-scale constructed wetland systems in place throughout the world. This number although large is perhaps on the low side with many wetland scientists projecting as many as 10000 CWs operating throughout the world. Although there are many CWs in operation throughout the world, with regards to their functionality they are for the most part consistently treated as a black box. The specific mechanisms and the underlying fundamental variables affecting the functionality of treatment wetlands have been given little attention. This fact can be mainly attributed to the lack of feasible testing methods and associated systems (Stottmeister *et al.,* 2003) which has hampered confidence and support for the technology. There has long been a fundamental inability to make *a priori* predictions in terms of the effectiveness of a proposed wetland for a given flow and input water chemistry (Wieder *et al.*, 1989).

1.1.2 Constructed Wetland Design

CW systems are complicated in their chemistry, hydraulics, and distribution of specific removal mechanisms; therefore, simplified relationships and models for contaminant removal in these systems have been sought for ease of design. Contaminant removal in CW systems has been shown to correlate well with hydraulic retention time (HRT). Since CW size is proportional to HRT, contaminant removal in CWs has been modeled based on volume and different aspect ratios.

Hydraulics, kinetics and other design criteria such as minimum depths, maximum superficial velocities, minimum HRTs and wetland slopes all need to be accounted for when designing a CW system. For general considerations regarding wetland design see Kadlec and Knight (1996). Wetland systems are most often modeled as plug flow reactors incorporating both dispersion and kinetics into the model (Cronk, 1996). Hydraulics are quantified through hydraulic conductivity, porosity, and in some cases, specific values for dispersion. Kinetics are almost always accounted for by using first-order rate constants. Other design criteria are based largely on past engineering experience and include but are not limited to a maximum superficial

velocity of 8.6 m/d to avoid damage to plant root systems (Sauter and Leonard, 1997), a minimum specific surface area of 1 m^2/population-equivalent for tertiary treatment of municipal wastewater in vertical flow systems (Schoenerklee *et al.*, 1997), and a gradient of 1% for proper water movement.

1.1.3 Treatment Applications

Constructed wetlands (CWs) can be used to treat organic farm waste (Cronk, 1996), food processing waste (Burgoon *et al.*, 1999), storm water (Kadlec *et al*., 2008), acid mine drainage (Mitsch & Wise, 1998), and many different types of industrial wastewaters (Kadlec *et al*., 2008). However, the majority of CW treatment systems have been built for the treatment of human wastewater (Decamp & Warren, 2000), which may include pathogenic organisms. Studies have shown effective secondary and tertiary removal of pathogens such as fecal coliforms, *Cryptosporidium, Giardia* (Gerba *et al*., 1999; Neralla *et al*., 2000).

The use of CW systems to retrofit existing wastewater treatment plants, as a form of tertiary treatment, is becoming increasingly popular. The cost effectiveness of treatment wetlands makes them an attractive option for small communities. Conventional methods of wastewater and pathogen treatment are both effective and reliable. Activated sludge, trickling filter and slow sand filtration are some of the more commonly cited methods. All of these treatment methods, when properly used, are effective at removing many different types of pathogens with typical removal efficiencies around 99-99.99% (Vymazal, 2005). These processes are most often employed in conjunction with a tertiary step for further pathogen treatment. Although chlorination is a popular tertiary disinfection step, free chlorine when in contact with a significant amount of natural organic matter can form trihalomethanes (THM's) or other organo-chlorine compounds which are known to be carcinogenic (Kadlec & Knight, 1996). This possible formation of harmful by-products is the main reason for the development of other tertiary (or polishing) conventional-type pathogen treatment methods such as UV and ozonation. Low energy non chemical methods of pathogen treatment have been used when

receiving waters are non potable or disinfection requirements are relaxed. Constructed wetlands have low principle and operating costs making them popular in remote areas and developing countries. The wetland concept has become an attractive cost-effective wastewater treatment alternative compared to conventional or tertiary treatment processes (Morsy, 2007). For an in-depth discussion regarding various CW treatment applications see Kadlec *et al*. (2008).

1.1.4 Treatment Prediction and Modeling

Constructed wetland models have been developed for industrial wastewater, agricultural run-off, human wastewater, and storm water applications to name a few. Constructed wetland modeling can be separated into five main categories: rules of thumb, regression equations, first order equations, variable order or Monod type equations, and mechanistic or compartmental type models. All five model types have positive and negative aspects associated with their use.

1.1.4.1 Rules of Thumb

Strictly speaking rules of thumb are not a type of modeling technique. Rules of thumb are developed to give easy to use design criteria for constructed wetland creation. They are based on a broad spectrum of data and resultant designs are quite conservative. Kadlec and Knight (1996) and Wood (1995) offer subsurface constructed wetland design criteria based on general rules of thumb. In the design of subsurface flow constructed wetlands, Kadlec and Knight (1996) suggest designing to achieve a hydraulic loading rate of 8-30 cm/day and a hydraulic retention time of 2-4 days.

1.1.4.2 Regression Equations

The second type of constructed wetland model is based on regression equations. Regression equations are created from historical data for numerous contaminants at many different field sites. Rousseau *et al.* (2004) summarizes many different regression equations for BOD, COD,

TSS, TN and TP from many sources. The standard regression equation is usually linear and of the form:

$$C_i = \alpha C_0 + \beta \qquad (1.1)$$

where:

- C_0 - effluent concentration
- C_i - influent concentration
- α - treatment coefficient/slope
- β - treatment coefficient/intercept

Some regression equations offered by Kadlec and Knight (1996) for example, contain exponential terms. Despite the form of the regression equation, the objective is to fit the data with the best possible relationship. Published regression equations summarized by Rousseau *et al.* (2004) have R^2 values ranging from 0.02 to 0.97. R^2 values of approximately 0.6 are commonly found and accepted as appropriate.

Regression equations can provide an estimate of the common removal rates found in wetland systems treating similar contaminants; however, the range of different removal efficiencies and rates cannot be overlooked. When constructing a wetland many factors play a role in the expected removal efficiency. Regression equations are useful for describing removal in a specific wetland, however they provide no insight into what design aspect may improve contaminant removal in the design. To attain this end a more descriptive mechanistic model is needed.

1.1.4.3 First Order Equations

Similar to regression equations, the use of first order equations is another way to describe specific contaminant removal in a constructed wetland system. Instead of creating a regression equation that best fits the data, a standard first order equation is used and the rate constant is adjusted to fit the data. The standard first order equation is of the form:

$$\ln\left[\frac{C_0}{C_i}\right] = -\frac{k}{q} \qquad (1.2)$$

where:

k - first-order rate constant

q - hydraulic loading rate

This equation is helpful in that it adjusts for the hydraulic loading rate which most regression equations do not include. In addition to the simple first order equation another first order equation commonly called the k-C* equation is utilized:

$$\ln\left[\frac{C_0 - C^*}{C_i - C^*}\right] = -\frac{k}{q} \qquad (1.3)$$

where:

C* - background concentration

Including the background concentration has been shown to improve data fits and better describe BOD and COD removal (Noorvee *et al.,* 2005). The rate constant is often adjusted for temperature effects by the equation:

$$k_T = k_{20}\theta^{T-20} \quad (1.4)$$

where:

k_{20} - reaction rate coefficient at 20°C

k_T - reaction rate coefficient at temperature (T)

T - temperature (°C)

θ - temperature factor

The temperature factor θ is distinct for different contaminants. Kadlec and Knight (1996) show the temperature factor θ for TSS and TP to be 1, with θ for TN to be 1.05.

Similar to regression equations, first order equations are useful in describing contaminant removal rates in wetland systems; however, they provide no insight into better design or optimization.

1.1.4.4 Variable Order and Monod type Models

First order rate models describe the removal rate as continually increasing as loading increases. This description is often found to be limiting as there is usually a maximum removal rate in any system. Monod type equations put a ceiling on the reaction rates, better describing actual removal rates over varying loading rates. If the loading rates are not high enough to observe close to the maximum reaction rate, then first order rate equations can be considered valid. In fact, actual loading rates have been shown to be much lower than the maximum loading rates in the majority of field cases (Rousseau *et al*., 2004). Kemp and George (1997) reported that in some cases with low loading rates, Monod type equations have been shown to better describe the data.

1.1.4.5 Mechanistic Models

Modeling of constructed wetlands can be said to serve two purposes: understanding the underlying mechanisms, and the optimization of existing design criteria (Langergraber, 2003). In contrast to the previously discussed contaminant removal models, mechanistic models try to include all of the major physical, biological and chemical processes occurring in the wetland system. The interdependence of many processes such as oxygen transfer and oxidation of organic matter (removal of BOD) is ignored in the single contaminant removal models. Mechanistic models offer a framework to compare processes between sites and a basis for understanding and optimization of the many processes occurring in the wetland. The range of processes included in mechanistic models varies substantially. The number of processes as well as the accuracy with which the processes are connected plays a major role in how well a mechanistic model will perform.

Mechanistic models often contain several sub-models describing contaminant removal processes including sedimentation, filtration, precipitation, sorption, plant uptake, microbial decomposition and oxidation. The more advanced mechanistic models contain water and oxygen balances, carbon and nitrogen cycles, both heterotrophic and autotrophic microorganism models and a plant growth/death model. Of all the mechanistic models presented to date, the model of Wynn and Liehr (2001) is perhaps considered the most inclusive and complex, although many other models offer improved sub models (McGechan *et al*., 2005; Langergraber, 2003; Giraldo and Zarate, 2001).

1.1.5 Removal Efficiencies

CW removal efficiencies upwards of 99.99% have been reported for virtually all types of water contaminants (Kadlec & Knight, 1996). Configuration has been correlated with different efficiencies for specific contaminants. For example, Vymazal (2005) presented removal efficiencies and first-order aerial rates recorded for different CW systems in-use at the time of the study, for 4 different indicator organisms (pathogen surrogates). Removal efficiencies

ranging from 65% to 99% were observed with the highest removal rates observed for hybrid systems (more than 1 CW configuration type used), followed by HSSF, and lastly FWS systems. VF systems were not included in the survey. Although broad based correlations between removal efficiency and configuration have been observed, there still exists significant amounts performance variability for similarly designed CW systems.

1.1.6 Pre-Treatment

Pre-treatment has been cited as a requirement when using CW systems to treat domestic wastewater or any other type of contaminated water with a large amount of particulate matter (Anderson *et al.*, 1996; Cronk, 1996; Kern and Idler, 1999; Perfler *et al.*, 1999; Peterson, 1998; Sauter and Leonard, 1997; Williams *et al.*, 1999). Suspended solids can temporarily or permanently clog CW systems (Cronk, 1996; Schoenerklee *et al.*, 1997). Clogged systems are subject to short circuiting, creating unwanted flow patterns and reduced contact time between the contaminant and the wetland substrate (Tanner *et al.*, 1998).

Sedimentation is cited as the most common method of pre-treatment for CW systems (Anderson *et al.*, 1996). Sedimentation can be performed through the use of lagoons, equalization basins, ponds, settling tanks or septic tanks (Frostman, 1996; Philippi *et al.*, 1999; Sauter and Leonard, 1997). Mechanical aeration can also be used as a pre-treatment process (Cronk, 1996); however, utilizing this technology can significantly increase the cost of the treatment system in question. Another form of pre-treatment is to use a hybrid “FWS to HSSF system”. Processing wastewater first through a FWS CW can effectively reduce the number of particles found within the water (Rochfort *et al.*, 1997). Use of a pre-treatment system can also help in equalizing flows over long periods of time, reducing the negative effects of low or high flows (Frostman, 1996).

1.1.7 Constructed Wetland Design and Implementation

As previously mentioned there are many drawbacks to using first-order decay kinetics when sizing a wetland based solely on area and inlet concentrations. These methods are based on the assumption that effluent concentrations are directly related to inlet concentrations and flow, and that removal efficiency will be based on wetland area and flow rates. As seen with the wide range of removal efficiencies for similarly designed CWs, performance cannot be accurately described based solely on these variables and doing so can lead to erroneous conclusions (Kadlec, 1997; Werker *et al*., 2000). Simple system changes such as a change in water levels will affect the concentrations of contaminants within the system (Kadlec, 1997; Neralla *et al.*, 2000). As well, seasonal cycles affecting inlet flows and concentrations, solar radiation, temperature (air, soil and water), precipitation, evapotranspiration, and biomass in wetlands will all affect system performance (Kadlec, 1999). Other factors to be considered in CTW design include but are not limited to local climate, site topography, site geology, loadings, local drainage areas, land availability, cost, size and extent of receiving water body and water quality objectives (Shutes *et al.*, 1999).

1.1.8 Design Limitations

1.1.8.1 Hydraulic Challenges

Channeling or short-circuiting has been shown to impact treatment performance (Rash and Liehr, 1999; Sauter and Leonard, 1997; Scholes *et al*., 1998). As discussed previously, pre-treatment can greatly reduce the chances of clogging and channeling. As such, there are a number of aspects related to hydraulics that should be considered when building CW systems. These include: 1) The presence of plant roots has been shown to direct water below the root zone in wetland systems (Rash and Liehr, 1999), creating a vertical hydraulic stratification. In some cases there is evidence that plants may cause short circuiting, although this may be limited to start-up periods (Frostman, 1996); 2) Wetland width can also have an effect on channeling as edge effects have been shown to influence CW hydraulics (Tanner *et al.,* 1998) and 3) The position of effluent removal has been shown to have an effect on channeling.

Collecting effluent from the bottom of the CW system has been shown to result in vertical stratification (McNevin *et al.*, 2000).

1.1.8.2 Water Level Maintenance

Water levels need to be maintained in wetland systems to ensure plant health, consistent microbial activity, and consistent microbial ecology within the CW system. A number of design considerations have been recommended in the literature to ensure consistent water levels in CW systems. These include: 1) Implementation of a preceding system to equalize variable or seasonal flows to ensure a CW system does not dry out or overflow (Anderson *et al.*, 1996); 2) Continuously flowing water is needed to avoid pipe freezing during winter (Wittgren and Maehlum, 1997) and 3) Dry periods in arid geographical regions can effectively dry out CW systems through lack of inlet flow and increased evapotranspiration.

As previously discussed use of a pre-treatment system can help to maintain equalized flows throughout short and long-term time periods. One simple way to help ensure a CW system does not dry-out due to reduced inlet flow is to place the effluent pipe at a height that ensures that the minimum necessary water level is maintained. This however may not be possible if treating water with a high level of particulate matter as this may result in clogging.

1.1.8.3 Cold Climates

Cold climates present another challenge for CW design. Decreased microbial activity, plant dormancy, and freezing of the water column can all occur due to cold temperature operation (Werker *et al.*, 2002). As such, there are a number of considerations when designing CWs for cold climates. 1) HSSF systems are often used in cold temperature climates to eliminate free water freezing and make use of warmer ground temperatures in the winter (Dusel and Pawlewski, 1997; Revitt *et al.*, 1997). 2) Vegetation can be used to help produce an insulating layer of mulch on top of the CW system (Smith *et al.*, 1997). Emerging vegetation can also

entrap snow, further insulating the system during winter operations (Wittgren and Maehlum, 1997). 3) CW systems could be made deeper, to avoid surface freezing. This may have a disadvantage in cases where higher levels of dissolved oxygen are required as installing deeper CW system can hinder treatment performance.

1.1.9 Contaminant Treatment Mechanisms

Through research studies on small scale systems and insightful characterization and study of full scale wetlands, the fundamental variables and mechanisms involved in water treatment can be identified to provide a better understanding of CW systems. With a better understanding, enhanced design, implementation and monitoring of in-use systems will ensue, potentially allowing for improved treatment performance for CWs of all sizes and configurations, and the ability to project long-term performance characteristics.

Suggested mechanisms for organic, inorganic and pathogen treatment in CWs include sedimentation, natural die-off, inactivation or death related to temperature, oxidation, predation, inactivation or death related to unfavorable water chemistry, biofilm interaction, mechanical filtration, exposure to biocides, UV radiation, precipitation and biotransformation (Kadlec and Knight, 1996; Vymazal, 2005; Borisko *et al.*, 2000; Cronk, 1996; Gerba *et al.*, 2000). Table 1 has been generated from a review of the literature and lists removal mechanisms along with some suggested design parameters which may have an effect on each respective mechanism. Also included are rough estimates of the time required for each mechanism to have an effect on contaminant treatment.

Table 1.1: Summary of contaminant treatment mechanisms in constructed wetlands

Removal Mechanism	*Design Factor*	*Effective Time*
Sedimentation	bed media type, configuration	minutes-years
Temperature	location, plant presence, microbial activity	days
Oxidation	plant presence, configuration	hours-days
Adhesion to biofilm	microbial ecology and activity	minutes-hours
Mechanical filtration	bed media type, configuration	minutes-hours
UV radiation	configuration	seconds-minutes
Biological transformation	microbial ecology and activity	minutes-hours
Precipitation	bed media type, configuration	seconds-minutes
Pathogen specific mechanisms		
Exposure to Biocides	plant type	minutes-hours
Unfavorable water chemistry	bed media type	minutes-days
Predation	microbial ecology and activity	minutes-days
Natural die off	hydraulic retention time	days-weeks

1.2 Experimental Research

Case studies involving large scale in-use wetland systems give practical information for design and implementation. Pilot studies involving both the treatment of contaminated waters in conjunction with comparison of wetland design orientations aim at understanding the effect of different design aspects on treatment performance. When working with in-use constructed wetlands there is a lack of practical replication and environmental control, and extended response times hinder true comparative or quantitative studies. Most constructed wetlands used for water treatment have been installed for non-research purposes (Wieder, 1990); therefore, most advances in the field can be attributed to large compilations of case study data. True experimental design and subsequent statistical analysis is not achievable for most constructed wetland research endeavors. Many case study reports do not take into account numerous factors which can vary between wetland systems over the study period due to the naturally divergent nature of many uncontrolled variables in wetland systems. These factors include microbiological activity, bacterial community changes, hydraulic dispersivity, porosity, and evapotranspiration effects. The resulting lack of understanding towards the specific

mechanisms and the underlying fundamental variables affecting the functionality of treatment wetlands has resulted in a wide variation in reported performance values for similarly designed systems (Kadlec and Knight, 1996), with little explanation as to the reasons for this variation.

Several challenges are apparent when designing experiments aimed at the study of CW removal mechanisms. 1) Large scale in-use wetland systems offer little control over environmental variables. 2) Removal mechanisms do not act independently, rather certain removal mechanisms may be correlated or act synergistically. 3) Large-scale in-use treatment wetland monitoring data often does not contain useful information regarding the action of specific contaminant removal mechanisms. 4) Appropriate statistical analysis is often unattainable using in-use wetland monitoring data.

1.2.1 Laboratory Scale Experimental Systems

Although laboratory scale wetland systems do not completely recreate or represent full scale in-use treatment wetland systems they do offer a number of advantages when investigating fundamental variables or mechanisms affecting treatment performance. Fundamental investigations are often not directed at accurately estimating removal coefficients in large scale systems, but rather at gaining an understanding of the quantitative comparison of certain variables or mechanisms on overall removal. In other words, trying to understand which mechanisms or variables have a significant impact on removal. To develop this understanding, it is important to assess factors that may influence these significant variables and mechanisms, and how these mechanisms or variables relate to each other. Although overall removal rates will not be representative in small scale systems, these systems offer research-related advantages not available in large scale-systems.

Laboratory scale treatment wetland systems are often referred to as mesocosms or microcosms. Examples of small scale mesocosm system studies can be found in the literature

(Kappelmeyer *et al.*, 2001; Prado, 2004; McHenry and Werker, 2005; Werker *et al.*, 2007; Weber *et al.*, 2008). Configurations, flow rates, feed entry points, and recycle rates can all be varied to achieve different internal system conditions. To reflect the inherently diverse nature of in-use CW treatment systems, the experimental conditions investigated within laboratory scale mesocosm systems can be varied to suit specific investigations. Figure 1.4 shows an example of the type of mesocosm system used for the research described in this thesis.

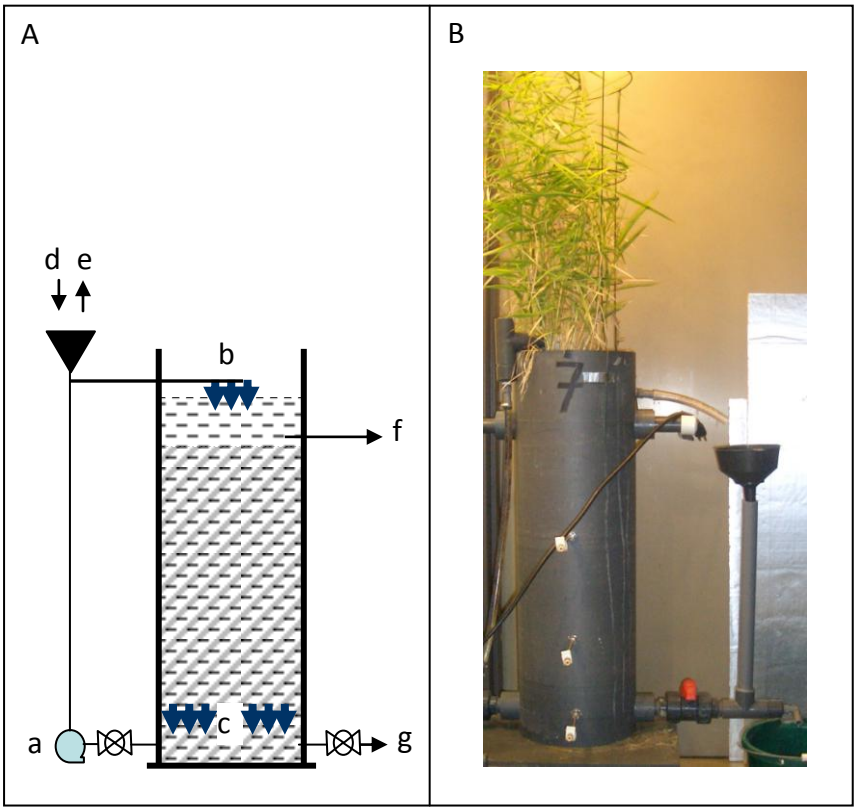

Figure 1.4: Mesocosm schematic (A) and representative picture for the system planted with Phragmites australis (B). In (A) water is fed into the mesocosm (b) and allowed to percolate through the pea gravel bed to be collected at the bottom (c), where a small centrifugal pump re-circulates the water (a). An atmosphere exposed port serves as an injection (d) and sampling (e) point. Drainage ports are located near the top to prevent overfilling (f), and near the bottom (g) for mesocosm drainage.

The constructed wetland mesocosm approach was developed as a quantitative method to gain insight into wetland performance, and ultimately use this insight to assist in the design and

treatment optimization of constructed wetlands by applying principles of tracers, reactor theory, modeling, and enzymology, while undertaking experiments to critically assess factors that could influence performance and reliability of treatment wetlands (Werker *et al.,* 2004). Through system replication, environmental control and shorter response-times, wetland mesocosms allow for the implementation of factorial designed experiments and subsequent quantitative and comparative statistical analysis. The mesocosm approach has been described as a powerful way to test research hypotheses using quantitative experimentation (Perrin *et al.*, 1992). Several mesocosm studies have recently been used in undertaking a quantitative approach to the study of constructed wetland systems (Kappelmeyer *et al.*, 2001; Prado, 2004; McHenry and Werker, 2005; Werker *et al.*, 2007; Weber *et al.*, 2008).

1.2.2 The Study of Biologically Related Removal Factors

It is generally accepted that constructed wetlands contain a biological regime associated with the wetland substrate (Truu *et al.,* 2009; Wynn and Liehr, 2001). The role of the biological regime, and the related mechanisms associated with contaminant treatment, have been largely overlooked in favor of using hydrodynamic and simple first-order removal rate models to describe water treatment in constructed wetlands. The role and influence which the biological regime has on specific aspects within constructed wetlands has been given little to no attention.

The abiotic removal mechanisms in wetland systems can be said to be similar to those in sand or gravel filters and are well documented and quantified (Stevik et. al, 2004), but the biotic removal mechanisms in wetland systems are what make them unique and more effective. Both the planted regions and the internally developed bacterial community within wetlands can be considered as biotic or “living” components of the wetland system. These biotic components are more difficult to study and quantify leading to a gap in our knowledge surrounding the associated removal mechanisms. Of further note is the likely interdependence of abiotic and biotic removal mechanisms. For example, physical filtration removal rates are closely related to

biofilm type and size, and biofilm type and size will be based on the overall activity of the microbial population within the wetland system and the different bacterial species populating the media.

Different communities will create different microenvironments including different biofilm environments (Faulwetter *et al.,* 2009). As previously discussed the existence of, and general integrity and make-up of these biofilms, can have an effect on contaminant removal in wetlands (Broadbent *et al.,* 1971; Richardson and Rusch, 2005; Vacca *et al.,* 2005; Larsen and Greenway, 2004). The different bacterial communities in wetlands play a vital role in water treatment performance and ecosystem health (Parkinson and Coleman, 1991; Aelion and Bradely, 1991). Although the bacterial community in CWs has been recognized as having a large influence on water treatment performance, little attention has been given to understanding the microbial ecology in these systems.

A number of microbial-based pollutant cycles/transformations have been well documented in the literature, including organic matter degradation, nitrogen transformations, and sulfur removal (Kadlec and Knight, 1996). Understanding how microbial processes can effect these cycles and transformations is important to furthering the scientific understanding of constructed wetlands (Faulwetter *et al.*, 2009). Realizing the simplicity or complexities behind the differing cycles is often the first step in beginning research with regards to microbial-based pollutant transformations. For example organics can enter wetland treatment systems in a variety of forms; many different types of bacteria are then able to use these organics as either a carbon or an energy source, releasing CO_2 into the atmosphere. The nitrogen cycle in CWs is however more complex; for complete nitrogen removal a number of different bacterial types are required. Nitrogen usually enters the wetland system as ammonium. Ammonium is then transformed into nitrite by ammonium oxidizing bacteria, and then into nitrate by nitrite oxidizing bacteria. These steps are both aerobic and require specific autotrophic bacteria to progress. Nitrate can then either be assimilated by plant roots or go through a multistep

anaerobic denitrification process producing gaseous N_2. Denitrification requires specific facultative or anaerobic chemoheterotrophs to occur. Therefore when designing a CW for nitrogen removal it is important to understand the different oxygen requirements of the bacterial cultures at each step of the nitrogen removal process, and also realize the carbon source requirement in the denitrification process.

The interaction between plants and the bacterial communities found in the wetland substrate is another major factor affecting bacterial community dynamics in wetland systems. Aquatic plants, such as *Phragmites australis*, have the ability to transfer oxygen from their aerial tissues and release it into the rhizosphere region (Karathanasis and Johnson 2003; Batty *et al.,* 2000). Plant root systems also provide mechanical support and perform many roles including the synthesis, accumulation, and secretion of compounds (Flores *et al.*, 1999). The chemicals secreted into the surrounding rhizosphere by roots are referred to as root exudates. Plants have been shown to exude 5-21% of all photosynthetically fixed carbon into the surrounding rhizosphere as root exudates (Walker *et al.*, 2003; Marschner, 1995). Through this exudation, roots can often influence the microbial community structure within the surrounding rhizosphere (Walker *et al.*, 2003; Nardi *et al.*, 2000). Recent work has gone so far as to take advantage of this bacteria-rhizosphere interaction to help promote plant growth using engineered bacteria (Reed *et al.*, 2005).

Biotic factors can be difficult to quantify in wetland systems. Two factors which can be studied with relatively little difficulty are microbial activity and microbial community structure. The activities of different bacterial species and the overall community structure affect treatment performance of constructed wetland systems. By gaining better insight into bacterial community activity and diversity, improvements to existing wetland models should be possible.

1.2.2.1Microbial Activity

Constructed treatment wetlands are used to treat any number of different wastewaters. In treating these wastewaters a number of mechanisms are at work. However, it has long been recognized that the treatment of the majority of water born contaminants in CWs is largely due to microbial processes of transformation and degradation (Faulwetter *et al.*, 2009). Therefore the rate at which these microbial-based transformations occur is of fundamental importance in understanding treatment efficiencies in constructed wetlands. If fundamental research can uncover the relationships between treatment efficiency and the activity of bacteria in CWs, perhaps the large performance differences between similarly designed CWs could be better understood. With a greater understanding in hand, further work into the study of specific bacterial "functional groups" responsible for specific pollutant transformations would represent a natural progression of the science (Faulwetter *et al.*, 2009).

One of the most commonly utilized microbial activity measures is the 5 day biochemical oxygen demand (BOD_5). Other more easily applied methods include carbon utilization measures (Weber *et al.*, 2008; Tietz *et al.*, 2008), and microbial-related enzymatic activity measures based on the conversion of FDA to FL (Schnürer and Rosswall, 1982; McHenry and Werker 2005; Weber *et al.*, 2008).

1.2.2.2 Microbial Community Assessment

Another important factor in wetland systems which has recently received attention is the bacterial community structure (Vacca *et al.*, 2005; Hallberg and Johnson, 2005; Weber *et al.*, 2008). Both the genetic diversity and functional adaptation of bacterial communities in wetland systems allow for improved long term treatment performance (Kadlec and Knight, 1996). Genetic diversity can give an idea of the number and distribution of species within a community while the study of the functional response or functional diversity, takes a more holistic approach, and yields an idea of the overall community response or function without cataloging

specific species. A brief look at some of the more commonly used bacterial community assessment methods follows.

1.2.2.2.1 Non-Molecular Methods

1.2.2.2.1.1 Light Microscopy

Traditional non-molecular methods include microscopy and culture based identification. Microscopy has advantages due to its ease of use and quick assessments, making microscopy a convenient and dependable method when monitoring communities of fixed or similar species distribution. Light microscopy allows for qualitative-heavy identification based on morphology however it is not possible to distinguish between living and dead organisms (Madigan *et al.*, 2002). Other drawbacks include the need for specialized expertise and the fact that different organisms can share similar morphology (Ferris *et al.*, 1996; Duineveld *et al.*, 2001).

1.2.2.2.1.2 Traditional Microbial Plating

Culture-based identification can be used to identify some microorganisms. By using previously developed expertise, species identification can be accomplished through sequential plating with different nutrient sources (Cullimore, 2000). These methods require a large amount of time, resources, and expertise. As well, many organisms may not be cultureable under plating conditions (Amman *et al.*, 1995); it has been suggested that plate count techniques account for a meager 0.1-20% of the original population (Muyzer *et al.*, 1993).

1.2.2.2.1.3 Community Level Physiological Profiling

Community level physiological profiling (CLPP) is an approach used to characterize microbial community function based on sole carbon source utilization patterns (CSUPs). CLPP can be used as an indicator of the metabolic characteristics and overall stability of a specific microbial community over time. Recent work in the area of microbial soil ecology has utilized BIOLOG™

plates as a tool for CLPP. BIOLOG™ plates consist of 96 wells, each well containing a different carbon source and a redox dye indicator (tetrazolium violet) which changes colour in response to carbon utilization. Garland and Mills (1991) were the first to use BIOLOG™ plates for characterizing heterotrophic soil bacteria communities through principle component analysis (PCA). A number of subsequent studies are discussed in Konopka *et al.*, (1997). Most recently Weber *et al.* (2008) successfully used BIOLOG™ ECO plates to profile the interstitial bacteria in constructed wetland mesocosms. In contrast to the original BIOLOG™ plates with 96 different carbon sources, ECO plates by the same manufacturer are based on 31 different carbon sources with built-in triplicates allowing for better replication. CLPP has advantages over both classic cell culturing techniques and molecular level RNA/DNA amplification techniques as these other techniques are time consuming and require specialized expertise (Garland, 1997).

Limitations pertaining to the CLPP approach using BIOLOG^{TM} ECO plates have been discussed in the literature (Garland, 1997; Konopka *et al.*, 1998; Preston-Mafham *et al.*, 2002). Limitations and pitfalls pertaining to data analysis have also been recently described (Weber *et al.,* 2007). Some of the most pertinent limitations include the bias in the technique toward rapidly growing bacteria, the need to ensure similar sample sizes, the need to reduce time between sampling and inoculation of the BIOLOG^{TM} microplates, and difficulties with meaningful data analysis and interpretation.

The CLPP approach has not been used widespread within either CW or natural wetland systems. Only 3 different CLPP studies can be found within the literature. Hadwin *et al*. (2006) studied the effect of different naphthenic acid treatments on CW resident communities in the Athabasca oil sands of Alberta, Canada. However using the CLPP method no definite trends could be seen. Hench *et al.* (2004) performed a study looking at the bacterial community of pilot scale planted and unplanted HSSF CW treatment systems over several seasons using the CLPP method. It was found that the bacterial communities in the CW systems were variable over the different seasons, and that a distinct difference in the bacterial communities of

planted and unplanted systems could be observed. Weber *et al*. (2008) used the CLPP method in the study of resident communities within laboratory scale wetland mesocosms and found distinct differences between the communities found within unplanted and planted mesocosms. This same study was also able to track community divergences and recovery trends based on an AMD perturbation. To the best knowledge of the author no studies applying the CLPP method to natural wetland bacterial communities have been published. However, based on personal communications at the 2nd International WETPOL conference (Tartu, Estonia) some scientists have attempted to apply the CLPP method to natural wetland communities, but have had only limited success. This lack of success could be due to the nature of the systems, or could also be due to data analysis difficulties as described by Weber et al. (2007). The CLPP method is a powerful method which has yet to be truly tested on either CW or natural wetland systems. Therefore there are still many questions surrounding the methods utility and feasibility in these systems.

1.2.2.2.2 Molecular Methods

With the advent of the polymerase chain reaction (PCR) and a growing library of genetic information on bacterial species, molecular identification methods have become increasingly popular in the field of bacterial community analysis. Although many of the molecular techniques developed for community analysis could be used to study the bacterial community in wetlands, only a small number of molecular-based community studies have been performed (Vacca *et al.*, 2005; Hallberg and Johnson, 2005; Faulwetter *et al*., 2009).

1.2.2.2.2.1 Denaturing Gradient Gel Electrophoresis (DGGE)

DGGE separates PCR amplified bacterial community rDNA gene segments by electrophoresis on a denaturing gradient gel. PCR is a method of DNA or RNA amplification using a heat stable polymerase, an excess of nucleotide bases (dNTPs), and an excess of 2 (20-base pair or smaller) primers (Tozeren and Byers, 2004). These 2 primers match highly conserved regions on the DNA, most commonly in the gene encoding bacterial 16s ribosomal RNA (rRNA). The primers

are selected with some *a priori* knowledge of what bacterial species may be expected in the samples. PCR has been shown to have a number of limitations including contaminants present in the samples, suboptimal reaction conditions, lack of primer specificity, and differential annealing (Suzuki and Giovannoni, 1996).

To perform a DGGE analysis, PCR amplified rDNA segments are run on a gel containing urea and formamide. The urea and formamide denatures the rDNA as it runs on the gel, completely restricting its movement in the gel at a specific location based on the original sequence. Therefore different proportions of nucleotides in the original species sequence will allow rDNA to move a characteristic distance unique to the original sequence (species). Each band present on the gel is then said to be representative of a specific bacterial species in the original sample (Nadarajah, 2007).

1.2.2.2.2.2 Terminal-Restriction Fragment Length Polymorphism (TRFLP)

Either one or both of the primers used in the PCR amplification is labeled with a fluorescent marker. These primers are again most often targeted at the 16s rRNA encoding region of the DNA, which has a highly conserved length (50-200 bp). A restriction enzyme is then added which cleaves the rDNA at a specific site dependant on the species sequence. The lengths of the fluorescently labeled fragment can then be determined using capillary electrophoresis (Dunbar *et al.*, 2001). Two electropherograms are then generated with two sets of colour peaks, usually blue for TRFs created from the 5' end and green created from the 3' end (Osborn *et al.*, 2000; Nadarajah, 2007). The resulting fingerprint gives a measure of phylogenetic diversity (Liu *et al.*, 1997). Biases for TRFLP originate from the same limitations discussed for PCR.

1.2.2.2.2.3 Fluorescent In-Situ Hybridization (FISH)

A library of fluorescently labeled probes is designed based on the expected 16s ribosomal RNA sequences of the bacterial species in the sample (MacDonald and Brozel, 2000). These single

stranded probes bind with the denatured DNA of the respective species within the samples. Fluorescence is then detected using fluorescence microscopy. FISH allows the visualization of the spatial distribution of organisms in a sample (Karp, 1999). FISH limitations include problems with cell permeability, target site accessibility, and a possible lack of *a priori* knowledge regarding species within a sample.

1.2.3 Experimental Design

When trying to ascertain the quantitative contributions of different mechanisms, design factors, or operating variables on system performance, a well designed experiment is of crucial importance. Many experimental regimes are based on single factor experiments where one variable is varied while all other controllable variables are kept constant. This method allows for the quantification of the effect a single independent variable has on a dependant variable, such as a system removal rate. In wetland systems many variables which may at first seem to be independent may in fact be interdependent or act synergistically. Therefore it is important to allow for the quantification of both single factor and synergistic variable effects on system performance.

One such experimental design method which allows for the quantification of both single factor and the interdependent effects of variables on the final dependant variable (system performance) is the factorial design. An x^n factorial design is performed at x number of levels for n number of variables. For example, an experimental design could be performed at 2 levels looking at the effect of plant presence and bed media type on overall pathogen removal performance in constructed wetland mesocosms. The 2 levels for the plant presence could be -1 for “no plants present”, and +1 for “plants present”. The two levels for the bed media could be -1 for sand, and +1 for gravel. This experimental regime would require 4 different mesocosm setups, and would require duplication for statistical analysis purposes giving a total of 8 experiments. Subsequent analysis is analogous to performing a multiple linear regression.

Statistical analysis of the results for a 2^2 factorial design experimental regime yields an equation of the form:

$$Y = \alpha_0 + \alpha_1 x_1 + \alpha_2 x_2 + \alpha_3 x_1 x_2 \quad (1.5)$$

where: Y = dependent variable (removal performance)

α = coefficient

x = independent variable

This equation once attained is not for use in predicting removal performance in the studied systems, rather it is simply used to quantitatively compare the magnitudinal effect of each studied independent variable (x_i) on the dependent variable (Y). The size of the $|\alpha|$ signifies the magnitude of that variable's effect on the dependent variable. The α terms can be positive or negative depending on the effect each independent variable has on the dependent variable. One of the numerous benefits of using a factorial design is the final "cross" term that is attained. The $\alpha_3 x_1 x_2$ in the final general linear equation signifies any synergistic or "cross" effects the two independent variables may have on the dependent variable (Y). Quantifying this cross term can significantly increase the fit of data and should be considered in wetland system experiments due to the synergistic nature of the inherent variables. Although quantifying the cross term does not give information as to the nature of the synergistic effect it does allow research to be directed in the proper direction. For example, if a cross term is found to be quite large in comparison to the single factor terms, perhaps more consideration in design and further research into the nature of this synergistic effect is needed.

Many statistical packages offer extensive analysis options for factorial experiments. Model fit evaluation and error propagation analysis are both needed to evaluate the validity of the general linear model. Data transformations may be needed for proper statistical analysis. For further details regarding factorial experiments and experimental design see Montgomery (2001). Other statistical methods such as multivariate analysis have been shown to be useful in evaluating microbial ecology in CW systems (Weber *et al.*, 2007) and could be similarly utilized in looking at other large CW data sets.

Through the use of small scale systems, active and inactive tracers, proper experimental design and statistical analysis, investigations regarding the fundamental mechanisms and variables affecting overall contaminant removal performance in CW systems can be performed. Biotic variables such as microbial activity and microbial community structure should be evaluated as these variables may have a large influence on removal performance. Greater research effort into understanding microbial population density and diversity, both spatially and temporally, would help to further optimize design of constructed treatment wetland systems (Faulwetter *et al.,* 2009). Including these variables in constructed treatment wetland research studies could perhaps help to better characterize and explain the large performance variations reported for similarly designed wetland systems.

1.3 Objectives

The overall objective of this work was to study the temporal and spatial bacterial community dynamics in wetland mesocosms. Several study objectives were identified to achieve this end:

A) Develop and apply the CLPP method using BIOLOG^{TM} Eco plates for the characterization of bacterial communities in constructed wetland mesocosms
B) Develop a metric for tracking community divergence using CLPP data
C) Develop a method for the detachment of viable bacteria from wetland gravel

D) Investigate fixed film bacterial communities associated with both wetland mesocosm gravel media and root systems at varying subsurface depths
E) Monitor changes in the interstitial bacterial communities in wetland mesocosms following the simultaneous start-up of multiple systems
F) Appraise the significance of plants and microbial seeding community origin on bacterial community development in CW mesocosms

1.4 Thesis Organization

This thesis consists of a five manuscript series preceded by an introduction (Chapter 1) and post-ceded by conclusions (Chapter 7). Each chapter includes a short introduction complementing the general introduction for the thesis.

Chapter 2 describes the community level physiological profiling (CLPP) method that was adapted and developed for CW mesocosms, and which is used throughout the post-ceding chapters to characterize the bacterial communities found within the experimental wetland mesocosms. This chapter has been published in *Methods in Molecular Biology: Bioremediation* (Weber & Legge, 2009).

Chapter 3 describes the development of a metric used to measure bacterial community divergence based on carbon source utilization patterns (CSUPs) gathered using BIOLOG^{TM} ECO plates via the CLPP method as described in Chapter 2. This chapter is currently in print in the *Journal of Microbiological Methods* (2009).

Chapter 4 summarizes a study describing the development of a technique for the detachment of viable bacteria from wetland mesocosm gravel media. This method was newly developed as

no previously published method for the detachment of viable bacteria from gravel or similar substrate was available in the literature.

Chapter 5 describes an investigation of the bacterial communities residing within both the interstitial water and within the mesocosm bed media of planted (*Phragmites australis*) and unplanted systems. Bacterial communities at various depths, associated with the plant roots and the bed media were characterized using the CLPP method to garner an understanding of spatial bacterial community dynamics in the CW mesocosm systems. Six mature mesocosms were sacrificed for the purpose of this investigation.

Chapter 6 describes the temporal bacterial community dynamics of 8 new mesocosm systems during the first 8 months following the start-up. The effect of initial microbial seeding population and plant presence on bacterial community development was studied by seeding the mesocosms with an initial bacterial inoculum from either A) the Waterloo wastewater treatment plant or B) a dairy farm wastewater holding tank. Half of the mesocosms were left unplanted with the remaining mesocosms planted with *Phragmites australis*.

Chapter 7 is a summary of the principle outcomes from the work presented. Future work and recommendations are also presented in this final chapter.

Appendix A summarizes the abbreviations and nomenclature used throughout the thesis.

Appendix B summarizes common tap characteristics for the region of Waterloo.

Appendix C is the contributor's agreement for Chapter 2.

Appendix D is the journal publishing agreement for Chapter 3.

Figure 1.5 recounts the research regime summarized in this thesis.

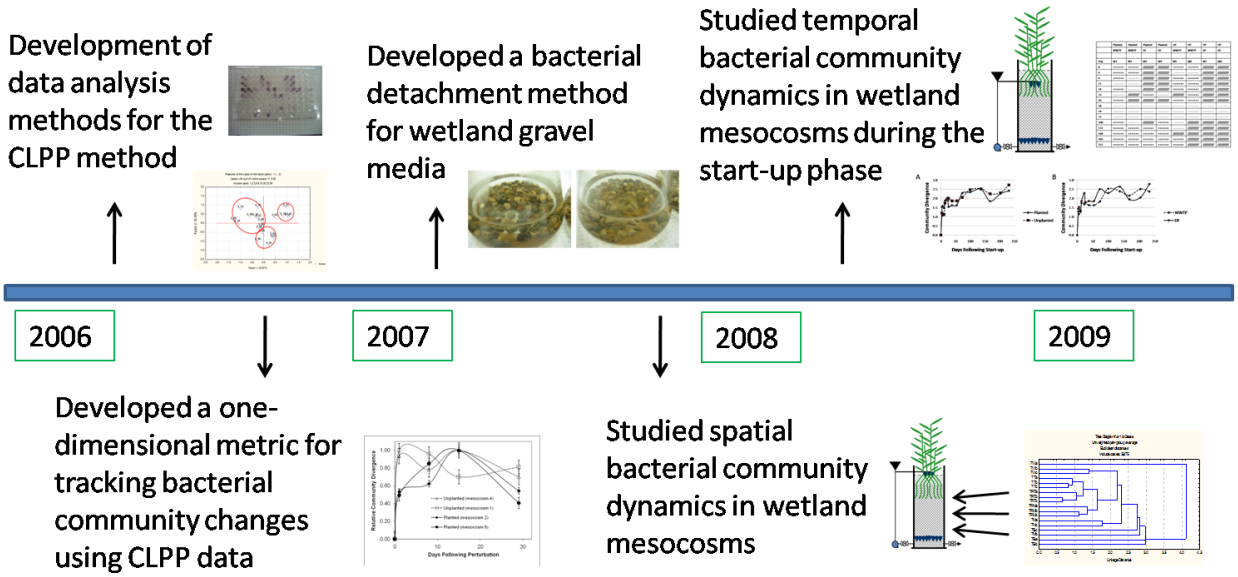

Figure 1.5: Research timeline.

Chapter 2: Community Level Physiological Profiling

Overview

Community level physiological profiling (CLPP) is a technique which offers an easily applied protocol yielding information regarding mixed microbial community function and functional adaptations over space and time. Different communities can be compared and classified based on sole carbon source utilization patterns (CSUPs) gathered using BIOLOG™ microplates. One of the most challenging aspects associated with the CLPP method is in the data analysis. This chapter describes the relatively simple CLPP laboratory protocol and provides a detailed description of different data analysis techniques. A quick reference guide to the method is provided at the end of the chapter.

Keywords: community level physiological profiling (CLPP), BIOLOG™, carbon source utilization pattern (CSUP), microbial community, microbial ecology, multivariate analysis, principle component analysis (PCA)

This invited chapter was coauthored with Dr. Raymond Legge and is currently in press as a contribution to "Methods in Molecular Biology - Remediation" (Cummings, S.P. ed), The Humana Press Inc., New Jersey. The *Methods in Molecular Biology* textbook series focuses on cataloging popular molecular biology protocols, with an emphasis on practical application through the incorporation of author *NOTES* into the final publications. See Appendix C for the contributor's agreement.

2.1 Introduction

The term community level physiological profiling (CLPP) was first coined by Lehman *et al.* (1995) to describe the characterization and classification of heterotrophic microbial communities based on sole carbon source utilization patterns (CSUPs). Although CLPP is considered a broad term which could cover many different types of studies undertaken using a number of different assays, currently the term CLPP is used almost exclusively in reference to data collected using BIOLOG™ microplates. BIOLOG™ microplates are 96 well plates where each well contains a different carbon source and a redox dye indicator, most often tetrazolium violet. When a mixed microbial community sample is inoculated into each of the wells the production of NADH via cell respiration reduces the tetrazolium dye to formazan, resulting in a colour change within each individual well, which can be detected photometrically.

There are a number of different microplates manufactured by BIOLOG™ for CLPP use with the three most popular being the GN2, GP2 and EcoPlates. The GN2 plate is the most recent version of the GN plate and is suitable for characterizing or identifying Gram-negative bacteria. The GP2 plate is the most recent version of the GP plate, and is suited to characterizing or identifying Gram-positive bacteria. The GN2 and GP2 plates both contain 95 different carbon sources with one of the 96 wells serving as a blank. Both the GN2 and the GP2 plates were originally developed for species identification (Insam, 1997) but are now commonly used for CLPP. The BIOLOG™ EcoPlate contains 31 different carbon sources and a blank in triplicate. Use of triplicates allows for increased confidence in statistical analysis of the resulting plate data. The EcoPlate was developed for environmental applications, which dictated the selection of carbon sources, with at least nine substrates considered constituents of plant root exudates (Campbell *et al*., 1997; Preston-Mafham *et al.,* 2002).

BIOLOG™ offers a number of other plates suitable for CLPP studies. SF-N and SF-P microplates are alternatives to the GN2 and GP2 plates, as they provide the same corresponding substrates,

but without the tetrazolium dye. Turbidity or a different metabolic indicator can be added to assess activity. MT microplates contain the same redox chemicals as the GN2 and GP2 plates but do not contain any substrates. These plates allow for the creation of customized plates by adding suitable substrates for specific ecological studies. FF plates, which have been recently introduced by BIOLOG™ for the study of fungi and yeasts, contain a unique set of carbon sources and use both turbidity and/or reduction of tetrazolium as activity indicators. The GN plate and its corresponding successor, GN2, have been favored in CLPP studies although other plates may offer greater relevance and analytical options (Preston-Mafham *et al*., 2002).

Garland and Mills (1991) were the first to use BIOLOG™ plates for characterizing heterotrophic soil bacterial communities and a number of studies have since followed [see Preston-Mafham *et al.* (2002), and Konopka *et al.* (1998) for examples]. The advantage of CLPP over both classic cell culturing and molecular level RNA/DNA amplification-based techniques is its relatively simple protocol and ease of use. Both classic cell culturing and molecular level RNA/DNA amplification-based techniques can be time consuming and require specialized expertise (Garland, 1997).

Limitations pertaining to the CLPP approach using BIOLOG^{TM} microplates have been discussed in the literature (Preston-Mafham *et al.,* 2002; Konopka *et al.,* 1998; Garland, 1997). Limitations and pitfalls pertaining to data analysis have also been recently described (Weber *et al.*, 2007). Some of the most pertinent limitations include the bias in the technique toward rapidly growing bacteria, the need to ensure similar inoculum sample sizes in the wells, the need to reduce time between sampling and inoculation of the microplates, and difficulties with meaningful data analysis and interpretation.

Some of the limitations surrounding the CLPP method pertain to the long incubation times, the indirect measurement of microbial activity, and the use of high substrate concentrations. The

use of lower substrate concentrations would allow for less selective enrichment within the wells as growth and incubation conditions would be more akin to those from which the sample originates. To allow for the use of decreased substrate concentrations, more sensitive and direct activity measurements would then be required. Newly developed CLPP methods include those of Degens *et al.* (2001) and Garland *et al.* (2003). Degens *et al.* (2001) developed a method for the detection of CO_2 generation from mixed microbial communities utilizing a range of carbon substrates. Although Degens *et al.* (2001) refer to their measurements as microbial catabolic diversity; the basis behind the study is similar to that of CLPP. Garland *et al.* (2003) developed a fluorescent-based method of measuring O_2 consumption for mixed microbial communities utilizing a range of carbon substrates. Using this method Garland *et al.* (2003) found that incubation times could be reduced to less than 24 hrs, and the substrate concentrations could be reduced by a factor of 10-100 when compared to BIOLOG™ plates. Currently the term CLPP almost exclusively refers to the use of BIOLOG™ microplates. Studies, such as those conducted by Garland *et al.* (2003) and Degens *et al.* (2001), point to the expanding usage of the term CLPP. Improvements to the CLPP method both with and without the use of BIOLOG™ plates will no doubt lead to the evolution and changing and/or broadening of the term CLPP and its associated methods in the future.

CLPP is a technique which offers an easily applied protocol yielding large amounts of information regarding mixed microbial community function and functional adaptations over space and time. Carrying out the CLPP laboratory protocol is a relatively simple process, which has lead to its recent increase in popularity. However, the data analysis aspect associated with CLPP can be challenging, often requiring a background in multivariate analysis methods. Following is a description of the CLPP laboratory protocol and an in depth description of the data analysis procedure.

2.2 Materials

1. Suspended mixed microbial community sample
2. BIOLOG™ microplate(s)
3. Microplate Reader equipped with a 590 nm filter

Optional:

1. Buffer solution – May be needed if a) performing a detachment protocol from a sediment sample to generate a suspended microbial community sample or b) performing serial dilutions before plate inoculation (see Section 2.3.2).
2. Incubator – May be needed if incubating samples at a temperature other than room temperature (see Section 2.3.3).

2.3 Methods

*2.3.*1 CLPP Protocol - General Description

Each well of the BIOLOG™ plate is inoculated with 150 µL of the sample of interest and incubated at temperatures generally ranging from 20-30°C. Absorbance readings (590-600 nm) are performed as necessary using a microplate reader over an incubation period ranging from 10-200 hrs. The sample should be a uniform suspension, so if sampling sediment or biofilm, an appropriate detachment and/or homogenization protocol is necessary.

*2.*3.2 Inoculation

150 µL of a suspended mixed microbial community sample is inoculated into each of the 96 wells of the BIOLOG™ microplate. An undiluted sample is recommended as dilution of samples containing a mixed population has been shown to affect the resulting CLPPs (Franklin *et al.,* 2001; Garland *et al.,* 2001). Analytical methods for dealing with small differences in inoculation densities are discussed later, however it is important to ensure similar cell densities of the

samples to reduce any error in the CLPP analysis (**see Note 1**). Although not recommended in all cases, if cell densities in the original samples are exceedingly high, serial dilutions may be needed before microplate inoculation. If the plates are inoculated at high cell densities, colour development may proceed at a rate where capturing meaningful data is difficult. It has been suggested that formazan production does not occur until cell densities between 10^5 - 10^8 cells/mL are reached (Konopka *et al.,* 1998; Garland *et al.,* 2001). Lastly, the time between sampling and inoculation should be kept to a minimum in order to reduce cell death or structural community changes of the sample prior to plate inoculation.

*2.*3.3 Incubation

Plates can be incubated over a range of temperatures with room temperature being the most common. Incubation periods tend to range between 10-200 hrs; and standard incubation temperatures between 20-30° C. Incubation temperatures similar to those from which the sample was collected are ideal. There is some debate surrounding the effect that incubation temperature has on the resulting CLPPs. Christian and Lind (2006) showed that temperature had an effect on the calculated CSUPs whereas Classen *et al.* (2003) showed CLPPs to be relatively insensitive to incubation temperature. Room temperature incubation has not been criticized in the literature, as the resulting CLPPs have proven useful and reasonable for most published studies.

The plates do not need to be agitated during incubation due to the relatively long incubation times. Stationary incubation at room temperature is the most common method, although shaking is required prior to plate reading to ensure uniform distribution of the formazan. Incubation periods will vary for different studies but generally range from 10 to 200 hrs depending on the study and the inoculation density. Choice of a specific incubation time is not obvious, and is largely dependent on the subsequent data analysis (see Section 4). Figure2.1 is a picture of a BIOLOG™ EcoPlate after a 20 hr incubation period following inoculation with interstitial water from a wetland mesocosm (Weber *et al.,* 2008).

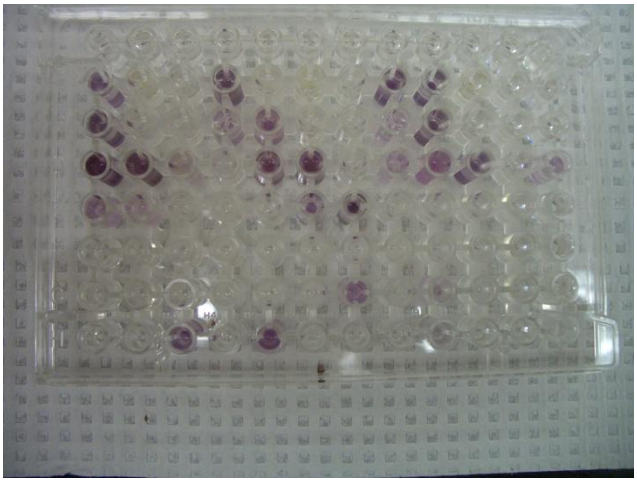

Figure 2.1: BIOLOG™ EcoPlate 20 hr after inoculation with interstitial water from a wetland mesocosm system. Plate used in the study of Weber *et al.* (2008).

*2.*3.4 Data Collection

Absorbance readings (590 nm) for all 96 wells are collected throughout the incubation period to reveal the kinetic profiles for each of the carbon sources. Reading frequency will vary based on the nature of the inoculum and the type of metric chosen for analysis (**see Note 2**), if data analysis does not require the fitting of kinetic profiles and a single time point is used for analysis (see Section 2.4), less frequent readings are reasonable. Plates should be agitated before each reading to ensure sufficient colour distribution in each well.

An example of the type of data that can be collected when following the described protocol is provided in Figure 2.2. Colour development for individual wells for a single set of EcoPlate replicates is presented (31 different carbon sources and 1 blank); colour development curves show a general sigmoidal shape.

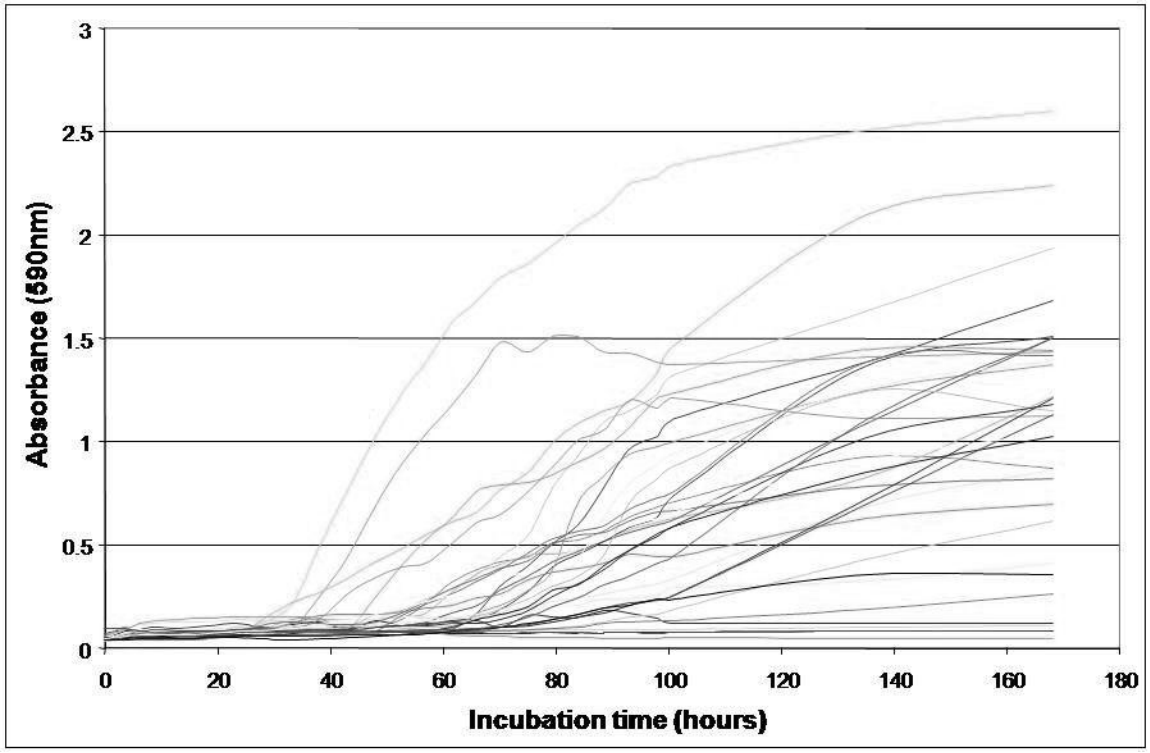

Figure 2.2: Individual well colour development curves for a single set of EcoPlate replicates (31 carbon sources and 1 blank). Data from Weber *et al.* (2008).

*2.*3.5 Anaerobic Protocol

A small number of earlier studies indicated that formazan is not produced in regular aerobic-usage BIOLOG™ plates incubated under anaerobic conditions (Preston-Mafham *et al.,* 2002; Winding and Henrikson, 1997). However, a number of recent studies have shown formazan production does occur under anaerobic conditions (Christian and Lind, 2006; Mills and Garland, 2002; Beaumont, 2007). BIOLOG™ manufactures an AN microplate for identification of anaerobic bacteria although a number of anaerobic CLPP studies have favoured the use of GN or ECO microplates as these plates have been previously shown to be suitable for ecological and exploratory CLPP studies for mixed microbial systems. The anaerobic CLPP protocol is identical to the aerobic protocol described here with a few changes focusing on minimizing the exposure to oxygen throughout the procedure (**see Note 3**). This can be accomplished through any number of standard anaerobic culturing and testing techniques.

2.4 Data Analysis

A large amount of data can be collected with a single BIOLOG™ plate; when numerous plates are used the amount of data can then become overwhelming. To deal with the large amount of data and large number of variables to be processed, some form of multivariate analysis is required. Data analysis will be largely discussed with direction towards the use of principle component analysis (PCA) as this is the most commonly and easily used method for analyzing and visualizing the CLPP data. A number of other techniques will also be discussed later.

*2.*4.1 Standard Analysis Method when using Principle Component Analysis

Before reaching the point of performing a multivariate analysis technique (such as PCA), a number of steps should be followed:

1. Decide what metric will be used for the data analysis
2. Standardization of the data
3. Assess heterogeneity, normality, and the underlying factor structure of the data
4. Perform a data transformation if required

2.4.1.1 Selecting a Metric

First a metric needs to be selected and extracted from the BIOLOG™ plate data to represent activity in each well. The 3 most commonly utilized metrics are:

A. An absorbance value for each well for a specific incubation time point
B. An absorbance value for each well taken from a time point representing a specific average well colour development (AWCD) for that plate
C. Some type of logistic curve fitting value such as lag, maximum utilization rate (slope), area under the curve, or an asymptote value

2.4.1.1.1 Selecting a Specific Incubation Time-Point

To evaluate all plate data within a study, a specific incubation time point can be chosen as a metric, but choosing this time point may not be obvious. An increase in the differences (or variation) between well absorbance values indicates an increase in the amount of information contained within the data set. Using absorbance values taken early in the incubation time would yield little information, for at early stages of growth (for the example 10 hrs in Figure 2.2) the difference between well absorbance values is too small to yield useful information. Using absorbance values taken later during the incubation can provide more information regarding the CLPP of the microbial inoculum as long as the values are not above a value of 2. As seen in Figure 2 there is an increase in the dispersion of (or differences between) well absorbance values as the incubation proceeds. This dispersion of well absorbance values can be represented as the standard deviation calculated at each time point (Table 2.1).

Table 2.1: Calculated AWCD, number of values above an absorbance of 2, and standard deviations for absorbance values over the 168 hr incubation time for the plate shown in Figure 2. Data from Weber *et al*. (2008).

Incubation time (hrs)	AWCD	# values above absorbance of 2	Standard Deviation
0	0.00	0	0.01
4	0.01	0	0.02
6	0.01	0	0.02
9	0.01	0	0.02
13	0.01	0	0.02
18	0.00	0	0.02
24	0.00	0	0.02
28	0.01	0	0.02
34	0.02	0	0.04
38	0.04	0	0.09
43	0.06	0	0.14
48	0.09	0	0.20
53	0.12	0	0.25
57	0.16	0	0.29
62	0.19	0	0.33
66	0.24	0	0.37
70	0.27	0	0.40
75	0.31	0	0.40
80	0.37	0	0.43
84	0.42	0	0.45
89	0.47	1	0.46
93	0.53	1	0.49
98	0.57	1	0.50
101	0.60	1	0.52
135	0.85	2	0.59
168	0.99	2	0.63

Absorbance readings above 2 contribute to measurement error as they are outside the linear absorbance range. An appropriate time point will be the time point that preserves the greatest variance between well responses while retaining the maximum number of wells within the linear absorbance range. For example, for the data in Table 2.1, Weber *et al.* (2008) chose to use absorbance data from the 84 hr time point for subsequent multivariate analysis (**see Note 4**). This study included a number of BIOLOG™ plates comparing a number of different microbial samples. The data from all plates was considered before a time point was chosen. When using this simplified method of choosing a specific time point for all analyses, similar inoculation densities for all plates is essential (Garland *et al.,* 2001).

2.4.1.1.2 Selecting a Time-Point Related to a Specific AWCD Reference Value

As recommended by Garland *et al.* (2001) a specific AWCD value can also be chosen as a reference point for all plates analyzed. Absorbance values used for subsequent multivariate analysis are extracted from each set of plate data at the specific time point where the reference AWCD occurs. Garland (1997) showed that using AWCD reference values between 0.25-1.0 yields relatively similar CLPPs for use in community classification (**see Note 5**).

2.4.1.1.3 Kinetic Analysis

Kinetic analysis can also be performed on the well colour development data (for examples see Preston-Mafham *et al.,* 2002; Garland *et al.,* 2001; Haack *et al.,* 1995; Mondini and Insam, 2003). A number of different metrics can be chosen for use in multivariate analysis. Factors such as lag time, maximum utilization rate (slope), area under the curve, or asymptote values have been used. A large amount of data needs to be acquired for a logistic curve to fit the data, and not all data are suited to a logistic fit. Deviation in absorbance readings and non-characteristic responses in some wells can have a large effect on curve fitting making some data unfit for kinetic analysis.

Kinetic approaches have great potential, as a more detailed understanding of the nature of the color responses can be theoretically attained (Preston-Mafham *et al.,* 2002; Garland *et al.,* 2001). However, a general lack of understanding regarding physiological or ecological bases for differences in the derived kinetic parameters limits the amount of information that can be extracted when using a kinetic approach (Garland *et al.,* 2001). If the objective is to classify different microbial populations, using data from a single absorbance point reading may be more useful. Garland *et al.* (2001) found that using a single absorbance point reading corrected by the AWCD was more successful than using kinetic parameters for classifying different soil bacteria populations, and remarked that the use of kinetic parameters for CLPP may provide some additional information, but only if the influence of inoculum density is carefully considered (**see Note 6**).

The overall incubation time for any one study is dependent upon what type of metric will be used and cannot be easily determined. It is preferable to run a number of test plates inoculated with microbial community samples similar to those to be characterized in the overall study before starting an experimental regime. Using these test plates, careful determination of which type of metric will be used and the resulting incubation time can be assessed.

2.4.1.2 Standardization of the Data

When performing a CLPP analysis numerous plates are often used to study different mixed microbial communities in space or over time. As recommended by Garland (1997) if the choice of metric is to use a single time point absorbance, and not perform a kinetic analysis of the data, an initial standardization of the data helps to reduce any bias due to inoculum density differences between samples. Standardization of the data involves correcting each absorbance value by its corresponding blank and then dividing by the AWCD for that time point. The standardized absorbance for well *k* can be calculated as:

$$\overline{A}_k = \frac{A_k - A_0}{\frac{1}{31}\sum_{i=1}^{31}\left(A_i - A_0\right)} \qquad (2.1)$$

where A_i represents the absorbance reading of well *i* and A_0 is the absorbance reading of the blank well (inoculated, but without a carbon source). Where there is very little response in a well, negative values of standardized absorbance may occur and, since this is physically meaningless, they are coded as zeros for further analysis. Standardization of the data may not be needed when performing certain types of kinetic analyses.

2.4.1.3 Assess the Suitability of the Data Set for Multivariate Analysis

Efficient and meaningful statistical methods for dealing with a large number of interdependently correlated variables is needed when evaluating CSUPs from BIOLOG™ plates; most researchers have turned to some form of multivariate technique and, in particular, principal component analysis (PCA) (Glimm *et al.,* 1997). In performing PCA, each plate (p number of plates) is considered an object, with n variables (31 for EcoPlates, 95 for GN2 or GP2 plates) giving a matrix with p rows and n columns. The transformation of BIOLOG™ plate data is an important aspect of multivariate analysis techniques such as PCA. Weber *et al.* (2007) provide an in depth study on data preparation techniques briefly described in the following section.

Many multivariate analysis techniques assume two fundamental properties of a data set: normality and homoscedasticity (that is, homogeneity of variance: all variables are assumed to have the same variance). In PCA, the dimensionality of the data set is reduced by extracting an orthogonal set of principal components (PCs) made up of linear subsets of the original ordinates; the extraction is designed so that the maximum amount of variance is concentrated in the first PC, with the second largest amount of variance contained in the second PC, and so on. This analysis technique is most powerful if the data have an underlying factor structure; that is, it is dependent on linear correlations between the different variables (Legendre and Legendre, 1998). Weber *et al.* (2007) concluded that if homoscedasticity, normality and the number of linear correlations within a data set are not evaluated and the possibility of transforming the data is not considered, erroneous analysis and misleading conclusions may arise when performing multivariate analysis on microplate data (**see Note 7**). Following is a short summary of useful data transformation techniques and data assessment methods used when working with BIOLOG™ microplate data.

2.4.1.3.1 Normality

Normality of BIOLOG™ microplate data can be evaluated through formal statistical tests. The kurtosis and skewness of each variable can be calculated and the standard errors found according to Equations (2.2) and (2.3) for kurtosis and skewness, respectively:

$$SE_{kurtosis} = \sqrt{\frac{24}{n}} \tag{2.2}$$

$$SE_{skewness} = \sqrt{\frac{6}{n}} \tag{2.3}$$

where SE_{kurtosis} and SE_{skewness} are the standard errors for kurtosis and skewness, respectively, and n is the number of observations. The corresponding z values can also be calculated as:

$$z_{kurtosis} = \frac{kurtosis}{SE_{kurtosis}} \tag{2.4}$$

$$z_{skewness} = \frac{skewness}{SE_{skewness}} \tag{2.5}$$

Either z value may be used in a formal statistical test of a null hypothesis that the data is normally distributed versus an alternative that it is not. A two-tailed test is used and the null hypothesis rejected with 95% confidence if $|z| > 1.96$.

Normality according to Weber *et al.* (2007) can be assessed using both kurtosis and skewness, by calculating the mean value of the statistics across all variables as well as testing the

individual variables. Note that the 95% significance level applies to the individual tests and not to the global set of tests of all variables; therefore, the number of significant results is considered to be indicative of the number of significantly non-normal variables tested. For a more detailed example see Weber *et al.* (2007).

2.4.1.3.2 Homoscedasticity

Homoscedasticity is perhaps best assessed by a scatter plot of pairs of variables (a characteristic oval appearance will result for homoscedastic pairs); however, this is not feasible for so many variables and instead a variance ratio can be calculated:

$$\text{variance ratio} = \frac{\text{highest variance}}{\text{lowest variance}} \tag{2.6}$$

This is adapted from the concept that a lesser degree of variation in separate variances contributed by many variables will constitute a lower ratio between the highest variance of any one variable and the lowest variance of any one variable in that data set (Lo and MacKinlay, 1998). This cannot be tested formally and should only be considered indicative of the relative homogeneity of variance between data sets (lower values being relatively more homogeneous).

2.4.1.3.3 Underlying Factor Structure - Linear Correlations

The number of linearly correlated variables within a data set can be calculated by obtaining the correlation matrix and counting the number of correlation coefficients greater than Pearson's critical *r* value for the specified number of observations. This corresponds to a pairwise formal test of the null hypothesis of no correlation between variables versus an alternative of (positive or negative) correlation at a 95% confidence level. Again, in making multiple comparisons the global confidence level of the test (over all pairs) is lower than the nominal pairwise level but

the number of significant results can, for the sake of this assessment, be interpreted as an indicator of the suitability of the data for PCA. If a transformation significantly reduces the number of linear correlations between variables, then it can be suspected that this may cause a problem in subsequent analysis.

2.4.1.3.4 Perform a Data Transformation if Required

As presented in Weber *et al.* (2007) two transformations commonly employed in ecological data analysis can also be used for BIOLOG™ microplate data: the Taylor power law transformation and the logarithmic transformation. The Taylor transformation (Taylor, 1961) is commonly used to stabilize variances and make data conform to the assumptions of parametric analysis such as normality (Legendre and Legendre, 1998). It is based upon the assumption that:

$$S^2 = a\bar{y}^2 \tag{2.7}$$

where, *S* is the standard deviation of a sample variable, $\bar{y}$ is the mean of a sample variable and *a* is the sampling factor. This leads to:

$$\log S^2 = \log a + b \log \bar{y}^2 \tag{2.8}$$

where the slope, *b*, may be obtained by linear regression of the data for all variables. This leads to the conditional transformation:

$$y_i' = y_i^{(1-b/2)} \qquad \text{for } b \neq 2 \tag{2.9}$$

or,

$$y_i' = \ln(y_i) \qquad \text{for } b = 2 \tag{2.10}$$

where y_i' is the value of the transformed variable.

A logarithmic transformation can also often serve to normalize skewed data (Legendre and Legendre, 1998). A common logarithmic transformation used in ecological data analysis is of the form:

$$A' = \ln(\overline{A}_k + 1) \tag{2.11}$$

where A' is the value of the transformed variable.

These two simple data transformation examples are given to provide a starting point for utilizing transformations when performing multivariate data analysis of microplate data. Many other data transformations exist and may be more suitable for specific data sets. See Legendre and Legendre (1998), and Montgomery (2001) for detailed discussions regarding different data transformation techniques.

2.4.1.4 Perform PCA on the Data Set

Principle component analysis (PCA) is the most commonly employed multivariate analysis technique when working with BIOLOG™ microplate data. PCA is based on an eigenanalysis of an R-mode (between variables) variance-covariance matrix (Legendre and Legendre, 1998). In short, PCA is able to take a high dimensional space (32 dimensions in this case) and ordinate samples (objects) on a two dimensional plane while preserving the maximum allowable amount of variance within the data set. PCA is most commonly used to visualize data plotted on the first two principle component (eigenvector) axes for interpretation. Common uses include the study of ecological shifts over time and space (Legendre and Legendre, 1998). PCA analysis can preserve varying degrees of the original variance within the first two axes; values from 40% to 80% are commonly achieved. PCA ordinations allow the CSUPs from the bacterial community samples to be grouped and differentiated. PCs are most commonly extracted from the covariance matrix of the data. Use of the covariance matrix preserves scale.

PCA has been widely adopted for analyzing CLPPs based on CSUPs generated using BIOLOG™ microplates. As outlined in previous sections, attention needs to be paid to the distribution of the underlying variables and the possibility of applying a transformation to the data to improve the analysis. One of the significant advantages of PCA is that it is robust and analyses remain valid even if the assumptions of normality and homoscedasticity are not met; however, the analysis can be improved if the data can be transformed to meet these assumptions. Recent CLPP example studies utilizing PCA analysis of BIOLOG™ microplate data include Weber *et al.* (2008), He *et al*. (2008), and Farnet *et al.* (2008).

Attached at the end of this chapter is a “Quick Reference Guide”, which attempts to briefly summarize the steps in the CLPP protocol within a single page for laboratory use.

2.4.3 Other Analysis Methods

In addition to PCA a number of other analysis methods have been successfully utilized in garnering information from BIOLOG™ plates. Some of the more common methods include:

1) Clustering Analysis

2) Diversity Indices – substrate diversity, substrate richness, substrate evenness

3) Alternative Methods - factor analysis, PCoA, DCA, NMDS, DA, CCorA, RDA

2.4.2.1 Clustering Analysis

As recommended by Legendre and Legendre (1998), clustering analysis is often performed to verify and validate results obtained using PCA. Clustering analysis allows for the CSUP similarities to be visualized in a dendogram for any given number of plates. An unweighted pair-group method using arithmetic averages (UPGMA) clustering analysis is often recommended. See Weber *et al*. (2008), He *et al.* (2008), and De Paolis and Lippi (2008) for recent examples of clustering analysis using CSUPs from BIOLOG™ microplates.

2.4.2.2 Substrate-Related Diversity Indices

BIOLOG™ plates have also been used, in a more traditional ecological sense, to calculate diversity indices based on CSUPs (Zak *et al.,* 1994). The Shannon index or what is often called "diversity" is a common ecological metric used to track and understand shifts in communities over space and time. Using the CSUP gathered from a single BIOLOG™ plate, substrate diversity (H) can be calculated as:

$$H = -\sum p_i \ln(p_i) \qquad (2.12)$$

where:

H - substrate diversity

p_i - ratio of the activity of a particular substrate to the sums of activities of all substrates

activity - chosen metric for analysis (absorbance value, kinetic parameter, etc.)

Two other parameters associated with substrate diversity which can be calculated using CSUPs are substrate richness (S) and substrate evenness (E). Substrate richness is a measure of the number of different substrates utilized by a microbial population. Substrate evenness is defined as the equitability of activities across all utilized substrates; substrate richness is calculated as the number of wells with a corrected absorbance greater than 0.25. Substrate evenness is calculated as:

$$E = H / H_{\max} \tag{2.13}$$

Recent examples of studies utilizing the Shannon index include Weber et al. (2008), He *et al.* (2008), and Farnet *et al.* (2008).

2.4.2.3Multivariate Analysis Methods

Although PCA is the most popular approach, with proper data treatment essentially any multivariate analysis technique can be used to analyze the data matrix attained when applying the CLPP protocol to any number of mixed microbial community samples. A short list of reference studies utilizing some of the less popular and/or more recently introduced multivariate methods either examining or relating BIOLOG™ data to other data sets include:

factor analysis (Nikilinska *et al.,* 2005), principle coordinates analysis (PCoA) (Hackett and Griffiths, 1997), detrended correspondence analysis (DCA) (Houlden *et al.,* 2008; Garland, 1996), non-metric dimensional scaling (NMDS) (Classen *et al.,* 2003), discriminant analysis (DA) (Mondini and Insam, 2003), canonical correlations analysis (CCorA) (Leriche *et al.,* 2004), and redundancy analysis (RDA) (Farnet *et al.,* 2008). For an in-depth description of the mentioned multivariate methods, see Legendre and Legendre (1998).

2.5 Notes

1. Using a minimum inoculation density of 10^5 cells/mL is the best way to reduce lag times although smaller inoculation densities can be used. True cell densities can be difficult to determine, therefore an alternative inoculation approach is to dilute the sample to an optical density if ~0.2 at 420 nm for the suspended mixture. This inoculation approach may lead to inoculation density differences, but from a practical perspective is more easily controlled and implemented.

2. Reading frequencies can vary quite widely. For the data seen in Figure 2, a reading frequency of 4 hours was used. This reading frequency provided enough data to decipher the sigmoidal shape of the colour development curves. However, if a larger inoculum density was used a faster response would be observed, and therefore more frequent readings would be required in order to properly decipher and/or model the sigmoidal shape of the colour development curves. Preliminary trial runs using inoculum densities and bacterial communities similar to one's study samples is always a good idea. They can help one determine inoculum dilutions, reading frequencies and metric choices which are essential in gathering meaningful data.

3. Following anaerobic inoculation, plates can be covered with non-slit silicon plate seal, or simply sealed around the edges with a generous amount of parafilm and masking tape. Both procedures have been proven effective. Microplate absorbance readings are then periodically

taken without removing the plate lids. In the author's experience, overall profiles and readings have been shown to not be significantly affected by leaving the lid on during plate readings. Some plate readers require that the lids to be removed before absorbance readings can be taken. These plate readers unless equipped with or situated in a nitrogen purging area would not be suitable for anaerobic samples.

4. Each metric contains associated positives and negatives. Use of a single time point reading often guides the user towards using data points in an area where almost all carbon source utilization curves are in a stationary (steady-state) phase. This can be useful when comparing plates over extended time-periods as the basis for comparison is relatively stable. However, in interpreting this type of data, one should be aware that the activity levels of the community on specific carbon sources is not emphasized due to the carbon utilization curves being in the stationary phase.

5. When choosing to use a reference AWCD one should be aware that the carbon utilization curves can be in the lag, exponential growth, or the stationary phase. Therefore this method, although based on a fixed reference point, may not give stable comparison results over an extended time period for community monitoring studies. However, in comparison to a fixed time point, this method does emphasize activity in each well which may be of interest to the user. It should also be mentioned that using an AWCD reference point may not be appropriate in studies where some plates contain a large number of unresponsive wells.

6. Kinetic analysis allows one to compare many different aspects of the carbon utilization curves, allowing the user to tune the analysis to emphasize a specific aspect of the community. However, in modeling the data sometimes poor-fit can occur, and in many instances kinetic analysis is not feasible due to time or instrument constraints.

7. The authors have found that with larger data sets (100+ objects) normality, homoscedasticity and the underlying factor structure of the data do not have as large an effect on PCA results and subsequent data interpretation when compared to smaller data sets. However, it should be emphasized that assessing the data set for normality, homoscedasticity and the underlying factor structure, and considering an initial data treatment are necessary steps in the data analysis procedure. If a large difference between PCA results from transformed and untransformed data is not observed the data set was likely already suited for PCA or was of a size where a data transformation did not have a large effect on the PCA results.

CLPP Protocol - Quick Reference Guide

I - Inoculation

→ 150uL of a suspended mixed microbial sample into each of the 96 wells

NOTES: Time between sampling and inoculation should be minimized. Keep inoculation densities similar between plates.

II - Incubation

→ Incubate at room temperature on the desktop

NOTES: Incubation period selected based on type of metric to be used for data analysis (Step IV-A-1). Common incubation times between 10-200 hrs. Common incubation temperatures between 20-30° C.

III - Data Collection

→ Periodic absorbance (590nm) readings taken for all wells during incubation period

NOTES: Plates should be shaken before each reading.

IV - Data Analysis

A) PCA Analysis

1) Choose a metric
    i) An absorbance value for each well from a specific incubation time point
    ii) An absorbance value for each well taken from a time point representing a specific AWCD for that plate
    iii) Some type of logistic curve fitting value such as lag, slope, area under the curve, or an asymptote value.
2) Standardization of data if not performing a kinetic analysis
3) Check data set for
    i) Normality
    ii) Homoscedasticity
    iii) Underlying factor structure - liner correlations
4) Perform a data transformation if required
5) PCA Analysis

B) Other Analysis Methods

1) Clustering Analysis
2) Diversity Indices – substrate diversity, substrate richness, substrate evenness
3) Alternative Methods - factor analysis, PCoA, DCA, NMDS, DA, CCorA, RDA

Chapter 3: One-Dimensional Metric for Tracking Bacterial Community Divergence using Sole Carbon Source Utilization Patterns

Overview

Community level physiological profiling (CLPP) has become a popular method to characterize and track changes in heterotrophic bacterial communities. Although the CLPP method is a straight forward laboratory protocol which yields large amounts of functional information regarding bacterial communities, due to the large amount of data attained, some type of multivariate analysis method is required to allow ordination and interpretation of the data. Multivariate analysis can be challenging as it requires a significant statistics background along with an understanding of the inferences and biases each multivariate analysis method incurs. This paper presents and evaluates a new approach to analyzing sole carbon source utilization data. A method is described which provides a one-dimensional metric derived from standard CLPP data (Biolog™ EcoPlate data). The one-dimensional community metric was developed using normalized Euclidean distances and is compared against 1) PCA results, and 2) shifts in the carbon source guild grouping utilizations. The one-dimensional community metric did not provide all of the information of PCA or guild grouping analysis; however, it was found to be more easily implemented and interpreted when analyzing the plate data. Validation of this approach is demonstrated using data acquired to track the divergence of bacterial communities in wetland mesocosm systems after an experimentally controlled disturbance. If the objective is to investigate community shifts over time the one-dimensional community divergence metric can be a useful tool.

Keywords: Euclidean distance; mesocosm; *Phragmites australis;* community level physiological profiling (CLPP); principle component analysis (PCA), BIOLOG™ EcoPlate

This chapter was coauthored with Dr. Raymond Legge and is currently in press for the *Journal of Microbiological Methods* (2009). See Appendix D for the journal publishing agreement.

3.1 Introduction

Full characterization of microbial communities requires complete identification and enumeration of all organisms. Although identification can be useful it can also be exceedingly time intensive and in many cases yield information that, from an engineering context, may not be very useful. If one is looking only to extract an indicator of the metabolic characteristics and overall stability of a specific microbial community, community level physiological profiling (CLPP) may be attractive. Recent studies on microbial soil ecology have employed BIOLOG™ plates as a CLPP tool. Garland and Mills (1991) were the first to use BIOLOG™ plates for characterizing heterotrophic soil bacteria communities through principle component analysis (PCA). Zak *et al.* (1994) suggested simplifying CLPP data by grouping the different carbon sources into a smaller number of guilds (groupings). Both analysis methods reduce the dimensionality of the data for subsequent analysis and interpretation. Recent work has continued to utilize these analysis methods. Weber *et al*. (2008) successfully used this approach to profile constructed wetland mesocosms.

Methods are well developed for measuring aggregate community variates such as total biomass or total density (Collins *et al.*, 2000); however, more information is often needed to fully understand bacterial community dynamics. Community composition, and possibly ecological processes, may be altered without detectable changes in aggregate community variates (Collins *et al*., 2000; Frost *et al*., 1995; Micheli *et al.*, 1999). Different bacterial species will respond to disturbances in different ways and to varying degrees of success based on the metabolic and physiological characteristics of that species (Nester *et al*., 1983). It follows that in situations where perturbations occur, the original community structure can influence the magnitude and direction of the resulting shift. For example, the way in which wetland bacterial communities respond to perturbations can affect water treatment performance and long term ecosystem health. Large performance variations exist for similarly designed wetland treatment systems (Kadlec & Knight, 1996). Information regarding both long term and short term bacterial

community stabilities could help in understanding and predicting some of the these large performance variations.

Community variations can occur in many different ways. Variations can be cyclic, directional, stochastic, or chaotic (Collins *et al*., 2000). Understanding the type of variation occurring in a community can be useful in determining the underlying mechanisms involved in generating or alleviating the variation. A simple metric that would provide an indication of how similar or dissimilar a community is to its original state would be useful for understanding and analyzing the effect of perturbations on bacterial communities. One such method, first proposed by Collins *et al*. (2000), is to plot a similarity (or distance) measure over time to understand how the community is changing. The Euclidean distance between different communities can be calculated using species abundance data. In the same fashion Euclidean distances between carbon source utilization patterns for different bacterial communities can be calculated. Plotting a Euclidean distance measure with respect to the original community composition over time could give pertinent information regarding both bacterial community dynamics and robustness. Although this approach seems relatively straight forward it has not been attempted using carbon source utilization data.

The purpose of this study was to assess a proposed one-dimensional community divergence metric for analyzing standard CLPP data. Wetland mesocosm interstitial water was characterized using BIOLOG™ EcoPlates during a recovery period following an acid mine drainage (AMD) perturbation. The community divergence metric was compared to PCA and a guild grouping analysis methods. All three methods were employed using the carbon source utilization data to understand both overall community similarities and community shifts for planted and unplanted mesocosms 29 days following an AMD perturbation. Differences in the perturbation response patterns for each of the three different divergence tracking methods and the observed differences between planted and unplanted mesocosm bacterial communities is discussed.

3.2 Materials and Methods

3.2.1 Constructed Wetland Mesocosms – Experimental Regime

The experimental regime described here closely followed that described in Weber *et al.* (2008). The experimental set-up and operation of the mesocosms was as previously described (Weber *et al.* (2008); Werker *et al.* (2004, 2007)). The interstitial water of seven constructed wetland mesocosms was characterized using BIOLOG™ EcoPlates prior to treatment with simulated acid mine drainage water (day 0). After this initial characterization an AMD treatment consisting of draining and filling all 5 mesocosms with simulated AMD of pH of 3, [Fe^{+2}] of 100 mg/L and a [SO_4^{-2}] of 350 mg/L was implemented. Following AMD treatment the interstitial water was circulated for 24 h, after which all mesocosms were again characterized (day 1). Water was circulated with periodic topping-up to maintain a constant volume for a 4 week period. The mesocosms were sampled on days 8, 15 and 29.

Five mesocosms in total were studied: two mesocosms (2 and 6), were planted with *Phragmites australis* obtained from a local marsh and two mesocosms were unplanted (1 and 4). These four, unplanted and planted, mesocosms were seeded initially with activated sludge from a local municipal sewage treatment plant 3 years prior to the beginning of this study. Each of four six mesocosms contained a biotic regime associated with the pea gravel substrate. The seventh mesocosm (8) was kept abiotic by regular treatment with 0.0525 % w/v sodium hypochlorite, and subsequent flushing with 0.5 % w/v $Na_2S_2O_3$.

3.2.2 BIOLOG™ EcoPlates

BIOLOG™ EcoPlates (Biolog Inc., Hayward CA., USA) consisting of 96 wells, with 31 different carbon sources and a blank in triplicate, were used for CLPP analysis. The assay was conducted by adding 150 μL of interstitial water to each well followed by incubation under aerobic conditions at 25 °C. Absorbance readings were taken at 590 nm with a plate reader (Multiscan

Ascent, Labsystems) every 4 h for 168 h. As previously described (Weber *et al.,* 2008; Weber & Legge, 2009), a single time point at 84 h was used for well comparisons.

3.2.3 BIOLOG™ EcoPlate Data Preparation and Principle Component Analysis

The transformation of the BIOLOG™ EcoPlate data is an important aspect of multivariate analysis techniques such as principle component analysis (PCA). Weber *et al.* (2007) describes the data preparation technique employed in this study and the approach for PCA. In total, data for 27 plates was collected. All five mesocosms (1, 2, 4, 6 and 8) were characterized on days 0, 1, 8, 15 and 29.

Two different PCA ordinations were created for this investigation. The first ordination examined the data for day 0 (before the AMD perturbation) and day 1 (after the AMD perturbation). As only 5 mesocosms were characterized on day 1, only the data for 5 mesocosms (1, 2, 4, 6 and 8) was used for either day giving a total of 10 plates. Each plate contains 3 internal triplicates giving a total of 30 objects. Objects were then ordinated on a factor plane consisting of the first 2 extracted principle components following a PCA of the logarithmic transformed data. A second ordination involving the data from day 0 and 29 was also investigated. The data for 5 mesocosms (1, 2, 4, 6 and 8) was again used for either day giving a total of 10 plates and 30 objects. PCA of the Taylor transformed (b=0.9441) data was performed.

3.2.4 Guild Grouping

As described by Zak *et al.* (1994) BIOLOG™ EcoPlate data can also be analyzed by first grouping the different carbon sources into groups or "guilds". Zak *et al.* (1994) proposed organizing data from a 96 well (95 carbon source) GN plate into 6 guilds: 1) carbohydrates, 2) carboxylic acids, 3) amino acids, 4) amines and amides, 5) polymers and 6) miscellaneous. In this study the 31 carbon sources were organized into groups 1-5 as described by Zak *et al.* (1994). Carbon

sources originally grouped as miscellaneous by Zak *et al.* (1994), or those new to the BIOLOG™ EcoPlate, were grouped into one of the other five categories (Table 3.1).

Grouping the data into 5 guilds compresses a 31 dimensional data set into 5 dimensions, significantly reducing the complexity of the data and subsequent interpretation. To interpret any community shifts occurring in the mesocosms due to the AMD perturbation, the carbon source utilization % for each guild from each plate was plotted over the 29 d study. Observing a shift in the % utilization in the guild groupings signifies a functional shift in the mesocosm's bacterial community.

Table 3.1: BIOLOG™ EcoPlate carbon source guild groupings

Well No.	ID	C-Source	Grouping (Guild) Classification
Well1	C0	Water (Blank)	
Well2	C1	Pyruvic Acid Methyl Ester	*Carbohydrate
Well3	C2	Tween 40	Polymers
Well4	C3	Tween 80	Polymers
Well5	C4	Alpha-Cyclodextrin	Polymers
Well6	C5	Glycogen	Polymers
Well7	C6	D-Cellobiose	Carbohydrates
Well8	C7	Alpha-D-Lactose	Carbohydrates
Well9	C8	Beta-Methyl-D-Glucoside	Carbohydrates
Well10	C9	D-Xylose	Carbohydrates
Well11	C10	i-Erythritol	Carbohydrates
Well12	C11	D-Mannitol	Carbohydrates
Well13	C12	N-Acetyl-D-Glucosamine	Carbohydrates
Well14	C13	D-Glucosaminic Acid	Carboxylic & Acetic Acids
Well15	C14	Glucose-1-Phosphate	*Carbohydrate
Well16	C15	D,L-alpha-Glycerol Phosphate	*Carbohydrate
Well17	C16	D-Galactonic Acid-Gamma-Lactone	Carboxylic & Acetic Acids
Well18	C17	D-Galacturonic Acid	Carboxylic & Acetic Acids
Well19	C18	2-Hydroxy Benzoic Acid	Carboxylic & Acetic Acids
Well20	C19	4-Hydroxy Benzoic Acid	Carboxylic & Acetic Acids
Well21	C20	Gamma-Hydroxybutyric Acid	Carboxylic & Acetic Acids
Well22	C21	Itaconic Acid	Carboxylic & Acetic Acids
Well23	C22	Alpha-Ketobutyric Acid	Carboxylic & Acetic Acids
Well24	C23	D-Malic Acid	Carboxylic & Acetic Acids
Well25	C24	L-Arginine	Amino acids
Well26	C25	L-Asparagine	Amino acids
Well27	C26	L-Phenylalanine	Amino acids
Well28	C27	L-Serine	Amino acids
Well29	C28	L-Threonine	Amino acids
Well30	C29	Glycyl-L-Glutamic Acid	Amino acids
Well31	C30	Phenylethylamine	Amines/Amides
Well32	C31	Putrescine	Amines/Amides

*Addition or change from original groupings by Zak *et al.* (1994)

3.2.5 Dissimilarity Measure

The objective of the approach being proposed here is to decrease the complexity of the CLPP data set and provide a simple, single one-dimensional metric for comparative purposes. Such a metric would reduce the need for expertise in complex multivariate analysis methods and perhaps be more useful for engineering design, performance or modelling applications.

The approach adopted is similar to Collins *et al.* (2000) where a dissimilarity measure was plotted against a time lag. Collins *et al.* (2000) recommended using the Euclidean distance for community data due to the clear geometric properties of the metric. The Euclidean distance measure was used in this study as a measure of dissimilarity of the bacterial communities within a specific mesocosm over the 29 d time period following AMD perturbation. The Euclidean distance can be calculated in n dimensions, where in theis study n=31 (31 differnet carbon source utilization responses). Given the two points:

$$P = (p_1, p_2, ..., p_n) \tag{3.1}$$

$$Q = (q_1, q_2, ..., q_n) \tag{3.2}$$

The Euclidean distance can be calculated as:

$$\sqrt{(p_1 - q_1)^2 + (p_2 - q_2)^2 + ... + (p_n - q_n)^2} \tag{3.3}$$

The approach taken here is different to that of Collins *et al.* (2000) in that carbon source utilization data for bacterial communities was used as opposed to direct species counts. As well, where Collins *et al.* (2000) used regression analysis to look at community relationships from three unrelated data sets; the objective in this study was to compare the different recovery trends of similar mesocosm systems under the same experimental conditions. The Euclidean distance for any one mesocosm was also divided by the largest distance calculated for the time course of that mesocosm, giving values between 0 and 1 during the 29 d following the AMD perturbation. A normalized Euclidean distance of 1 means that the community within the respective mesocosm on that day is the most dissimilar to day 0, a normalized Euclidean distance of 0 means that the community on that day is exactly the same as the community on day 0 for the given mesocosm. The normalized Euclidean distance measure here does not give information as to how similar the communities in different mesocosms are, rather how similar the community in any one mesocosm is in comparison to the community in that mesocosm on

day 0. As the bacterial community within any 2 mesocosms is not entirely the same, normalizing the Euclidean distance helps in visualizing and comparing the bacterial community responses for different mesocosm systems following the AMD disturbance.

3.3 Results and Discussion

The intent of this study was to develop and assess a new metric for interpreting bacterial community shifts using BIOLOG™ EcoPlate data. Several methods are presented and discussed for comparison purposes. The first method uses PCA to assess for a shift in the bacterial communities. This method was presented in Weber *et al.* (2008) and serves as the basis point for comparing the different methods of assessing bacterial community shifts. The second method involves grouping the 31 carbon sources into 5 guilds and assessing changes in the % guild utilizations over the study period. Fundamentally this method compresses the 31 dimensional space of any one plate into 5 dimensions. The last method is a 1 dimensional metric which has not previously been utilized to assess for bacterial community shifts using this type of data. This method involves calculating normalized Euclidean distances for a set of BIOLOG™ plates for any 1 system (a constructed wetland mesocosm in this case) over a time period of interest. This method basically compresses the 31 dimensional space of any one plate into a single dimension for simple plotting and interpretation.

3.3.1 Principle Component Analysis

Figure 3.1A shows a PCA ordination determined as previously presented by Weber *et al.* (2008) for 5 mesocosms on day 0 before an AMD perturbation. The ordination shows the CLPP's for the interstitial bacterial communities of the planted mesocosms (2 and 6) to be quite similar forming a tight grouping. The unplanted mesocosm CLPP's (1 and 4) are seen to be different than the planted mesocosm objects but also dissimilar from each other. The abiotic mesocosm (8) CLPP is shown to be different than either the planted or the unplanted mesocosms. Figure 3.1B shows an ordination of the same 5 mesocosms on day 1 after the AMD disturbance. On

day 1 the mesocosm objects aggregate on the ordination plane and no discernable groupings can be made.

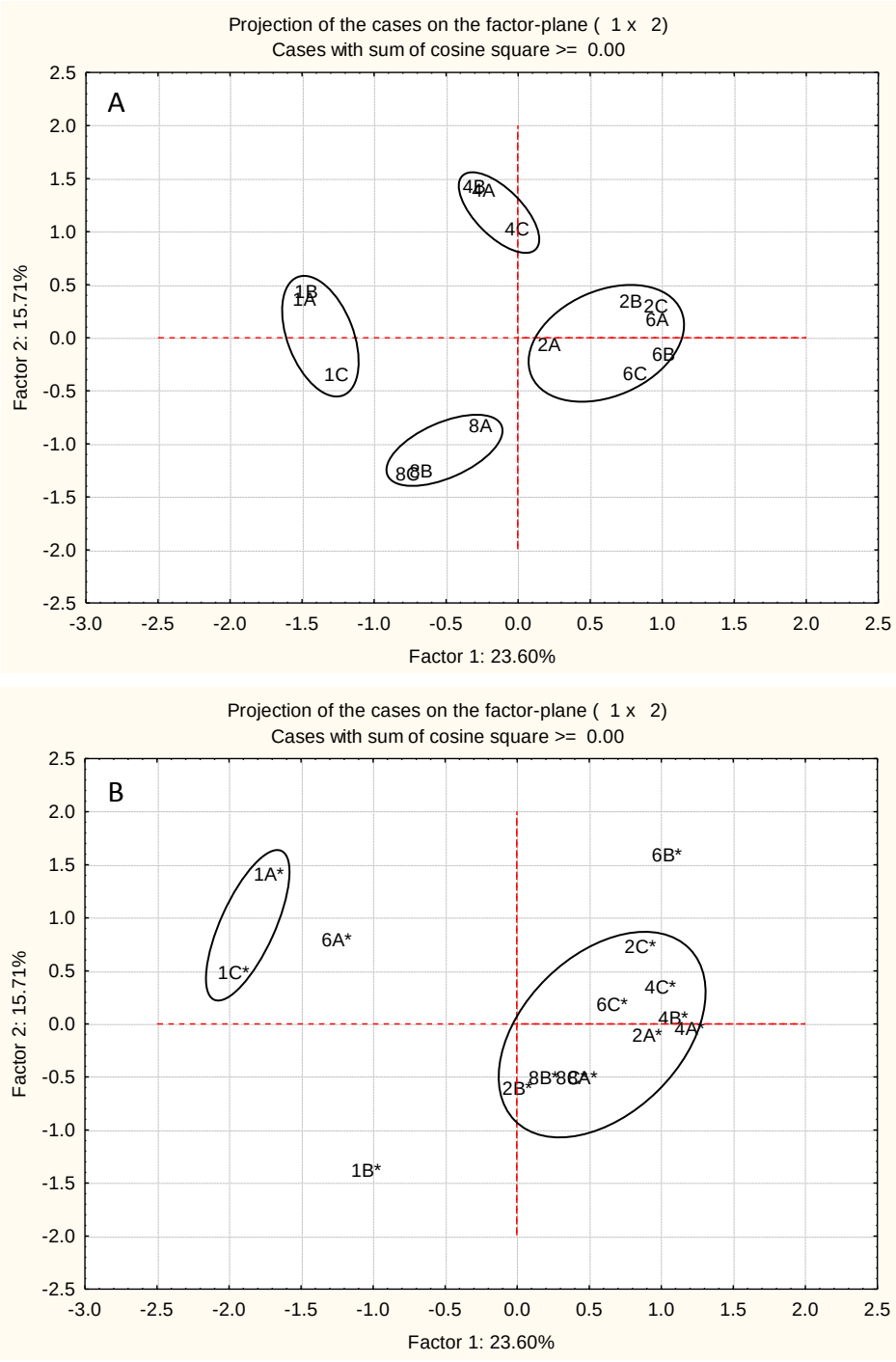

Figure 3.1: CLPP of the logarithmic transformed BIOLOG™ EcoPlate data before (A) and 24h after exposure to AMD (B) of 5 mesocosms, planted (2 and 6), unplanted (1 and 4), and abiotic (8), in triplicate (A–C). Output generated using Statistica 7.1

The defined groupings from day 0 shifted on the ordination plane to aggregate in a similar area on day 1 after the AMD disturbance (Figure 3.1). The shift of objects on the ordination plane is due to the mesocosm bacterial communities having different carbon source utilization patterns on day 1 as compared to day 0. Weber *et al.* (2008) showed evidence that detachment of the fixed biological regime may be occurring as a result of AMD treatment in the same mesocosm systems, lending support to the idea that a different bacterial community species structure may be populating the interstitial water after an AMD perturbation.

The differences in the community structure between the planted and the unplanted mesocosms, on day 0, is likely due to the relationship between the plant root system and the associated bacterial community, collectively called the rhizosphere. The role of reeds, such as *Phragmites australis* have been proven to be fundamentally influenced by the periphyton communities developing on the underwater surfaces of aquatic macrophytes (Acs *et al.*, 2003; Albay & Akcaalan, 2003; Gross *et al.*, 2003; Lakatos & Bartha, 1989; Neely & Wetzel, 1995). In a similar fashion the bacterial communities within the rhizosphere region will also be affected by plant activities. It is possible that the carbohydrates and other metabolites exuded by the plant roots into the rhizosphere provide an added and consistent food source for the bacterial community within the planted mesocosms (Walker *et al.,* 2003; Nardi *et al.,* 2000) and as a result affect their structure and sensitivity to disturbances.

Figure 3.2 presents PCA ordinations of the studied mesocosms on day 0 and day 29. Figure 3.2A portrays an ordination very similar to that presented in Figure 3.1A. The carbon source utilization patterns for bacterial communities of the planted mesocosms (2 and 6) form a tight grouping; whereas the unplanted mesocosm objects (1 and 4) are seen to be different than the planted mesocosm objects and also dissimilar from each other. The abiotic mesocosm object is again shown to be different than either the planted or unplanted mesocosms.

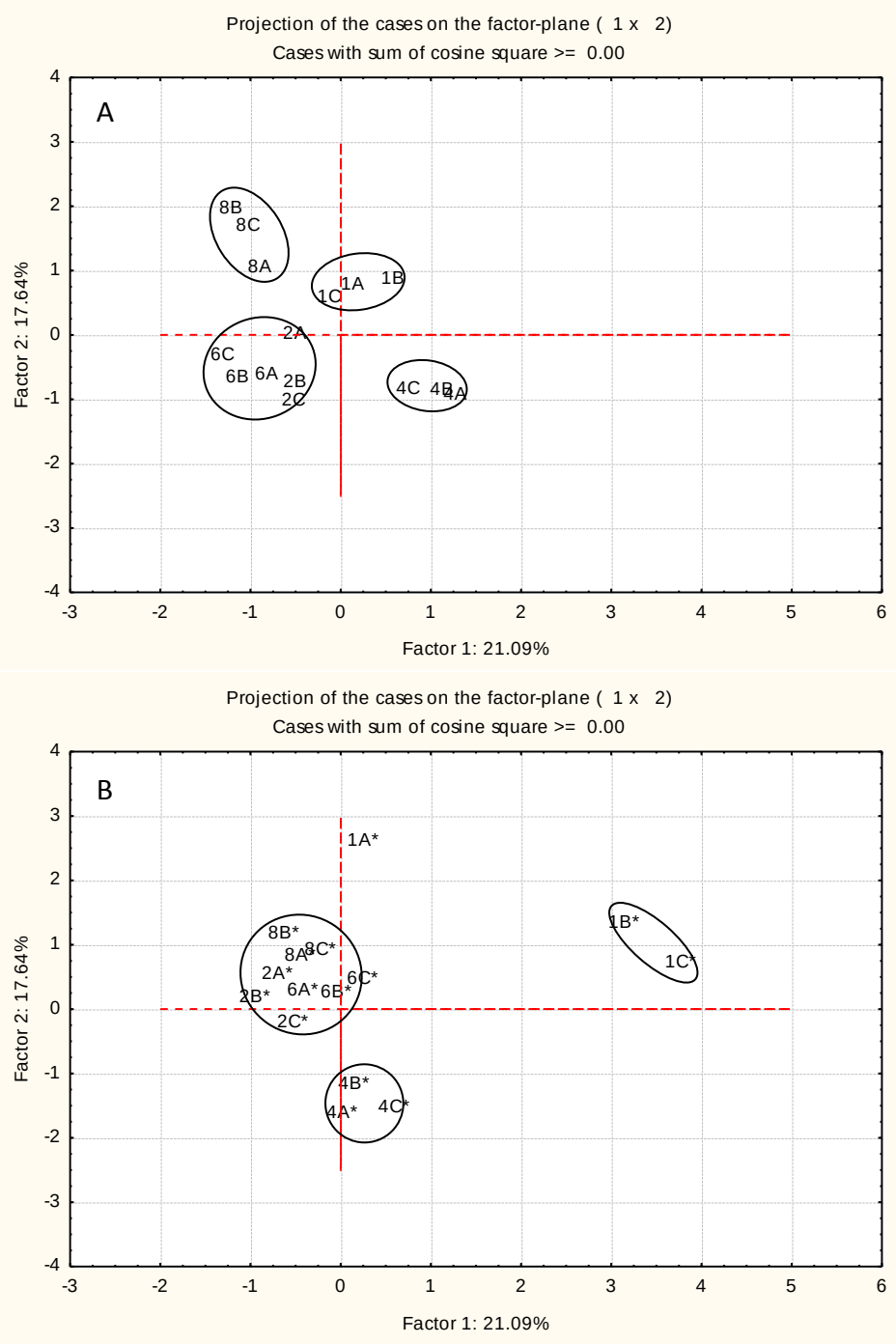

Figure 3.2: CLPP of the Taylor transformed (b=0.9441) BIOLOG™ EcoPlate data before (A) and 29days after exposure to AMD (B) of 5 mesocosms, planted (2 and 6), unplanted (1 and 4), and abiotic (8), in triplicate (A–C). Output generated using Statistica 7.1

Figure 3.2B presents an ordination of the 5 mesocosms after the 29 day period following the AMD perturbation. In this ordination it can be seen that the planted mesocosms (2 and 6) did not shift an appreciable distance on the ordination plane in comparison to their original positions (Figure 3.2A). As well, the planted mesocosms still form a tight grouping, meaning the carbon source utilization patterns of the planted mesocosms are still quite similar at day 29. Figure 2B shows mesocosm 4 to have shifted from its original position on day 0 (Figure 3.2A). Mesocosm 1 also shifted from day 0 to day 29 but to a greater extent than did mesocosm 4. The unplanted mesocosm communities diverged to a greater extent than did the planted mesocosm communities after the 29 day recovery period, and the divergence shown by mesocosm 1 was greater than that shown by mesocosm 4.

Plant root systems are known to provide mechanical support for bacterial communities and perform many roles including the synthesis, accumulation, and secretion of compounds (Flores *et al*., 1999). Through the exudation of compounds, roots systems have been shown to regulate the microbial community structure within the surrounding rhizosphere (Walker *et al*., 2003; Nardi *et al*., 2000). This may suggest that the plant root exudates in the interstitial water of the planted mesocosms help buffer the bacterial community from the AMD disturbance. The added carbohydrates and other metabolites exuded by the roots in the planted mesocosms are present before, during and after the AMD perturbation. This consistent source of nutrition would reduce a structural change in the community. In addition, the larger amount of organic matter from dead root matter (Kadlec & Knight, 1996) within the planted mesocosms may have helped buffer the fixed biological regime within the planted mesocosms from any initial low-pH stress associated with the AMD perturbation.

Recounting the main points extracted from the PCA analysis of the data thus far:

1) There is a definite shift from day 0 to day 1 as a result of the AMD perturbation in all mesocosms

2) The planted mesocosms seemed to recover to a state (Day 29) more similar to their original structure (Day 0) than did the unplanted mesocosms
3) The planted mesocosms are found to be quite similar to each other both before and after the recovery period
4) The unplanted mesocosms are found to be similar to each other before the disturbance, however are seen to be somewhat different from each other after the 29 day recovery period

3.3.2 Guild Grouping

Figure 3.3 presents the % of total carbon source utilization for each guild (see Table 3.1 for guild groupings) over the 29 day study period. As can be seen for the unplanted mesocosms, both mesocosm 1 (Figure 3.3A) and mesocosm 4 (Figure 3.3B) show a large shift in the guild utilization from day 0 to day 1. After day 1 the guild utilizations for both unplanted mesocosms do not seem to follow any set pattern; however, by day 29 the guild utilizations again begin to look somewhat similar to the pattern seen on day 0.

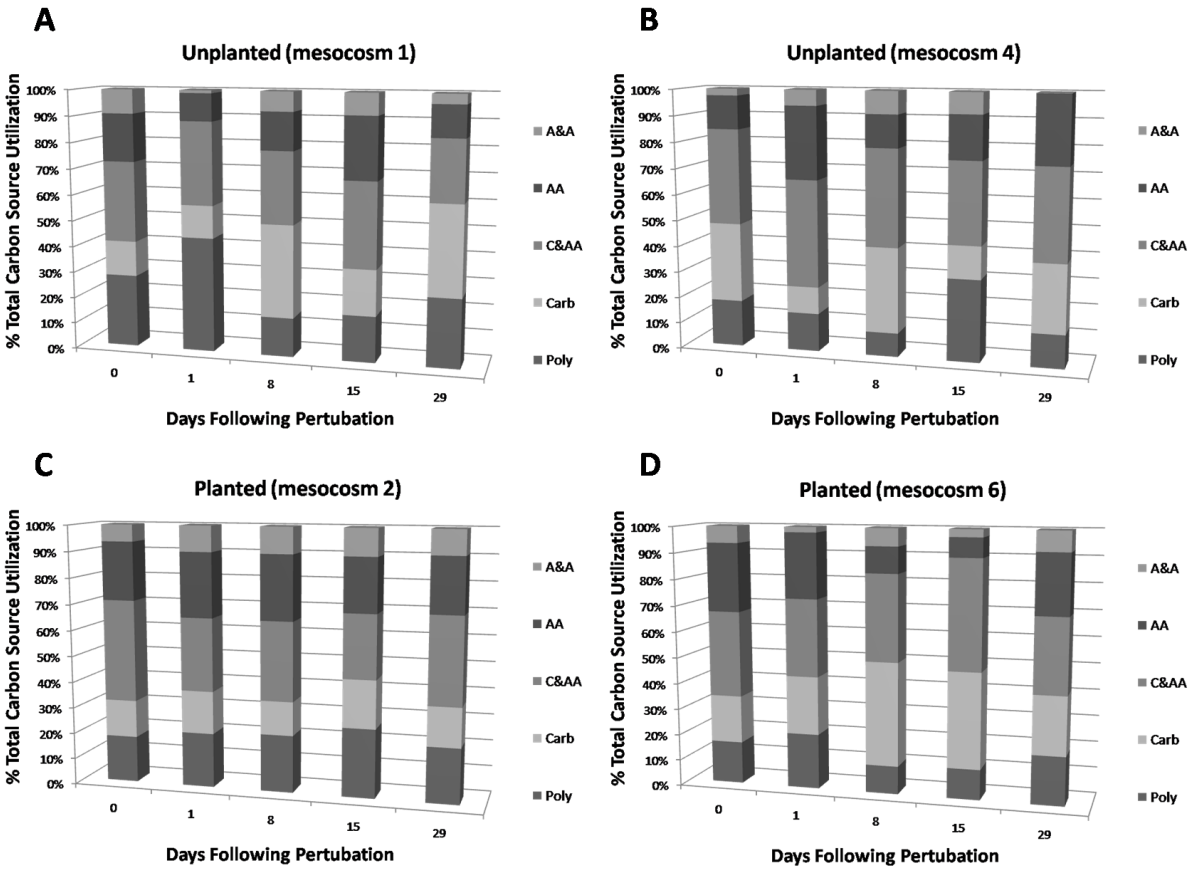

Figure 3.3: Percent of total carbon source utilization response, tracked over the 29 day study period, for the different guilds – amines and amides (A&A), amino acids (AA), carboxylic and acetic acids (C&AA), carbohydrates (Carb), and polymers (Poly). Four different mesocosm systems shown: (A) mesocosm 1 [unplanted], (B) mesocosm 4 [unplanted], (C) mesocosm 2 [planted], (D) mesocosm 6 [planted].

When looking at the guild shifts for the planted mesocosms, mesocosm 2 (Figure 3.3C) and mesocosm 6 (Figure 3.3D) also show an initial shift in the guild utilization patterns, however the shifts seen for planted mesocosms seems to show a pattern that is a dynamic "response" to the perturbation. At day 29 the planted mesocosm guild utilization patterns appear to be quite similar to the patterns seen on day 0.

Comparing the guild utilizations at day 0 and day 29 for the planted mesocosms (2 and 6) the guild utilizations are quite similar (Figures 3.3C and 3.3D) for the two different mesocosms,

suggesting that the bacterial communities of the planted mesocosms are quite similar to each other. However, comparing the guild utilizations of the unplanted mesocosms at day 0 (Figure 3.3A) guild utilizations are similar for day 0, however not quite as similar as was seen for the planted mesocosms. It also is also evident that the unplanted mesocosms are quite different from each other at day 29 (Figure 3.3B), suggesting that these bacterial communities are not as stable or robust as they are not able to recover to as great a degree as the planted mesocosm communities. These conclusions are the same as those made using the PCA method.

3.3.3 One Dimensional Relative Divergence Metric

Figure 3.4 provides the relative divergence (normalized Euclidean distance) of the mesocosms for 2 planted (2 and 6) and 2 unplanted (1 and 4) mesocosms over the 29 day period. The general trend for all 4 mesocosm systems can be said to be quite similar over the period following the AMD perturbation. After the initial AMD disturbance (day 0) the relative community divergence for all mesocosms was observed to increase (day 1) and remain high for the first 15 days followed by a decrease by day 29. Although the initial community divergence appears to be followed by a recovery phase, the mesocosm communities at day 29 do not completely recover to the day 0 state suggesting a possible long-term permanent community shift in response to the AMD perturbation.

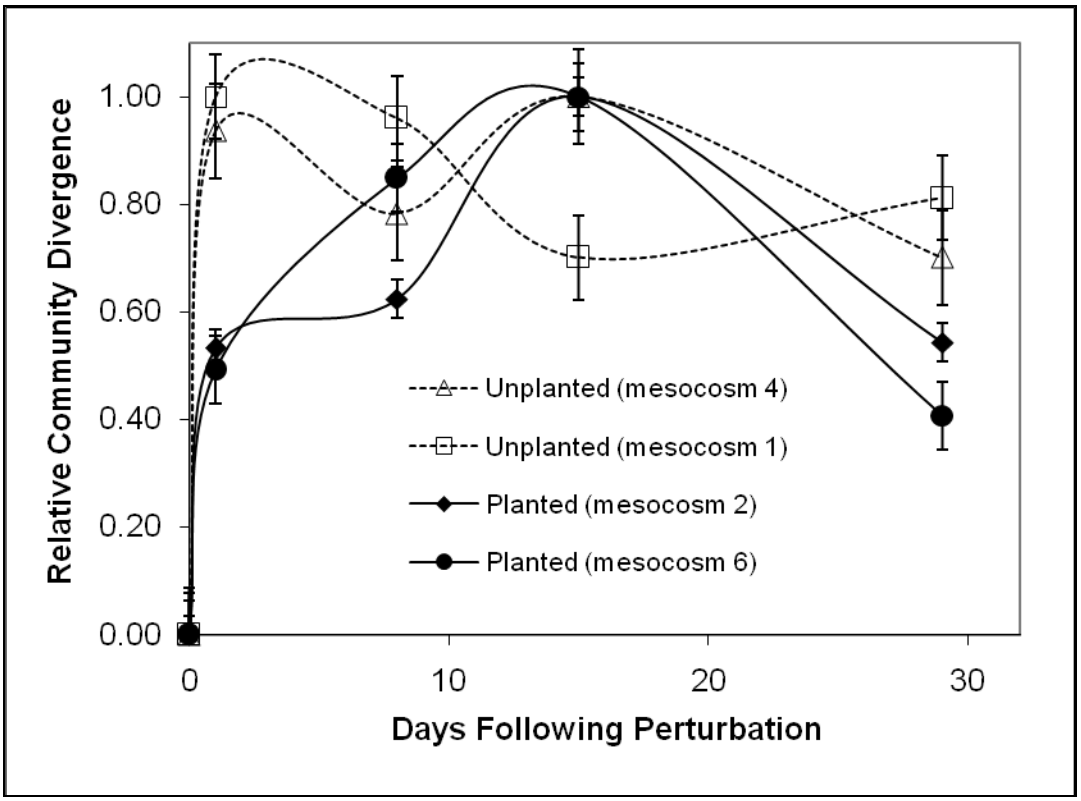

Figure 3.4: Relative community divergence (normalized Euclidean distance) of 4 mesocosms, planted (2 and 6), unplanted (1 and 4), for 29 days following an AMD perturbation (day 0).

In distinguishing the recovery trends for the planted and unplanted mesocosms several observations can be made. The initial slope of the divergence parameter for the unplanted mesocosms is approximately twice that of the planted mesocosms, suggesting that the initial divergence of the unplanted mesocosms is greater than that of the planted mesocosms. It is also evident that there is a difference in the recovery trend between the planted and unplanted mesocosms; the planted mesocosms recovered to a greater degree than the unplanted mesocosms. The unplanted mesocosms recovered to a relative divergence of 0.7 and 0.81, where the planted mesocosms recovered to a relative divergence of 0.41 and 0.54.

A difference between the response trends of the planted and unplanted mesocosms was seen at day 15 (Figure 4). The planted mesocosms reached a maximum relative divergence on day

15, whereas the unplanted mesocosms reached a maximum relative divergence on day 1. As well, the 2 unplanted mesocosms were quite different at day 15. The difference in the 2 unplanted mesocosms (1 and 4) may be due to different communities within the 2 mesocosms. The unplanted mesocosms were discerned to be slightly different on day 0 (Figure 3.1A). It is probable that the different trends shown by the 2 unplanted mesocosms around day 15, as seen in Figure 4, is a result of the differences in the initial communities and their ability to return to a comparable structure following the AMD perturbation.

These results are in agreement with the analysis presented using both PCA and the guild grouping methods. The relative divergence measure showed that:

1) There is a definite shift from day 0 to day 1 as a result of the AMD perturbation in all mesocosms

2) The planted mesocosms seemed to recover to a state (Day 29) more similar to their original structure (Day 0) than did the unplanted mesocosms

However the relative divergence measure was not able to definitively show that:

3) The planted mesocosms are found to be quite similar to each other both before and after the disturbance and recovery period

4) The unplanted mesocosms are found to be somewhat similar to each other before the disturbance, however are seen to be somewhat different from each other after the 29 day recovery period

This method was not able to compare the similarities of different mesocosms. Rather the relative divergence metric presented here was suitable for comparing the divergence responses of the different mesocosms. Instead of gathering information regarding the community

population in a static state, this method reveals information regarding the community response or divergence over time. In this case the method was used to monitor community divergence in response to an AMD perturbation in several mesocosm systems. However, this method could be used on a broader basis to monitor communities during a start-up phase, for possible failure monitoring, or to gather information regarding community shifts in response to unknown environmental factors such as pollution.

The relative divergence measure is quite useful in that it requires limited knowledge of multivariate statistics to perform. As shown by Weber *et al.* (2007) when performing multivariate methods such as PCA, an in depth knowledge of the assumptions and biases of the method being utilized is required to analyze a multivariate data set. The relative divergence measure is also easy to interpret. As a one dimensional metric, the divergence measure can be used with any number of classical plotting or statistical methods. However, if performing any kind of advance statistics on data such as the relative divergence data as shown here, use of non-normalized data should be considered and may be preferred. Data herein was normalized in order to compare the divergence trends of different communities and to also compare the method to other widely used analysis methods. If monitoring a single system over time, use of non-normalized data may prove more useful.

3.4 Conclusions

The BIOLOG™ EcoPlate method yielded useful data regarding differences in communities and differences in the divergence trends shown by planted and unplanted mesocosms systems over a 29 day time period following an AMD perturbation. Using all three of the described analysis methods, PCA, guild utilization, and the proposed relative divergence measure, a number of interesting observations were made from the data: 1) There was a definite shift in the bacterial communities in all mesocosms in response to the AMD perturbation; 2) The planted mesocosms were able to recover to a state more similar to their original structure than did the

unplanted mesocosms; 3) The planted mesocosm bacterial communities were found to be quite similar to each other both before and after the 29 day recovery period and 4) The unplanted mesocosms bacterial communities were found to be similar to each other before the disturbance, however were seen to be somewhat different from each other after the 29 day recovery period. In comparing the three different analysis methods it was found that in using either the PCA or guild utilization methods, both methods yielded the same conclusions. The proposed relative divergence measure was able to support points 1 and 2, however not points 3 and 4. The relative divergence measure cannot by definition yield information regarding communities in a static state; rather this method reveals information regarding the community response or divergence over time. The community divergence method as presented is easy to use as it does not require a background in multivariate statistics as does PCA. This metric is also one dimensional, which makes the divergence trend over time easy to interpret and provides an opportunity for standard curve evaluation and statistical methods. If looking to monitor community shifts over time the community divergence method does not provide all of the information that PCA or guild grouping analysis does, however is a more easily implemented and interpreted evaluation method when analyzing BIOLOG™ EcoPlate data, and could be considered a useful addition to the toolbox of scientists and engineers interested in bacterial community divergence.

Acknowledgements

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Chapter 4 - Method for the Detachment of Viable Bacteria from Wetland Gravel

Overview

The study of bacterial communities in microbially mediated water treatment systems is becoming increasingly popular. Aquatic bacterial communities are often found in fixed film environments, residing within a matrix of extracellular polymeric substances often referred to as a biofilm. To either enumerate or characterize these bacterial communities a method for detaching the biofilm is required. Bacterial detachment methods include scraping, swabbing, shaking, sonication, blending, and digestions. Herein, various shaking technique protocols are evaluated for effectiveness in the detachment of viable bacterial communities from the biofilm surrounding pea gravel from constructed wetland mesocosms. Three different shaking technique protocol factors were investigated via a triplicated 2^3 factorial design in an attempt to find the most effective detachment protocol. Factors studied include: the use of either tap water or phosphate buffer as the shaking/detachment solution; the use of either manual-shaking at room temperature or mechanical shaking at 30°C, and the addition of either no enzyme to the shaking solution or the addition of lipase, β-galactosidase and α-glucosidase (maltase). Resulting suspensions from the different protocols were characterized for organics, inorganics, viable bacteria, community level physiological profile (CLPP) and several BIOLOG™ ECO plate substrate related diversity indices. Using these metrics for the evaluation of the differing protocol treatments the most effective protocol was found to be the use 10 mM pH 7 phosphate buffer solution with mechanical shaking for 3 hr at 30°C and the addition of the enzymes lipase, B-galactosidase and α-glucosidase to the detachment solution.

4.1 Introduction

Bacteria can attach to solid surfaces by creating an adhesive biofilm to promote irreversible adhesion to particles (Bockelmann *et al.*, 2003). This biofilm primarily consists of extracellular polymeric substances (EPS), which are composed mainly of polysaccharides, proteins, lipids and nucleic acids (Flemming and Wingender, 2001; Bockelmann *et al.*, 2003). For general characterization and study of bacterial communities, various methods have been developed to detach bacteria from this EPS biofilm. Bacterial detachment methods include scraping, swabbing, shaking, sonication, blending, and various digestion approaches.

Several studies have been performed to compare the effectiveness of different bacterial detachment techniques. Camper *et al.* (1985) studied homogenization, blending and sonication as bacterial detachment methods using bacterial biofilms grown on granular activated carbon (GAC). Results showed that sonication killed cell cultures on all sonicator settings. Due to heat of mixing problems blending was shown to only be effective if used for less than 3 min (up to temperatures of 45 °C). Where, homogenization (stomacher method) was shown to be the best method for bacterial detachment maintaining almost 100% cell viability after detachment. Gagnon and Slawson (1999) evaluated scraping, swabbing and a stomacher method for detachment of bacteria from polycarbonate coupons. This study found stomaching at room temperature for 2 min was most effective. Grove *et al.* (2004) found that shaking biofilter peat in a buffer solution for 3 hr at room temperature to be an effective method for the detachment of representative bacterial communities.

Another recent study also looked at the effect of adding specific enzymes during the detachment procedure to facilitate the breakdown of the EPS matrix entrapping attached bacteria. Bockelmann *et al.* (2003) performed a bacterial detachment study using sonication in a sodium pyrophosphate buffer preceded by an enzymatic digestion of the bacteria/biofilm

matrix with α-glucosidase, β-galactosidase and lipase. Results showed the enzymatic digestion improved cell detachment, presumably due to the EPS destabilization and degradation.

The study of bacterial communities in wetland systems is still fairly new. Most studies have been restricted to the interstitial bacterial communities (Hadwin *et al.,* 2006; Hench *et al.,* 2004; Stoeckel and Miller-Goodman, 1998; Weber *et al.,* 2008). This is likely due to difficulties with sampling wetland sediments and the lack of published bacterial detachment methods focusing on wetland sediments/supports.

A number of bacterial community profiling techniques exist. Some of the more popular molecular methods include denaturing gradient gel electrophoresis (DGGE), terminal restriction fragment length polymorphism (TRFLP), and fluorescent in situ hybridization (FISH). Non molecular techniques include microscopy-based identification, culture-based identification, and community level physiological profiling using BIOLOG™ plates. Community level physiological profiling (CLPP) is an approach used to characterize microbial community function based on sole carbon source utilization patterns (CSUPs). CLPP can be used as an indicator of the metabolic characteristics and overall stability of a specific microbial community over time. Recent work in the area of microbial soil ecology has utilized BIOLOG™ plates as a tool for CLPP (Hadwin et al., 2006; Hench et al., 2004; Weber et al., 2008). BIOLOG™ plates consist of 96 wells, each well containing a different carbon source and a redox dye indicator, tetrazolium violet, which changes colour in response to carbon utilization. Garland and Mills (1991) were the first to use BIOLOG™ plates for characterizing heterotrophic soil bacteria communities through principle component analysis (PCA). A number of subsequent studies are discussed in Konopka *et al.*, (1997). Most recently Weber *et al.*, (2008) successfully used BIOLOG™ ECO plates to profile the interstitial bacteria within constructed wetland mesocosms. In contrast to the original BIOLOG™ plates with 96 different carbon sources, ECO plates by the same manufacturer are based on 31 different carbon sources with built-in triplicates allowing for better replication (see Section 3.2.4 for the specific carbon sources). CLPP has advantages over

other molecular and non molecular techniques as it is relatively rapid, does not require specialized expertise, and allows for functional community characterization. In characterizing bacterial communities in constructed wetland systems used for water treatment, functional characterization is often more useful than a characterization based on either a full or partial species identification. Functional characterization yields information regarding substrate consumption which in the case of bacterial communities in treatment wetlands, can be directly related to the treatment of the organics contained in wastewater.

The fixed bacterial communities and the interstitial community in constructed wetland systems are assumed to be at least partially related; however, this hypothesis has not yet been extensively tested. Development of an efficient and effective bacterial detachment technique would help verify this assumption. Weber *et al.* (2008) investigated bacterial community dynamics in the interstitial water of wetland mesocosms; this study showed that the bacterial community within the constructed wetland mesocosms shifted, based on CLPP analysis using BIOLOG™ ECO plates, in response to acid mine drainage exposure. It was not clear from this study whether the interstitial community underwent an ecological shift in response to the AMD disturbance, or whether a detachment of the fixed biological regime occurred thus altering the bacterial community species distribution in the interstitial water. Study of the attached bacteria in the EPS structure surrounding the mesocosm sediment, in addition to the CLPP of the interstitial water, would have given a more encompassing picture of the mesocosm community profiles.

To further study bacterial communities in wetlands a detachment method would be valuable. The objective of this study was to develop a method for the detachment of viable bacterial communities from pea gravel from constructed wetland (CW) mesocosms. Focus was given to the detachment of representative and viable bacteria that would enable use of the CLPP method for bacterial community characterization. Based on the results of Camper *et al.* (1985), Gagnon and Slawson (1999), Grove *et al.* (2004) and Bockelmann *et al.* (2003) this study

investigated a shaking technique. The simple shaking technique was chosen as it has shown promise in previous studies (Grove *et al.*, 2004) and can be used with little expertise and does not require specialized equipment. Three different shaking technique factors were investigated via a triplicated 2^3 factorial design. Factors studied included: the use of either tap water or phosphate buffer as the detachment solution, the use of either 5 second manual-shaking at room temperature or 3 hr mechanical shaking at 30°C, and the addition of either no enzyme or the enzymes lipase, β-galactosidase and α-glucosidase (maltase) to the shaking solution. Resulting suspensions from the different shaking technique protocols were characterized for organics, inorganics, viable bacteria, community level physiological profile (CLPP), and several BIOLOG™ ECO plate substrate related diversity indices to identify the most suitable method.

4.2 Materials and Methods

4.2.1 Experimental Design

Variations of a simple shaking method for the detachment of viable bacteria from constructed wetland mesocosm pea gravel were performed on replicate sediment samples to study the effect of 3 different variables associated with the detachment method on the total effectiveness of the detachment protocol. The effect of: A) type of shaking solution, B) shaking time, and C) enzyme addition on the effectiveness of the technique using a triplicated 2^3 factorial design was assessed (see Table 1). The overall effectiveness of the different shaking protocols were quantified using a number of different methods. All methods were applied to the resulting biomass suspensions following the different shaking treatments. Methods used for suspension characterization included organic content (volatile solids), inorganic content (ash content), colony forming units (CFUs), community level physiological profile (CLPP), and several diversity related metrics calculated using the CSUPs from BIOLOG™ ECO plates.

Table 4.1: Experimental Design - Bacterial Detachment Method

	Low level	High level
Shaking Solution	Tap Water	Phosphate Buffer
Shaking Time	5 sec manual shaking	3 hr at 100rpm
Enzyme Addition	No enzymes	Enzymes present

4.2.2 Mesocosm Systems and Sampling

The type of mesocosm used to develop the detachment method protocol is described in Chapter 1, Section 1.2.1, Figure 1.4. Samples were taken from 10 cm below the mesocosm surface in all cases and lightly mixed to produce representative samples for protocol development. See Figure 4.3 for a picture of the pea gravel media.

4.2.3 Detachment Protocol

The basis for the detachment protocol used was adapted from methods employed by Grove *et al.* (2004). For each 25 g sample of mesocosm pea gravel 100 mL of shaking solution was added in a 250mL Erlenmeyer flask and shaken at ~100 rpm for either 3 h at 30°C or manually for 5 sec. Manual shaking for 5 sec can be essentially described as re-suspension and is therefore referred to herein as “no shaking”. The different shaking solution treatments included: tap water without enzymes, phosphate buffer without enzymes, tap water with enzymes and phosphate buffer with enzymes added. The phosphate buffer solution (PBS) was 10 mM with a pH of 7 and 8.5 g/L NaCl made up from autoclaved deionised water. Tap water was also autoclaved before the detachment protocol applied. The enzyme mixture included: lipase (50 units/g pea gravel), β-galactosidase (10 units/g pea gravel), and α-glucosidase (2 units/g pea gravel) [Sigma-Aldrich®]. Average tap water characteristics in the region of Waterloo are summarized in Appendix B. The tap water used had an average hardness of ~350 (mg/L as

$CaCO_3$), and a pH of 7.6. Additional experiments were also performed as standards for the organic content and inorganic content characterization methods. Figure 4.1 summarizes the experimental regime followed.

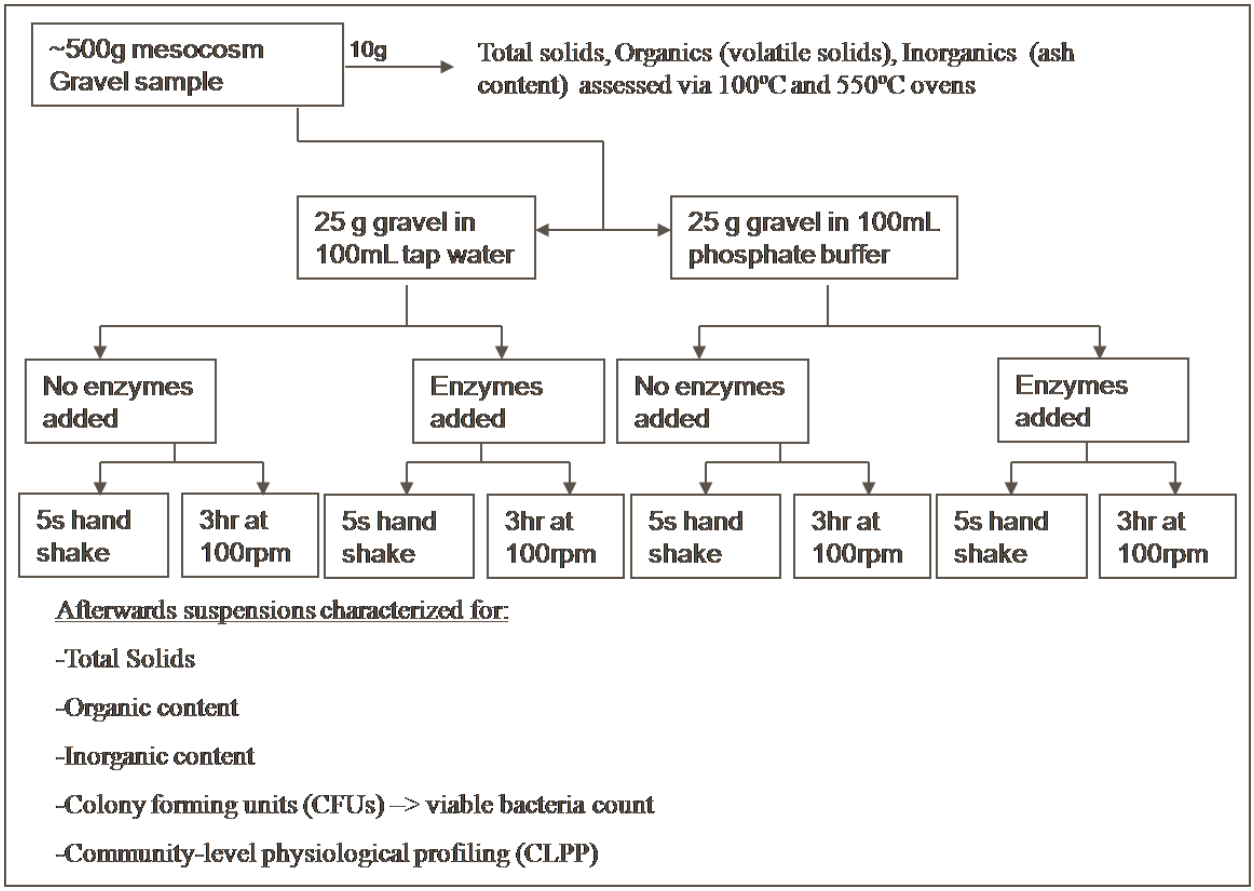


Figure 4.1: Experimental regime.

4.2.4 Total Solids, Organic and Inorganic Content

Total solids (TS) was determined as the dry weight of a sample after drying in ceramic crucibles at 105°C for 24 hr. Following the drying, samples were then characterized for organic and inorganic content. Organic content (volatile solids) of a sample was determined as the amount of sample weight lost after muffle furnace treatment at 550°C for 15 min. Inorganic content

(ash content) was then calculated as the weight of sample remaining after 15 min, 550°C treatment (Eaton *et al*., 1995).

Two different types of samples were characterized for TS, organic and inorganic content: solid wetland pea gravel media samples taken directly from the mesocosm systems, and suspension samples acquired post detachment protocol treatment. Pea gravel sample size was ~10 g, suspension sample size was 10 mL. For calculating % organics detached for any one detachment technique the amount of organics found in the suspension was divided by the amount of organics found to be on a representative sample of wetland pea gravel media.

4.2.5 Colony Forming Units

Viable bacterial counts in the post detachment suspensions were quantified by enumeration of colony forming units (CFU). Serial dilutions of the suspensions were grown on 2 % agar plates with $^{1}/_{10}$ LB-broth. Plates were incubated at 30°C and colonies counted 7 days after inoculation. Samples were prepared in triplicate; enumeration counts between 20 and 200 were considered for analysis. LB media was used to capture a population pertaining to a large number of species from the mixed mesocosm cultures. $^{1}/_{10}$ LB-broth was used as the mesocosms are operated under low nutrient loads, and therefore the bacterial populations were acclimatized to 'environmental' sample nutrient conditions rather than standard laboratory culture nutrient conditions. 1/10th LB media has been shown to gather a broad species spectrum by allowing slow growing bacteria to reproduce at a rate similar to the nutrient-hindered fast growing bacteria. Although R2A is a popular media used for environmental samples, 1/10th LB media is often used in harvesting or isolating bacteria in environmental water samples (Bollmann *et al*., 2007).

4.2.6 Community Level Physiological Profiling

The community level physiological profiling (CLPP) laboratory protocol was performed using the methods outlined in Chapter 2. BIOLOG™ ECO plates were inoculated with 150 μL of a 100 X's diluted post-detachment protocol suspension, and incubated at room temperature. As recommended by Weber *et al.* (2009) inoculates of this dilution step gave an OD of ~0.19. The BIOLOG™ ECO plates were then read at an absorbance of 590 nm after an 84 hr incubation period.

4.3 Data Analysis

4.3.1 Community Level Physiological Profiling

The community level physiological profiling (CLPP) data analysis was performed using the methods outlined in Chapter 2. Absorbance readings (590nm) at 84 hrs were identified as the metric of choice for further CLPP data analysis. BIOLOG™ ECO plates (Biolog Inc., Hayward CA., USA) consist of 96 wells. The wells contain 31 different carbon sources, and a blank in triplicate. 2 plates were used for each of the 8 experimental design points (see Table 1). For each plate 3 replicate carbon source utilization patterns (CSUPs) were attained giving a total of 48 objects (data sets) used for data analysis.

The absorbance values at 84 hr were initially standardized by first correcting by the corresponding blank value and then dividing by the average well colour development (AWCD) for that time point. Assessment of normality, homoscedasticity and linear correlations within the data set according to Weber *et al.* (2007), yielded a recommended Taylor transformation (b=1.171) for subsequent PCA. PCA was completed using Statistica 8.1 (StatSoft, Tulsa, OK).

4.3.2 Substrate Related Diversity Indices

BIOLOG™ plates can also be used, in a more traditional ecological sense, to calculate diversity indices based on carbon source utilization patterns (CSUPs). The Shannon index or what is often called "diversity" is a common ecological metric used to track and understand shifts in communities over space and time. Using the CSUP gathered from a single BIOLOG™ plate, substrate diversity (H) can be calculated as:

$$H = -\sum p_i \ln(p_i) \tag{4.1}$$

where:

H - substrate diversity

p_i - ratio of the activity of a particular substrate to the sums of activities of all substrates

activity - chosen metric for analysis (absorbance value (590 nm) at 84 hr)

Two other parameters associated with substrate diversity which can be calculated using CSUPs are substrate richness (S) and substrate evenness (E). Substrate richness is a measure of the number of different substrates utilized by a microbial population. Substrate evenness is defined as the equitability of activities across all utilized substrates; substrate richness is calculated as the number of wells with a corrected absorbance greater than 0.25. Substrate evenness is calculated as:

$$E = H / H_{\max} \tag{4.2}$$

Substrate diversity, evenness and richness we calculated using the average response from the three replicates on any one plate to give a single result for each plate. Recent examples of studies utilizing the Shannon index calculated from CSUPs gathered using BIOLOG™ plates include He *et al.* (2008), Farnet *et al.* (2008), and Weber *et al.* (2008).

4.3.3 Multiple Linear Regression

Multiple linear regression was performed using the software package Design Expert® 7.1 (Stat-Ease, Minneapolis, MN) to identify the significant factors ($p<0.01$) affecting a number evaluative metrics when using the shaking technique. The regression equations given herein were used for analysis purposes only and are not to be construed as suitable for design purposes. A description of similar experimental design approaches and the use of coding for quantitative analysis can be found in Montgomery (2001). All factors were coded as -1 for low levels and +1 for high levels as given in Table 4.1. Low levels of the three controlled variables were the use of tap water for "shaking solution" type, 5 sec manual shaking for "shaking time" and no enzymes present for "enzyme addition". High levels of the three controlled variables were the use of phosphate buffer for "shaking solution" type, 3 hr shaking at 30°C 100 rpm for "shaking time" and enzymes present for "enzyme addition".

General linear models were generated to ascertain the relationship between the 3 controlled (dependant) variables, and the detachment method performance based on: % organic detachment, inorganic detachment, viable bacteria detachment, substrate diversity, substrate evenness, substrate richness, and average well colour development (AWCD). The general linear models were generated through a backward step-wise method, keeping only variables with $p<0.05$. Two and three factor interactions were included in the model estimation method. Final model results were verified by performing the same analysis using a forward step-wise method. No statistical outliers (i.e. studentized residual greater than 2.5) were found within any of the seven data sets.

4.4 Results and Discussion

The simple shaking technique used for the detachment of viable bacteria from CW mesocosm pea gravel was effective in all cases. Even in the case of a simple 5 sec manual shaking a large amount of solid matter was detached from the pea gravel, creating a brown coloured suspension. Due to the brown colour of the suspensions no discernable difference in any of the different protocol treatments could be seen with the naked eye. Figure 4.2 shows 4 representative sample suspensions following the detachment protocol. Some differences in the amount of detached solids could be discerned if the sample were allowed to settle for approximately 1 hr following the detachment protocol treatment. Figure 4.3 shows 2 different suspensions samples after a 1 hr settling period.

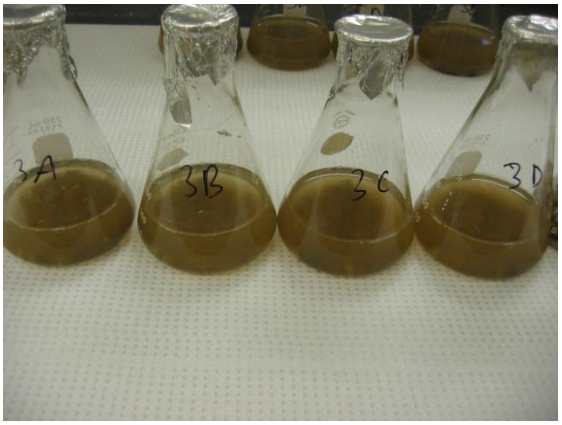


Figure 4.2: Sample suspensions – post detachment protocol.

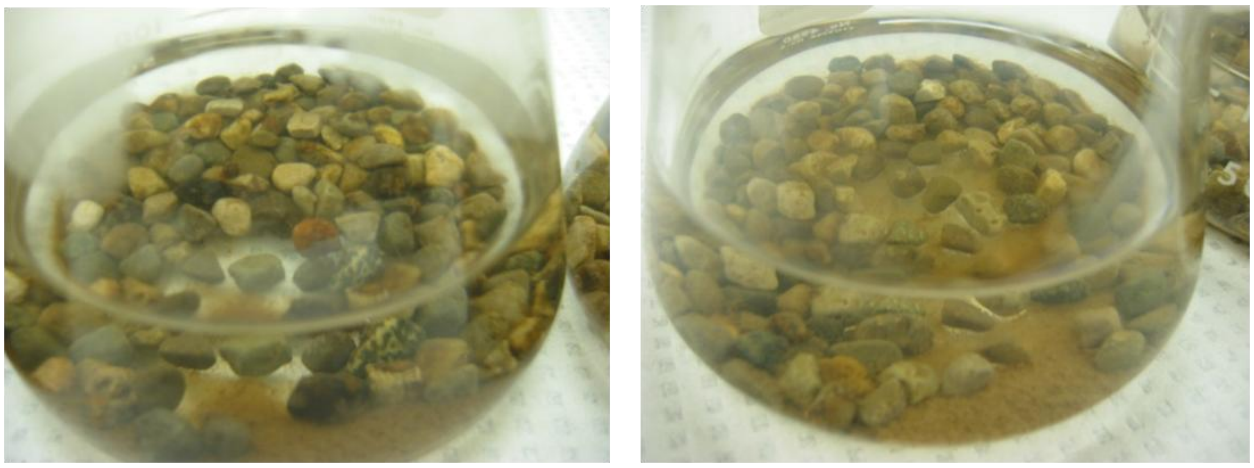


Figure 4.3: CW mesocosm pea gravel - post detachment protocol – following1 hr settling.

Following a 550°C oven treatment for organic and inorganic determinations for each sample, it was qualitatively obvious that the different treatments yielded different results based on sample colours in the ceramic crucibles. Figure 4.4 shows 21 dishes post 550°C treatment used for organic and inorganic determinations.

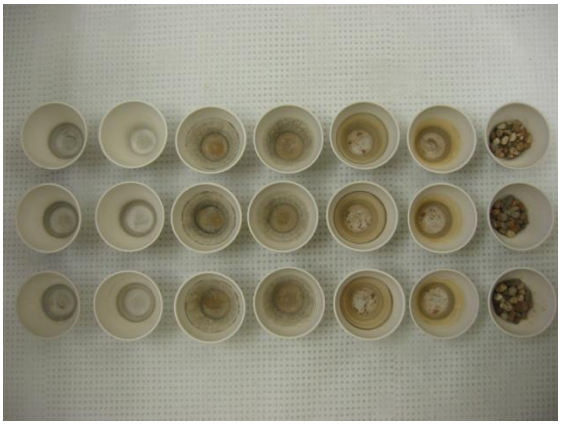


Figure 4.4: Spectrum of resulting samples in ceramic crucibles - post 550°C oven treatment.

4.4.1 Organic, Inorganic and Viable Bacteria Detachment Data

Using the data collected, the effect of the 3 different experimental design variables on the overall success of the detachment protocol was evaluated through several general linear models (GLMs) as described in section 4.3.1. Figure 4.5 summarizes the results found for the % of organics detached from the CW mesocosm pea gravel via the various detachment protocol treatments.

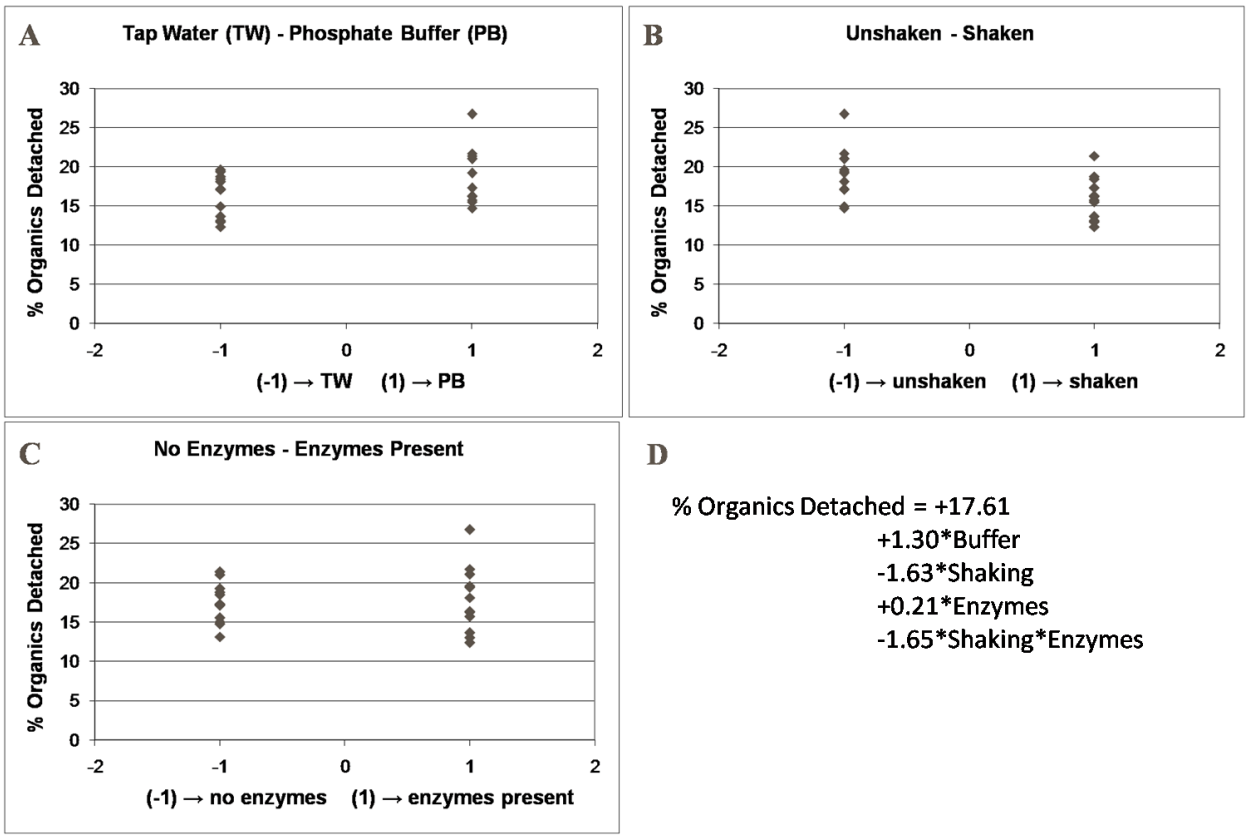


Figure 4.5: Results - organics detached. A) tap water vs. phosphate buffer; B) unshaken vs. shaken; C) no enzymes vs. enzymes present; D) general linear model.

The equation given in Figure 4.5D quantitatively compares and summarizes the effect of buffer use, shaking time and enzyme addition on the % organics detached. As can be seen from Figure 4.5A using phosphate buffer seems to have a positive influence on the amount of organics

detached from the pea gravel. Figure 4.5B shows that shaking the pea gravel samples for 3 hr has a negative effect on the amount of organics detached, while Figure 4.5C shows that there is essentially no difference in the amount of organics detached when enzymes are added to the shaking solution. Equation 4.5D shows the average amount of organics detached to be ~17.6% of the total available organics. This equation also shows that shaking had a large negative effect on overall organic detachment with a coefficient of -1.62591. Use of a buffer solution had a large positive effect on organic detachment yielding a coefficient of 1.30473 in the GLM. The effect of enzymes use on organic detachment was found to be insignificant (p=0.6471), however the cross effect between enzymes and shaking was found to be significant and therefore enzyme use was included into the GLM. The cross effect between enzymes and shaking yielded the largest coefficient (-1.65284) suggesting that the use of enzymes in conjunction with shaking had the greatest negative effect on organics detached of all the terms included in the GLM. All other 2 factor and 3 factor interactions were found to be insignificant at the 95% confidence level.

The equation given in Figure 4.6D quantitatively compares and summarizes the effect of buffer use, shaking time and enzyme addition on the amount of inorganics detached. The amount of inorganics detached is quite low with the average amount equal to about 0.17% of the total pea gravel weight. As can be seen from Figure 4.6A, 4.6B and 4.6C only the addition of enzymes to the detachment solution significantly changes the amount of inorganics detached. All other single factor, 2 factor and 3 factor interactions were found to be insignificant at the 95% confidence level.

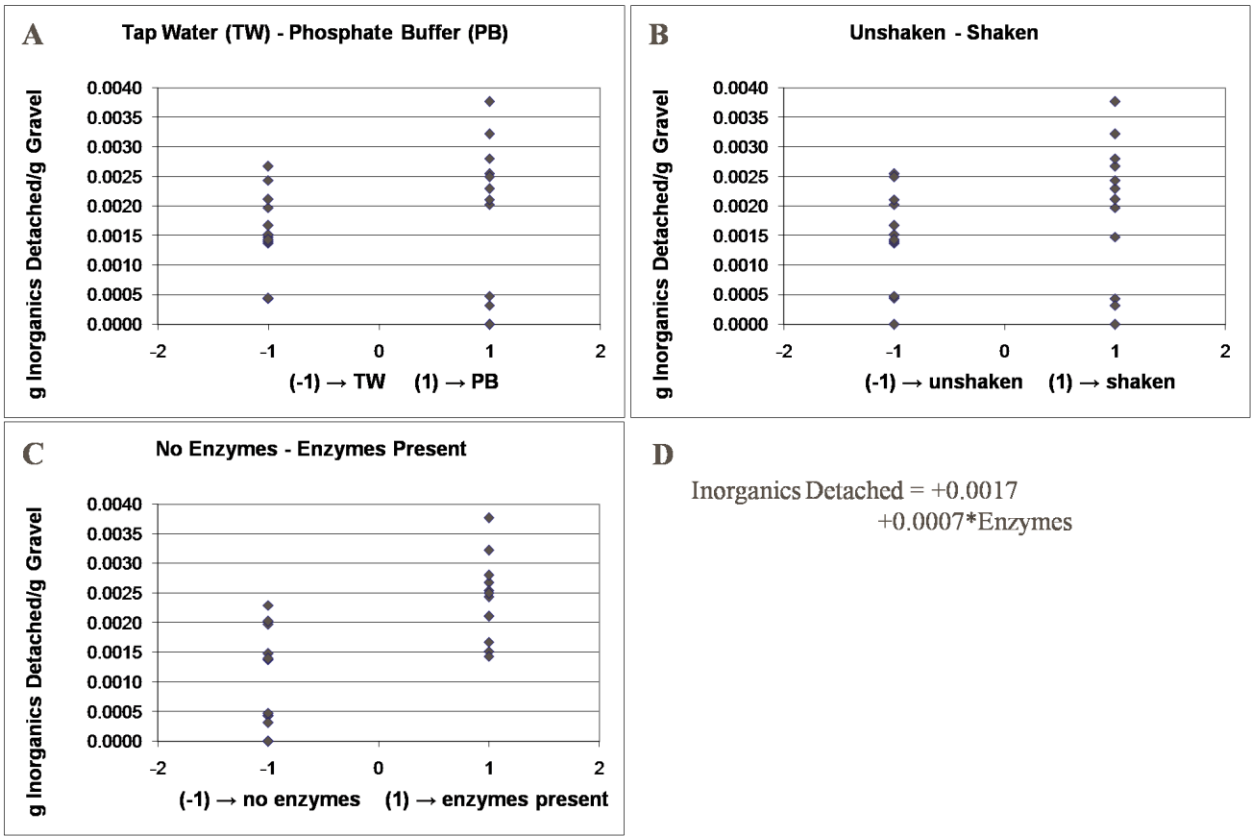


Figure 4.6: Results - inorganics detached. A) tap water vs. phosphate buffer; B) unshaken vs. shaken; C) no enzymes vs. enzymes present; D) general linear model.

The equation given in Figure 4.7D quantitatively compares and summarizes the effect of buffer use, shaking time and enzyme addition on the number of viable bacteria (CFUs) detached via the shaking protocol. As can be seen from Figure 4.7A, 4.7B and 4.7C the use of a phosphate buffer, shaking for 3hrs and adding enzymes to the detachment solution all significantly increased the number of viable bacteria detached from the CW mesocosm pea gravel. Equation 4.7D shows the average number of CFUs detached to be ~1.7×10^6 CFUs/g pea gravel. The coefficients associated with enzyme use, the use of a weak phosphate buffer, and shaking for 3 hours were 0.95, 0.86 and 0.73 respectively showing the use of enzymes to have the largest effect and the 3hr shaking time to have the smallest effect (although only slightly). The cross effect between shaking and enzyme use was also shown to be significant with an associated

coefficient of 0.68. All other 2 factor and 3 factor interactions were found to be insignificant at the 95% confidence level.

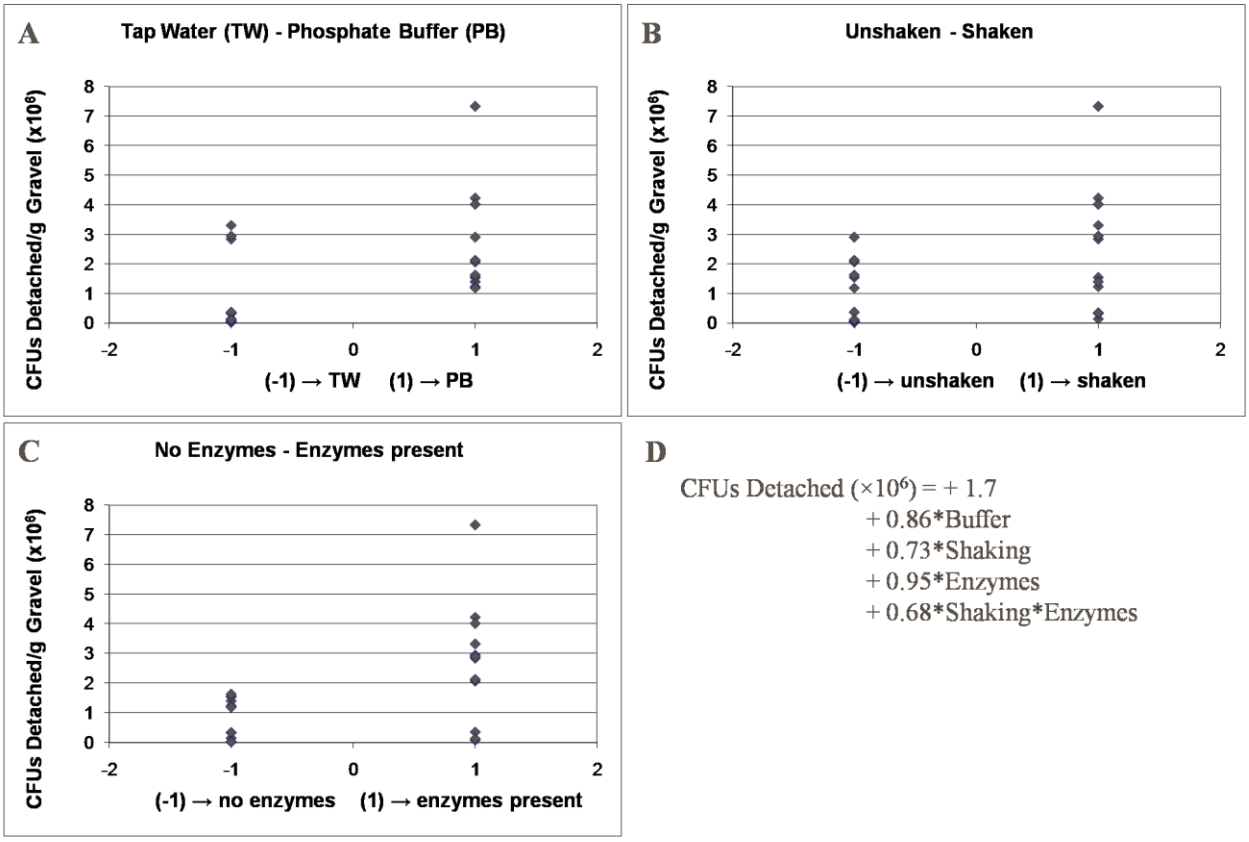


Figure 4.7: Results - colony forming units (CFUs) detached. A) tap water vs. phosphate buffer; B) unshaken vs. shaken; C) no enzymes vs. enzymes present; D) general linear model.

4.4.2 BIOLOG™ ECO Plate Data and Community Level Physiological Profiling

A number of metrics related to the CSUPs gathered using the BIOLOG™ ECO plates were also calculated and the effects of the 3 experimental design variables (ie. buffer use, shaking time, enzyme addition) on the different metrics determined in the same fashion as for % organics detached, inorganics detached, and CFUs detached. Average well colour development as described in Section 4.3.2, in addition to substrate diversity, evenness and richness as described

in Section 4.3.3, were all used as independent variables to measure the effectiveness of the different detachment protocols.

It was found that of all three experimental design variables studied none had a significant effect on AWCD, richness or evenness as measured using the CSUPs from the BIOLOG™ ECO plates (data not shown). It was found however that all three of the experimental design variables studied had a positive influence on the substrate-related diversity measure.

The equation given in Figure 4.8D quantitatively compares and summarizes the effect of buffer use, shaking time and enzyme addition on the diversity of substrates utilized by the detached bacterial community based on the BIOLOG™ ECO plate response at 84 hrs. As can be seen from Figure 4.8A, 4.8B and 4.8C the influence of phosphate buffer, shaking for 3hrs and adding enzymes to the detachment solution all significantly increased the diversity of the substrates utilized by the detached bacterial community. Equation 4.8D shows the average substrate diversity to be 3.112. The coefficients associated with enzyme use, the use of a weak phosphate buffer, and shaking for 3 hrs were 0.022, 0.015 and 0.013 respectively showing the use of enzymes to have the largest effect and the 3 hr shaking time to have the smallest effect (although only slightly). All other 2 factor and 3 factor interactions were found to be insignificant at the 95% confidence level.

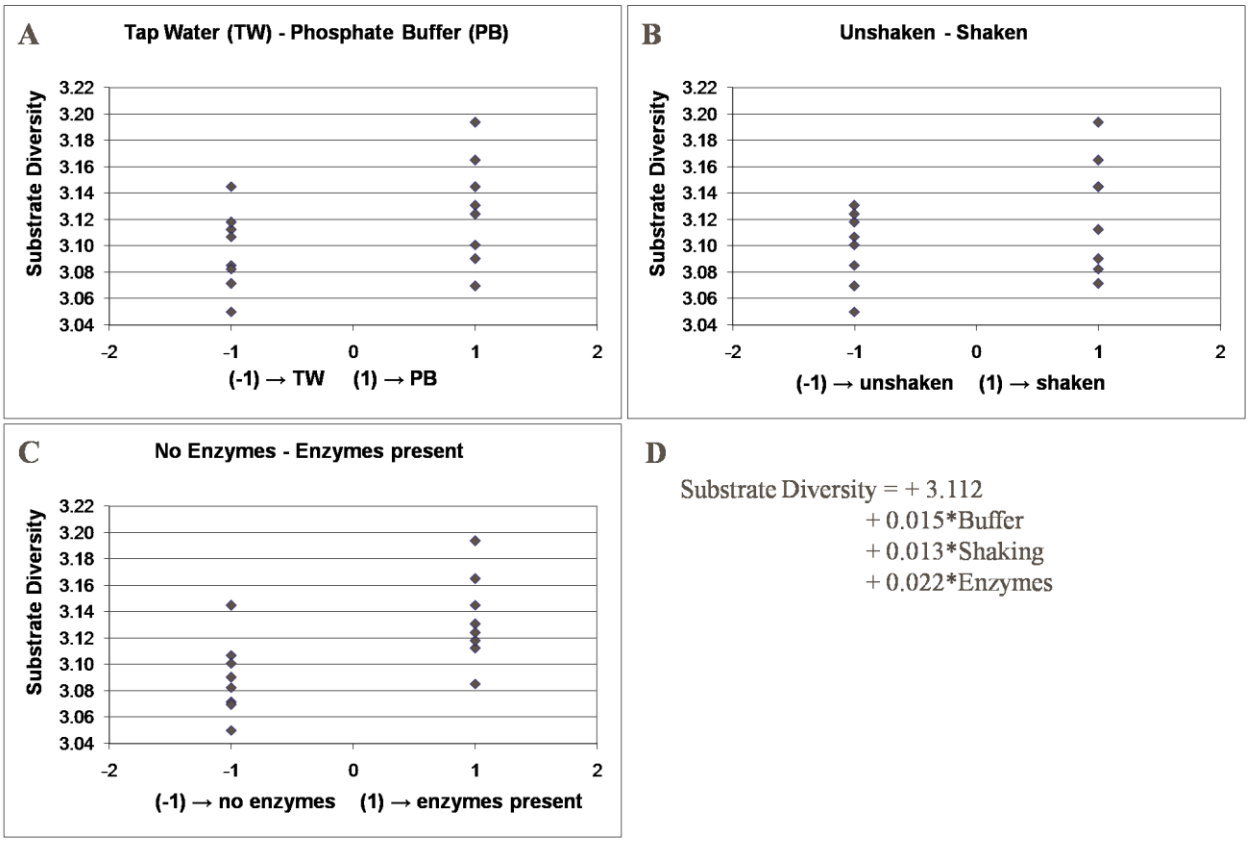


Figure 4.8: Results - substrate diversity based on BIOLOG™ ECO Plate Response at 84 hrs. A) tap water vs. phosphate buffer; B) unshaken vs. shaken; C) no enzymes vs. enzymes present; D) general linear model.

Table 4.2 summarizes the results obtained from the 2^3 factorial design study. This study was designed to investigate and develop a protocol for the detachment of viable bacteria from CW mesocosm pea gravel for enumeration and community profiling, therefore the two most important metrics considered, CFUs detached and community diversity, should be emphasized in Table 4.2. The number of CFUs detached gives an indication of the number of viable bacteria being detached. Community diversity is often used as an ecological surrogate measure for overall community health. Diversity measures are a mixed measure including both number of species (or responsive wells in this case) and overall evenness distribution of all species in the community (i.e. similarity of well responses in this case). A population with a higher diversity is able to more readily handle acute disturbances and positively adapt to changing environmental conditions. A larger community diversity, from a water treatment perspective, also increases

the chance that any one waste constituent will be degraded within a treatment wetland due to the increased functional (or metabolic) diversity associated with bacterial community.

Table 4.2: Summary of experimental outcomes (+ positive effect, - negative effect, / no significant effect). Significant effect measured at the 95% confidence level.

	Organics Detached	Inorganics Detached	CFUs Detached	AWCD	Community Evenness	Community Richness	Community Diversity
Phosphate Buffer	+	/	+	/	/	/	+
3hr Shaking	-	/	+	/	/	/	+
Enzyme Addition	/ (+)	+	+	/	/	/	+

The first row in Table 4.2 summarizes the effect of using phosphate buffer (as opposed to tap water) for the detachment solution on the different metrics used for evaluation. The use of phosphate buffer had a positive effect on the amount of organics detached, the number of viable bacteria (CFUs) detached and sustained, and the resulting community diversity. The use of phosphate buffer is therefore recommended for the detachment protocol.

This result was not completely unexpected. Although the mesocosms were filled with tap water and run using a nutrient-solution/tap-water mix the presence of limestone in the mesocosms was likely important. Even though tap water is used as the bulk solution in the mesocosm systems, limestone dissolution occurs, which likely increases the salt concentrations seen by the bacterial communities in the biofilm surrounding the support matrix. Therefore it is not surprising that a salt infused buffer seems to help in detaching and sustaining viable bacteria.

The second row of Table 4.2 summarizes the effect of adding a 3 hr shaking step (as opposed to a 5 second manual shake) on the different metrics used for evaluation. Adding the 3 hr shaking

step had a negative effect on the amount of organics detached, a positive effect on the number of viable bacteria (CFUs) detached, and a positive effect on the resulting community diversity.

Organic content of suspensions is a commonly used metric to evaluate detachment efficiency (Eaton *et al*., 1995). As can be seen for the results for the 3 hr shaking in Table 4.2, organic detachment may not always be the most appropriate measure to evaluate detachment efficiency. Adding a 3hr shaking step to the detachment protocol was found to decrease the amount of organic material detached. As the bacteria residing in the biofilm are closely associated with the organic EPS matrix, one would then expect the number of viable bacteria being detached to have also decreased when adding a 3hr shaking step. However, this was not the case. Adding a 3hr shaking step was found to increase the number viable bacteria (CFUs) detached from the pea gravel media. If the goal of the detachment method used is to detach organic material, and cell viability is not of concern organic detachment may be an appropriate metric for measuring overall detachment efficiency. However, if detachment of viable cells for enumeration or community profiling is required, as shown here, organic detachment may not be the most suitable metric as an evaluation criteria.

All suspended samples yielded a brown suspension mixture similar to the samples shown in Figure 4.2 and it was for this reason the “no shaking” (i.e. 5 second manual shake) was studied in the experimental design. A simple manual-shaking resuspension seemed to detach a large amount of solid material from the pea gravel media. It is proposed that the during the 3 hr mechanical shaking period the bacterial population may have utilized some of the detached organic material, accounting for both a measured decrease in organics detached and a possible increase in the number of bacteria enumerated. This explanation is however theoretical, to verify this statement further experimentation would be required.

As this study is focused on the detachment of viable bacteria it can be said that the positive influence of adding the 3hr shaking step outweighs the observed negative influence the shaking step had on organic detachment. For further use of this method it is recommended that a 3hr shaking step be included in the protocol.

The third row in Table 4.2 summarizes the effect of adding enzymes to the detachment solution (as opposed to no enzymes added) on the different metrics used for evaluation. Adding enzymes had a positive effect on the amount of organics detached, the number of viable bacteria (CFUs) detached, and the resulting community diversity. Therefore, the addition of enzymes to the detachment solution is recommended for the detachment of viable bacteria from CW mesocosm pea gravel.

The use of enzymes was studied in an effort to examine the capacity for increased EPS destabilization and degradation via enzymatic activity. Increased EPS destabilization and degradation should allow for a greater number and broader profile of bacterial species being detached from the substrate (Bockelmann *et al.,* 2003).

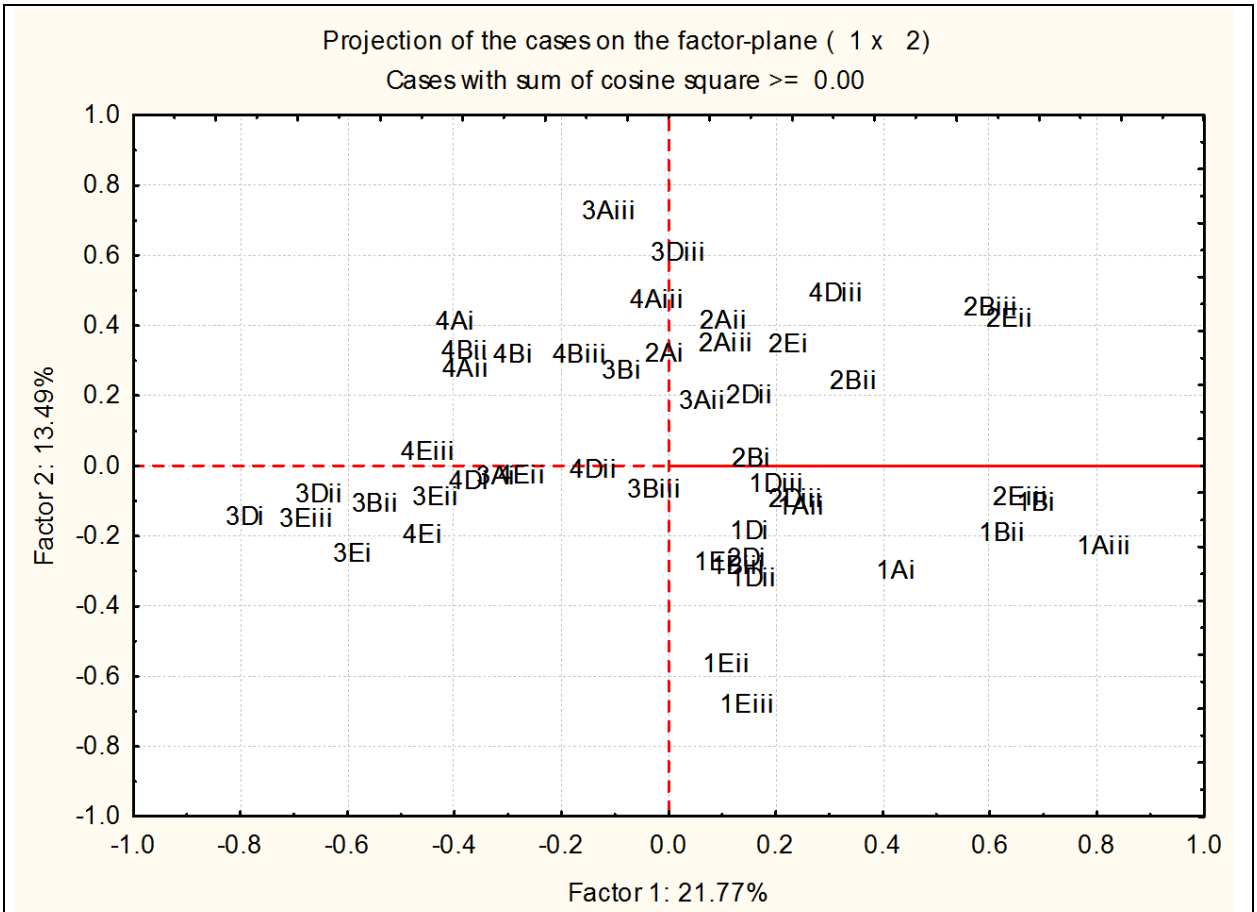
Bacterial biofilms are most often heterogeneous in nature (Noguera *et al.*, 2004), with different bacterial species residing in different areas of the biofilm. As the biofilm develops over time, so does the bacterial community profile in the biolfilm. As biofilms grow in size around particles the amount of oxygen and nutrients available to the bacterial species residing in the biofilm interior is reduced, creating a nutrient profile, which in turn creates a heterogenous bacterial species profile in the biofilm. If the inclusion of enzymes in the detachment solution allows for greater EPS destabilization and degradation, the bacterial species residing within the interior biofilm space are more likely to detach. The observations from Figures 4.5C, 4.7C and 4.8C support this hypothesis, as the increased amount of organics detached, and the increased number of viable bacteria detached can both be explained by a greater EPS matrix

destabilization and degradation in response to enzymatic activity associated with the added enzymes.

4.4.3 Community Level Physiological Profiling

In addition to the mentioned diversity related metrics calculated using the CSUPs, PCA analysis was also performed. All 48 CSUPs (objects) were ordinated on the same plane using the first 2 principle component axes. As described in section 4.3.1 the data was subjected to a Taylor transformation (b=1.171) prior to ordination to better meet the data restrictions associated with PCA.

Figure 4.9 shows a PCA ordination of the CSUPs gathered using BIOLOG™ ECO plates. As can be seen from the ordination there is a significant amount of grouping seen for each protocol treatment type. First, groups 1 and 2 which were protocols performed without enzyme addition can be found on the right hand side of the ordination, while groups 3 and 4 which contained enzymes are found on the left hand side of the ordination. Similarly, groups 1 and 3 which were protocols performed in tap water can be found in the bottom half of the ordination, where groups 2 and 4 which used phosphate buffer can be found in the top half of the ordination. The effect of 3 hr shaking on the CLPP results shown in Figure 4.9 are not as apparent, and therefore the effect of shaking seems to have less of an effect on the resulting CSUPs when viewed on a 2D ordination plane.



	1A,B	1D,E	2A,B	2D,E	3A,B	3D,E	4A,B	4D,E
Phos Buffer (y/n)	N	N	Y	Y	N	N	Y	Y
3hr Shaking (y/n)	N	Y	N	Y	N	Y	N	Y
Enzyme (y/n)	N	N	N	N	Y	Y	Y	Y

Figure 4.9: PCA of the CSUPs gathered using BIOLOG™ ECO plates. Detachment protocol treatments shown in triplicates (i, ii, iii). Output generated using Statistica 7.1.

CSUPs gathered using BIOLOG™ ECO plates are a metabolic fingerprint of the bacterial community inoculum. Samples with different CSUPs are regarded to have a different community structure. It is apparent from Figure 4.9 that the use of phosphate buffer changes the CSUPs suggesting that a different community is gathered when using PBS rather than tap water. As previously discussed this can likely be explained due to the increased salt concentrations within the CW mesocosm systems due to limestone dissolution.

Tap water appears to detach and sustain a different community structure than did the phosphate buffer solution. This could be due to differences in the bacterial species being detached or differences in the bacterial species sustained in solution. Keeping in mind the results seen in Figures 4.5 and 4.6, a larger portion of the community is being detached and sustained in the PBS solution. The results in Figure 4.7 also illustrate differences in the species surviving the detachment protocol when using the different shaking solutions. When PBS was used, a more functionally diverse bacterial community with a greater number of viable bacteria was attained.

Figure 4.9 shows the use of enzymes in the detachment protocol gives different CSUPs. This could be due to different bacterial species being detached from the biofilm or possible enzymatic activity within the wells of the BIOLOG™ ECO plate, degrading some carbon sources, which could in turn allow for preferential growth and metabolic activity of some bacterial species in select wells.

The use of enzymes was studied in an effort to examine the capacity for increased EPS destabilization and degeneration via enzymatic activity. Increased EPS destabilization and degeneration should allow for a greater number and broader profile of bacterial species being detached from the wetland substrate. The results in Figure 4.9, illustrates the different CSUPs obtained when adding enzymes to the detachment mixture. In trying to decipher whether the different CSUPs are due to different bacterial species being detached from the biofilm or possible enzymatic activity within the wells of the plate, a comparison of samples 3A,B with samples 3D,E can be made in Figure 4.9; from this it is quite clear that the two groups are distinctly different. Samples 3A and 3B use tap water, contain enzymes in the detachment solution, and are unshaken (i.e. 5 second manual-shake). Samples 3D and 3E use tap water, contain enzymes in the detachment solution, and are shaken for 3 hrs. As the detachment solutions are used as the inoculants for the BIOLOG™ ECO plates, the enzymatic mixture is present in all of the 3 series (i.e. 3A, 3B, 3C, 3D) samples. The only difference is the shaking

time. Time is required for the enzymes to destabilize and/or degenerate the EPS matrix, therefore in the case of samples 3A and 3B the enzymes will have had no time to cleave any of the molecular bonds within the EPS matrix. Therefore, samples 3A and 3B are essentially standards in this comparison allowing a clear view of what effect the enzymes had in the BIOLOG™ ECO plate wells. As the 3A,B group is quite different from the 3D,E group it could be concluded that the difference in the CSUPs gathered can be explained due to a larger breadth and number different bacterial species being detached from the EPS matrix due to enzymatic degradation of the EPS matrix.

However, it should also be noted that the differences between the CSUPs for samples with enzymes compared to samples without, is in part due to enzymatic cleavage of carbon sources in the BIOLOG™ plates. This is shown through the CSUP dissimilarities between samples 1A,B and samples 3A,B. Samples 1A and 1B use tap water, do not contain enzymes in the detachment solution, and are unshaken (i.e. 5 second manual-shake). Samples 3A and 3B use tap water, contain enzymes in the detachment solution, and are unshaken (i.e. 5 second manual-shake). The only difference between the samples is the addition of enzymes to the detachment solution for samples 3A and 3B. All samples are unshaken, therefore enzymatic cleavage of the EPS structure is non-existent in all samples. The only difference between samples is the presence of enzymes in the BIOLOG™ ECO plates for samples 3A,B. As can be seen in Figure 4.9, samples 1A,B form a distinctly different grouping than samples 3A,B. If the presence of enzymes within the BIOLOG™ plate had no effect on the final CSUPs, then these two groups should overlap; however, as seen in Figure 4.7 this is not the case. Therefore it can be assumed that the enzymes used in the detachment protocol solution also affect the CSUPs gathered using BIOLOG™ ECO plates.

4.5 Conclusions

A 2^3 factorial experimental design proved useful in evaluating the effectiveness of the different detachment protocols for the development of a biofilm detachment method for CW mesocosm pea gravel media. Several factors were evaluated for their effectiveness in detaching organics, inorganics, and viable bacteria. Further consideration was given to the bacterial community in the resulting suspension. CSUPs gathered using BIOLOG™ ECO plates were evaluated for AWCD, evenness, richness and diversity. Using these metrics for the evaluation of each protocol the following detachment protocol is recommended:

1. The use of a 10 mM pH 7 phosphate buffer solution for biofilm detachment and suspension
2. The inclusion of a 3 hr, 30°C, 100 rpm shaking period
3. The addition of lipase (50 units/g pea gravel), ß-galactosidase (10 units/g pea gravel), and α-glucosidase (2 units/g pea gravel) into the detachment solution

PCA analysis and subsequent 2D ordination of the CSUPs from the suspension samples showed that the inoculation of the BIOLOG™ ECO plates with the enzyme mixture significantly affected the resulting community level physiological profile. However, due to the structure of the experimental design employed in the study, it could also be confidently concluded that the use of the enzyme mixture in the detachment solution helped detach a functionally different and more diverse bacterial community.

Chapter 5: Bacterial Community Stratification in the Substrate of Constructed Wetland Mesocosms

Overview

The community level physiological profiling (CLPP) method was used to study and compare the bacterial communities in the substrate of planted and unplanted wetland mesocosm systems. Bacterial community samples associated with the pea gravel bed media taken from the top (10 cm depth), middle (30 cm depth), bottom (60 cm depth) of all mesocosms were profiled and compared. Interstitial water communities were also profiled and compared to the pea gravel-associated bacterial communities from all depths. The study of both planted and unplanted mesocosms enabled the comparison of the respective bacterial communities. Root associated (rhizospheric) bacterial communities were also gathered from varying depths within the planted mesocosm systems and compared to the media-related and interstitial community profiles. Activity determinations suggested that the communities within the lower depths of the bed media were less active than those near the mesocosm surface. Also, the activities of the root-associated (rhizospheric) bacterial communities were seen to be much higher than the activities seen for the gravel-associated bacterial communities. A decrease in community substrate richness and diversity values was seen with increasing mesocosm depth. A general difference in mesocosm bacterial communities based on plant presence/absence was seen from the interstitial water and all gravel samples at various depths suggesting that when trying to decipher general community differences between mesocosm systems via the CLPP method, interstitial water samples provide representative information of the intrinsic microbial population and that the presence of roots within at least part of the mesocosm system does not only have a localized effect on the attached bacterial population but on attached bacteria from all depths within the mesocosms. Differences in the bacterial community structure, as a function of gravel depth were seen for all mesocosm systems supporting the notion of vertical community structure stratification.

5.1 Introduction

It is generally accepted that constructed wetlands contain a biological regime associated with the wetland substrate (Truu *et al.*, 2009), and that microorganisms play a major role in ecosystem health and in the degradation of contaminants in the environment (Parkinson and Coleman, 1991; Aelion and Bradley, 1991; Wynn and Liehr, 2001; Faulwetter *et al.*, 2009). The role of the biological regime and the related mechanisms associated with contaminant treatment in wetland treatment systems is, however, not fully understood. Research into understanding microbial population density and diversity, both spatially and temporally, would help to further understand these systems and would be useful in the optimization and design of constructed treatment wetland systems (Faulwetter *et al.*, 2009).

The role of plants in constructed wetlands is still a debatable issue for some scientists. Certain studies have shown that plants help to effectively remediate contaminated waters (House *et al.*, 1994), whereas other studies have shown plants to have no effect on treatment performance (Gray *et al.*, 2000). Aquatic plants, such as *Phragmites australis* (the common reed), have the ability to transfer oxygen from their aerial tissues and release it into their rhizosphere (Karathanasis & Johnson 2003; Batty *et al.,* 2000). Plant root systems also provide mechanical support and perform many roles including the synthesis, accumulation, and secretion of compounds (Flores *et al*., 1999). The compounds secreted into the surrounding rhizosphere by roots are referred to as root exudates. Plants have been shown to exude 5-21% of all photosynthetically fixed carbon into the surrounding rhizosphere as root exudates (Walker *et al*., 2003; Marschner, 1995). Through this exudation of compounds, roots can often regulate the microbial community structure within the surrounding rhizosphere (Walker *et al*., 2003; Nardi *et al*., 2000). Study of the differences between rhizospheric and bed media associated bacterial communities in wetland systems has not yet been given significant attention (Faulwetter *et al.*, 2009).

With the growing use of community level physiological profiling (CLPP) (Weber *et al.*, 2009), along with advent of PCR and associated bacterial identification and community characterization methods, a number of recent studies have taken a general look at bacterial communities in wetland systems (Hadwin *et al.*, 2006; Hallberg and Johnson, 2005; Hench *et al.*, 2005; Weber *et al.*, 2008; Sleytr *et al.*, 2009). Most studies have tended to focus on the interstitial bacterial communities, operating under the assumption that changes in the interstitial community are related to changes in the media related biofilm bound bacterial communities. Although this assumption holds merit in many ways, this assumption has yet to be tested for mesocosm systems.

Attaining sediment bacterial samples or biofilm bound bacterial community samples can be a difficult task, and often results in a destructive disturbance to the wetland system being studied. Therefore, only a few studies have been performed looking at bacterial communities associated with the bed media in wetland systems (Sleytr *et al.*, 2009; Truu *et al.*, 2005). Several questions arise when considering bacterial communities in wetland systems. Do spatial bacterial community variations exist within wetland bed media? In what way are the rhizospheric communities related to the biofilm bound or media associated bacterial communities? If spatial community variations in the wetland bed media exist, what effect do plants have on the community variations?

The objective of this study was to spatially investigate bacterial community in wetland mesocosm systems. Community level physiological profiling (CLPP) was used to study and compare bacterial communities in three planted and three unplanted mesocosm systems. Bacterial community samples associated with the pea gravel bed media taken from the top (10 cm depth), middle (30 cm depth), bottom (60cm depth) of all mesocosms are profiled and compared. Interstitial water communities are also profiled and compared to the pea gravel associated bacterial communities from all depths. The use of both planted and unplanted mesocosms enabled the comparison of the respective bacterial communities. Root associated

(rhizospheric) bacterial communities were also gathered from varying depths within the planted mesocosm systems and compared to the media-associated and interstitial community profiles.

5.2 Materials and Methods

5.2.1 Experimental Design

This study was carried out to evaluate the spatial distribution of bacterial communities in planted and unplanted mesocosm wetland systems. The study required sacrificing six mature and functional mesocosms. Of the mesocosms sacrificed, three were planted and three were unplanted (Table 5.1).

Table 5.1: Experimental Design – mesocosm designations

	Unplanted	Planted
Mesocosm Designation	1,4,6	2,5,7

Each mesocosm was constructed of schedule 80, polyvinylchloride (PVC) columns (90 cm by 25 cm Ø) filled to ~ 80 cm with pea gravel (80% limestone) and filled to 70 cm with water (Figure 5.1A). Water was circulated through the mesocosm with a 1/200 HP, 3200 rpm, March (Glenview, Illinois) series 1 (1A-MD 1/2) centrifugal pump.

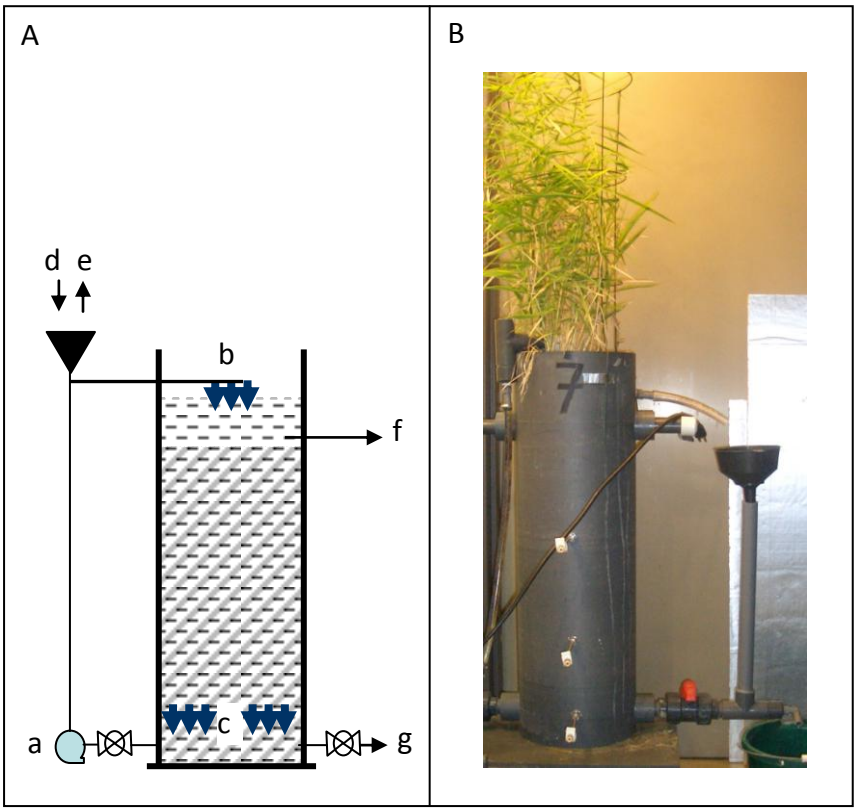


Figure 5.1: Mesocosm schematic (A) and photograph of a planted mesocosm (B). In (A) water (a) was circulated through the mesocosm via a small centrifugal pump and allowed to (b) percolate through the pea gravel bed to be collected at the bottom (c). An atmosphere exposed port served as an injection (d) and sampling (e) point. Drainage ports were located near the top to prevent overfilling (f), and near the bottom (g) for mesocosm drainage.

The six mesocosms studied contained a biotic regime associated with the pea gravel media. These six biotic mesocosms were originally seeded with activated sludge from the Waterloo Sewage Treatment Plant (Waterloo, ON) 5 years prior to the study. During the first 2 years the mesocosms underwent pathogen removal experimentation. The following year included AMD treatment experiments, with the last 2 years being a non-experimental years where simple operation and nutrient feeding occurred.

All six biotic mesocosms were fully developed microbiologically. Mesocosm maintenance and operation, prior to sacrificing, consisted of draining and refilling each mesocosm weekly with a

nutrient/water solution. The mineral nutrient solution used was according to Hoagland's and Arnon (1938). Nutrient solutions were mixed in regular tap water and fed to the unit wetlands giving interstitial concentrations of 28.75 mg/L $NH_4H_2PO_4$; 151.5 mg/L KNO_3; 236 mg/L $Ca(NO_3)_2 \cdot 4H_2O$; 123.25 mg/L $MgSO_4 \cdot 7H_2O$; 9.175 mg/L FeNaEDTA; 0.715mg/L H_3BO_3; 0.4525mg/L $MnCl_2 \cdot 4H_2O$; 0.055mg/L $ZnSO_4 \cdot 7H_2O$; 0.0125mg/L $CuSO_4$ and 0.005mg/L $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$.

Water was circulated at approximately 2.4 L/min, giving an average cyclic hydraulic retention time of approximately 4 minutes. All mesocosms were exposed to artificial illumination (14,000 lumens) with a 15 hour photoperiod. The mesocosms were housed in an indoor laboratory and subjected to common office room temperatures (20-25°C). Plants were sprayed daily with water to reduce drying

Wetland gravel samples were taken from a depth of 10 cm (top-T) (Figure 5.2A), a depth of 30 cm (middle-M) (Figure 5.2B), and a depth of 60 cm (bottom-B) (Figure 5.2C) in order to investigate the attached (fixed) bacterial communities associated with the wetland bed media in the top middle and bottom of all mesocosms. In the case of the planted mesocosms, representative root samples were also taken from depths of 10 cm, 30 cm and 60 cm although not all planted mesocosms had root mass at all depths. Mesocosm 2 contained root mass throughout the system, mesocosm 5 contained root mass to a depth of ~15 cm, and mesocosm 7 contained root mass to a depth of ~30 cm. Triplicate samples were taken from all regions. After collection, all samples were subjected to a detachment method in order to detach the bacterial community from the gravel or root samples for subsequent characterization.

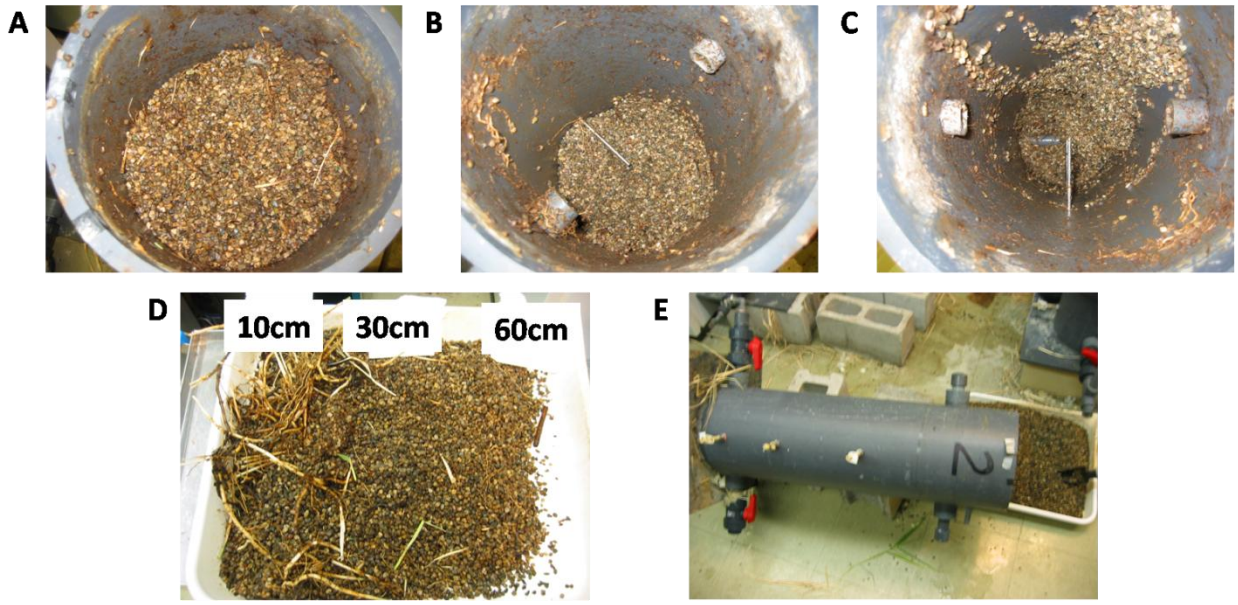


Figure 5.2: Mesocosm 2 gravel collected from depths of 10 cm (A) 30 cm (B) and 60 cm (C), with representative gravel and root samples (D). Mesocosm gravel was removed using a small gardening shovel and placed into large plastic buckets (E).

5.2.2 Detachment Method Protocol

The detachment protocol was performed using the method developed in Chapter 4. Each 25 g gravel sample of mesocosm pea gravel media was processed with 100 mL of shaking solution and shaken at 100 rpm in a 250 mL Erlenmeyer flask for 3 hr at 30°C. The shaking solution consisted of a 10mM phosphate buffer solution (PBS) at pH of 7 (1.547 g $Na_2HPO_4 \cdot 7H_20$ (BDH); 0.584 g $NaH_2PO_4 \cdot H_20$ (Sigma)) and 8.5 g NaCl (BDH) per liter of water. Shaking solution was made using an autoclaved deionized water. An enzyme mixture was also added that included lipase (50 units/g pea gravel), β-galactosidase (10 units/g pea gravel) and α-glucosidase (2 units/g pea gravel).

5.2.3 Total Solids, Organic and Inorganic Content

Total solids (TS) were determined as the dry weight after drying in ceramic crucibles at 105°C for 24 hours. Organic content (volatile solids) was determined based on the sample weight lost after muffle furnace treatment at 550°C for 15 min. Organics detached were determined for all

gravel and root samples. In general 25 g of gravel was used for the media samples; however, in most cases only 2-5 g of root mass was available for the root samples. Organic detachment is reported as a normalized value to the original sample weight of the gravel or root mass used [g organics detached/g of original sample (gravel or root mass)].

5.2.4 Community Level Physiological Profiling

The community level physiological profiling (CLPP) laboratory protocol was performed on all samples (detached and interstitial) using the methods outlined in Chapter 2. BIOLOG™ ECO plates were inoculated with 150 μL of mesocosm interstitial water and incubated at room temperature. The inoculants were set to an OD of ~0.19 as described by Weber *et al.* (2009). The BIOLOG™ ECO plates were then read at an absorbance of 590 nm after an 84 hr incubation period at room temperature.

5.3 Data Analysis

5.3.1 Community Level Physiological Profiling

The community level physiological profiling (CLPP) data analysis was performed using the methods outlined in Chapter 2 (Weber *et al.*, 2009). Absorbance readings (590nm) at 84 hrs were identified as the metric of choice for further CLPP data analysis. BIOLOG™ ECO plates (Biolog Inc., Hayward CA., USA) consist of 96 wells. The wells contain 31 different carbon sources, and a blank in triplicate. One plate was used for each interstitial water sample (6), for each wetland gravel sample from each of the three depths from all mesocosms (18), and for each root sample where appropriate (6), giving a total of 32 plates. Each plate represents triplicate samples, yielding a total of 96 objects used for analysis.

The absorbance values from 84 hr were initially standardized by first correcting by the corresponding blank value and then dividing by the average well colour development (AWCD)

for that time point. Assessment of normality, homoscedasticity and linear correlations within the entire data set was performed according to Weber *et al.* (2007), and yielded a recommended natural logarithmic transformation for subsequent PCA. At each step some points were designated as outliers and removed in order to analyze the remaining data. A maximum of 2 of the 96 objects were removed for any single analysis. Outliers occurred due to excessively high readings for blanks yielding negative responses for all wells. Negative well responses were coded as zeros during data treatment, and therefore the outlier data sets were simply removed from the analysis rather than keeping the discussed 0 response plates. PCA was completed using Statistica 8.1.

5.3.2 Clustering Analysis

UPGMA clustering analysis, based on the Euclidean distances, was performed on the logarithmic transformed BIOLOG™ ECO plate data in accordance with Legendre and Legendre (1998).

5.3.3 Substrate Related Diversity Indices

As first suggested by Zak *et al.* (1994), BIOLOG™ plates can also be used in a more traditional ecological sense, to calculate diversity indices based on carbon source utilization patterns (CSUPs). The Shannon index or what is often called “diversity” is a common ecological metric used to track and understand shifts in communities over space and time. Using the CSUP gathered from a single BIOLOG™ plate, substrate diversity (H) can be calculated as:

$$H = -\sum p_i \ln(p_i) \qquad (5.1)$$

where:

H - substrate diversity

p_i - ratio of the activity of a particular substrate to the sums of activities of all substrates

activity - chosen metric for analysis (absorbance value (590nm) at 84hrs)

Two other parameters associated with substrate diversity which can be calculated using CSUPs are substrate richness (S) and substrate evenness (E). Substrate richness is a measure of the number of different substrates utilized by a microbial population. Substrate evenness is defined as the equitability of activities across all utilized substrates; substrate richness is calculated as the number of wells with a corrected absorbance greater than 0.25. Substrate evenness is calculated as:

$$E = H / H_{\max} \tag{5.2}$$

Substrate diversity, evenness and richness were calculated using the average response from the three replicates on any one plate to give a single result for each plate. Recent examples of studies utilizing the Shannon index calculated from CSUPs gathered using BIOLOG™ plates include He *et al.* (2008), Farnet *et al.* (2008), and Weber *et al.* (2008).

5.4 Results and Discussion

Figure 5.3A provides the results for the amount of organics detached from the gravel samples from the top (10 cm), middle (30 cm) and bottom (60 cm) sections for each mesocosm. There does not appear to be a difference in the organic content from the different depths of the mesocosms (Figure 5.3A). However, it appears that there is a general decrease in organics

detached from 10 cm to 30 cm; this was not the case for all systems. No statistically significant (95% CL using a t-test at each depth) difference or qualitative (visual) difference was apparent between the amount of organics in the planted and unplanted systems (Figure 5.3A).

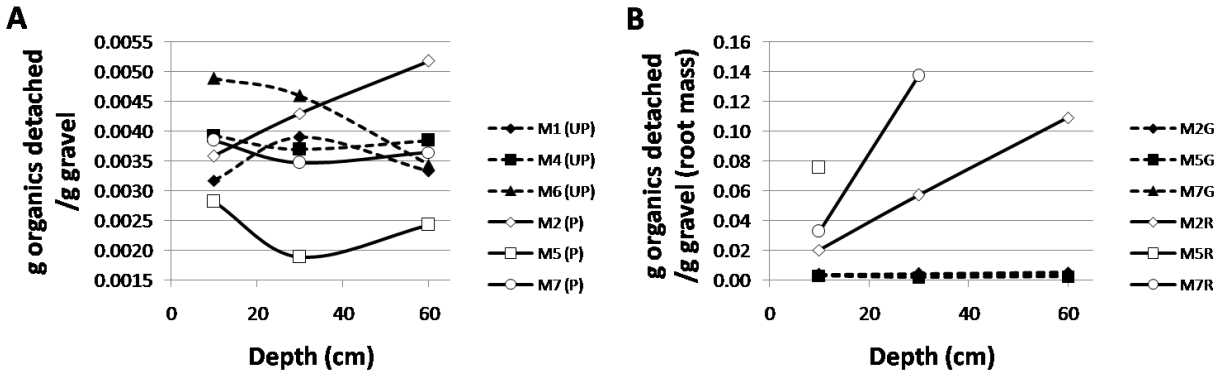


Figure 5.3: (A) Depth profiles for detached organic matter for all planted (P) and unplanted (UP) mesocosm systems. (B) Depth profiles for detached organic matter for all gravel (G) and root (R) samples for the three planted mesocosm systems.

A summary of the amount of organics from the root and gravel samples from different depths is presented in Figure 5.3B. Here a definite trend can be seen. The root samples from the lower depths showed a higher organic content than the root samples from the top. This is most likely due to a difference in the root types found at the different mesocosm depths. The lower sections tended to consist of a larger amount of fibrous roots where the upper sections consisted mostly of larger root stalks with less fibrous root material. The fibrous material possesses a substantially larger surface area than the larger root stalks, likely accounting for the greater organic detachment observed. There was also a large difference in the organics detached from the roots compared to the gravel samples. This may be due to the biofilm surrounding the root structures and a generally larger surface area due to the root structures associated with the samples.

Figure 5.4A summarizes the average well colour development (AWCD) collected via BIOLOG™ ECO plates from the top (10 cm) middle (30 cm) and bottom (60 cm) section gravel samples for

each mesocosm. AWCDs for gravel and root samples were normalized by the original sample weight used. No normalization was performed for the interstitial water samples. Figure 5.4B summarizes the AWCDs found for the gravel and root samples for the planted mesocosms at all depths.

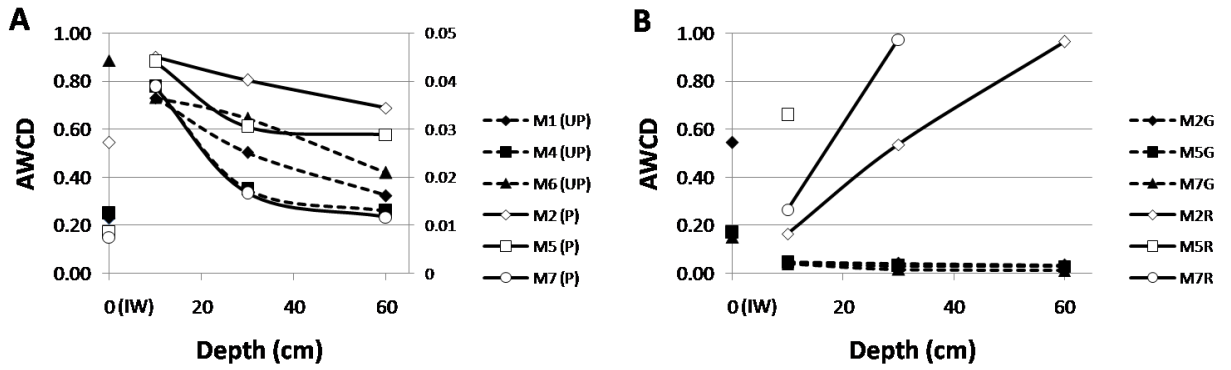


Figure 5.4: (A) Depth profiles for average well colour development (AWCD) for all planted (P) and unplanted (UP) mesocosm systems. Left axis (primary) corresponds to the interstitial water samples, right axis (secondary) corresponds to the gravel and root samples. (B) Depth profiles for average well colour development (AWCD) for all gravel (G) and root (R) samples for the three planted mesocosm systems. Zero cm represents the interstitial water.

A couple of trends were evident. The normalized AWCD generally decreased for all mesocosm gravel samples with increasing depth (Figure 5.4A). It is often found that the area of high microbial activity is found within the top 10 cm of laboratory wetland systems (Truu *et al.*, 2009; Tietz *et al.*, 2007). These findings agree with this general trend. It should be noted however that although the top 10 cm gravel samples had the highest reported activities, all depths within the mesocosm were relatively similar.

A marked difference in the AWCDs for the gravel and root samples was observed (Figure 5.4B). The root samples had much higher normalized AWCDs than the gravel samples, and the AWCDs for the root samples increased with increasing depth. The root samples likely have higher

AWCDs again due to the nature of the root environment. This result is an agreement with several other studies which have also shown densities and activities of bacterial populations to be higher in the plant rhizosphere regions (Collins *et al.*, 2004; Gagnon *et al.*, 2007). The favorable environment offered by increased oxygen and carbohydrates exudates associated with the plant roots are perhaps directly responsible for enhanced bacterial development in root environments (Karathanasis & Johnson 2003; Walker *et al*., 2003; Batty *et al*., 2000; Marschner, 1995).

Substrate richness values from the top, middle and bottom sections for each mesocosm are presented in Figure 5.5A. Figure 5.5B summarizes the richness values found for the gravel and root samples for the planted mesocosms at all depths. Richness results are essentially very similar for all mesocosm and sample types. There is a trend of decreasing richness with increasing depth which suggests that the number of bacterial species inhabiting the mesocosm media/biofilm environment is decreasing with depth in the system.

Also noteworthy (Figure 5.5) is the greater substrate richness for the gravel and root samples in comparison to the interstitial water samples. This suggests that there is a greater diversity of bacterial species in the fixed biofilm environment when compared to the interstitial water of the mesocosms.

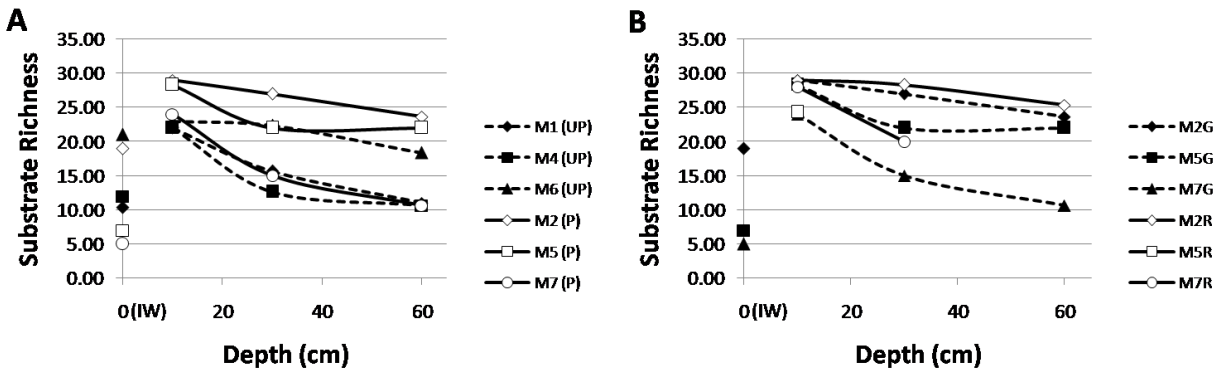


Figure 5.5: (A) Depth profiles for substrate richness for all planted (P) and unplanted (UP) mesocosm systems. (B) Depth profiles for substrate richness for all gravel (G) and root (R) samples for the three planted mesocosm systems. Zero cm represents the interstitial water.

Figure 5.6A summarizes the substrate evenness values for the top, middle and bottom sections from each mesocosm. Figure 5.6B summarizes the evenness values for the gravel and root samples for the planted mesocosms at all depths. Evenness results are essentially very similar for all mesocosm and sample types. A trend showing a slight decrease in evenness with depth is apparent for all mesocosm systems and all sample types. Figure 5.6 also shows the evenness of all interstitial water samples to be within the range found for the gravel and root samples.

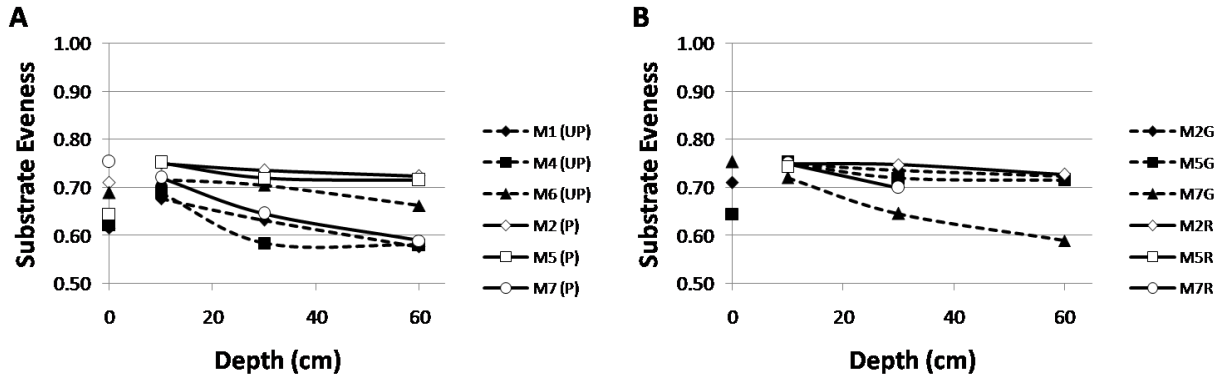


Figure 5.6: (A) Depth profiles for substrate evenness for all planted (P) and unplanted (UP) mesocosm systems. (B) Depth profiles for substrate evenness for all gravel (G) and root (R) samples for the three planted mesocosm systems. Zero cm represents the interstitial water.

Figure 5.7A summarizes the substrate diversity values collected via BIOLOG™ ECO plates from the top, middle and bottom sections for each mesocosm. Figure 5.6B summarizes the diversity values found for the gravel and root samples for the planted mesocosms at all depths. Diversity can be explained as a mixture of richness and evenness, and is often used as a surrogate measure for community health. Communities with greater diversities are often able to better adapt to changing and/or non ideal situations or environments.

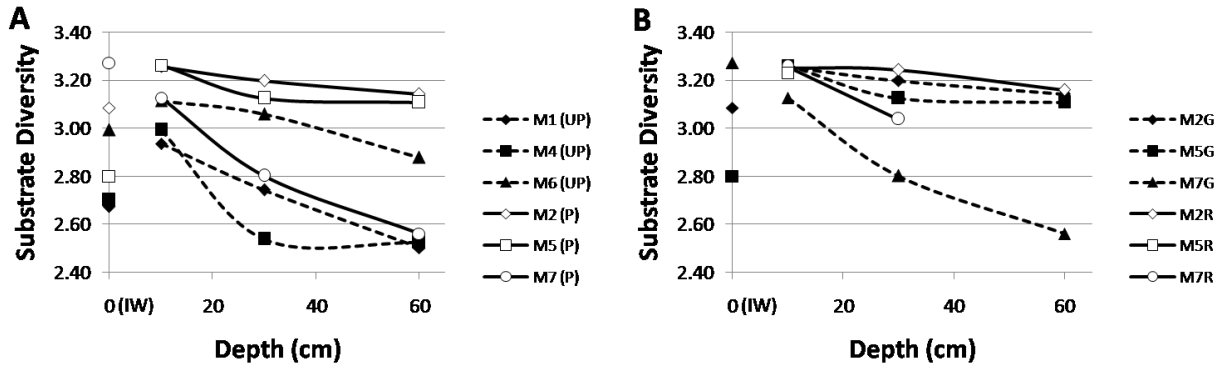


Figure 5.7: (A) Depth profiles for substrate diversity for all planted (P) and unplanted (UP) mesocosm systems. (B) Depth profiles for substrate diversity for all gravel (G) and root (R) samples for the three planted mesocosm systems. Zero cm represents the interstitial water.

The diversity trend results found here are essentially very similar for all mesocosm and sample types. In general, the diversity values for the different gravel and root samples decrease with increasing depth, suggesting that the health of the attached bacterial communities within the mesocosm systems is decreasing with increasing depth. This result is supported by the discussion regarding the trend of decreasing richness and evenness with increasing depth, and is in agreement with the results of Truu *et al.* (2005), who also found a decrease in bacterial diversity with increasing bed depth. Diversity results of the interstitial water samples tend (in most cases) to be equal to about that of the middle depth gravel samples.

In addition to the substrate diversity related information, the CSUPs derived from the plate data were also used to generate a number of different PCA ordinations. PCA is most often used

to investigate difference between objects. For example, the CSUPs from the top gravel samples of all mesocosms could be compared to the CSUPs of the middle gravel samples for all mesocosms. Alternatively, the top gravel samples from the planted mesocosms could be compared to the top gravel samples of the unplanted mesocosms, or to the middle gravel samples of either the planted or unplanted mesocosms. One needs to generate separate PCA ordinations for each type of comparison. The number of PCA ordinations which could be generated from this data set is extremely large and therefore not all ordination combinations will be presented here. A select number of representative ordinations and dendograms will be presented which underline the main relationships found after examining a total of 50 PCA ordinations and dendograms.

Figure 5.8 displays a PCA ordination of the interstitial water samples taken from all mesocosms before being sacrificed. As can be seen, the bacterial communities within the mesocosm interstitial waters can be separated into two groups: communities from planted mesocosms and communities from unplanted mesocosms. This separation is apparent although some overlap between groups does exist. This finding agrees with previous findings (Weber *et al.* 2008) and can be explained by the oxygen concentration and carbohydrates exuded into the rhizospheric space of the planted mesocosms affecting the resident bacterial communities.

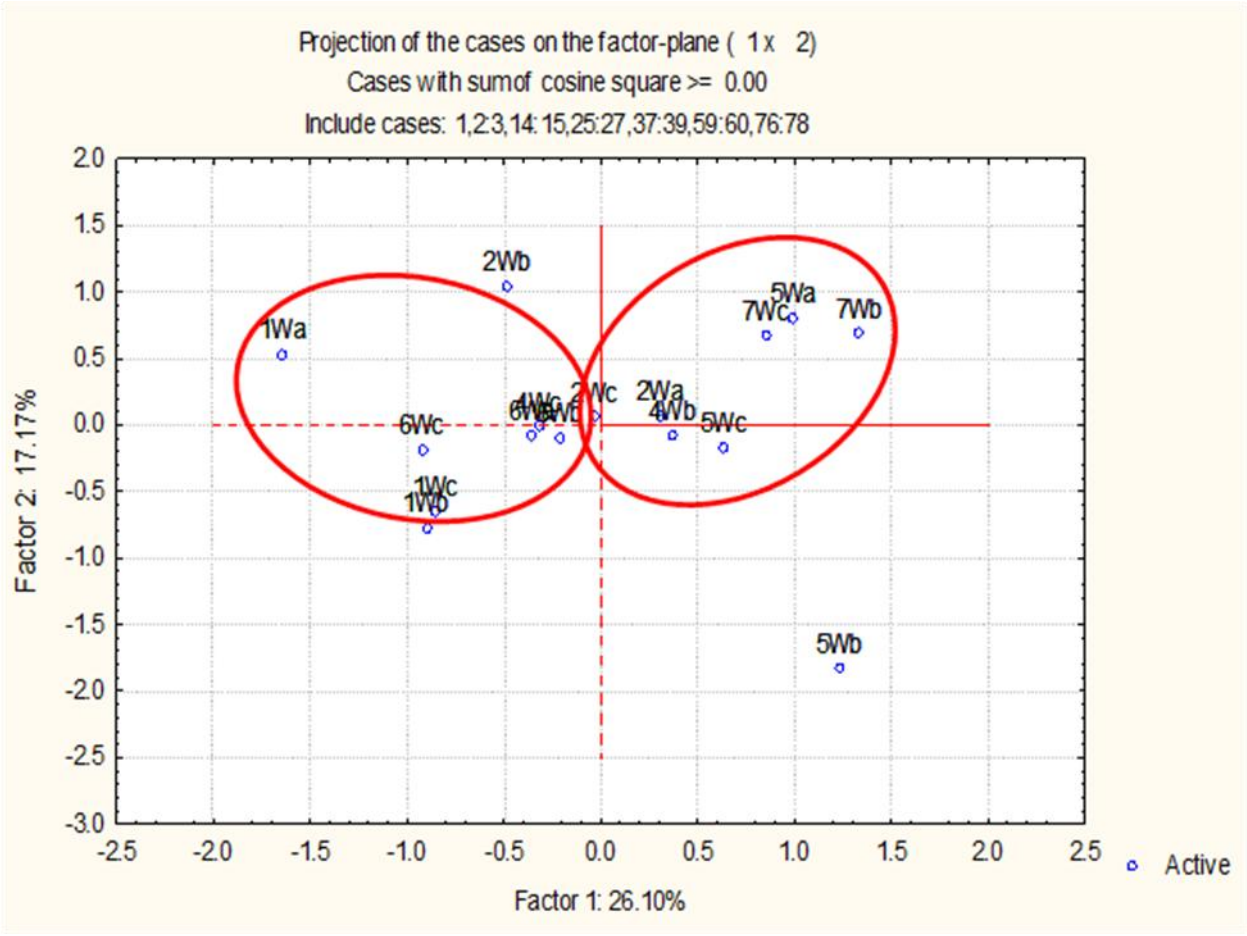


Figure 5.8: PCA ordination of the interstitial water (W) sample CSUPs gathered via BIOLOG™ ECO plates for all mesocosms (1,4,6 - unplanted), (2,5,7 - planted). a, b, c - triplicate sample designations. Ovals represent manual groupings. Output generated using Statistica 8.1.

Figures 5.9, 5.10, and 5.11 display PCA ordinations of the top, middle, and bottom gravel samples for all mesocosms, respectively. Similar to the interstitial water ordination, the bacterial communities at each depth can be separated into two groups: communities from planted mesocosms and communities from unplanted mesocosms. Again this separation is apparent although with some overlap between groups in each ordination. This finding is significant in a number of ways. First, it can be said that the general difference in mesocosm bacterial communities based on CSUPs is similar in the interstitial water and at all depths in the mesocosm based on the analysis of detached communities from the gravel samples. This suggests that when trying to decipher community differences between mesocosm systems via

the CLPP interstitial water samples can give the same type of information that gravel associated community samples can. Deciphering community differences using interstitial water samples has a number of advantages over the use of sediment/gravel samples; interstitial water samples take less time to gather, interstitial water sampling is non-disruptive and/or non-destructive, and interstitial water samples do not require any further processing such as a detachment protocol. Second, the general separation based on plant presence is found throughout the entire depth of the mesocosm gravel media; this observation holds despite the fact that the roots in mesocosms 5 and 7 did not visibly penetrate to the bottom of the mesocosm. This suggests that the presence of roots within at least part of the mesocosm system does not only have a localized effect on the attached bacterial population but on attached bacteria from all depths within the mesocosms. This may in part be due to the small size of the mesocosm, allowing for a relatively even distribution of plant exudates. This finding does suggest that the presence of plants in constructed wetland mesocosms, and perhaps in larger scale constructed wetland systems, may have a more profound influence on the bacterial populations than previously assumed.

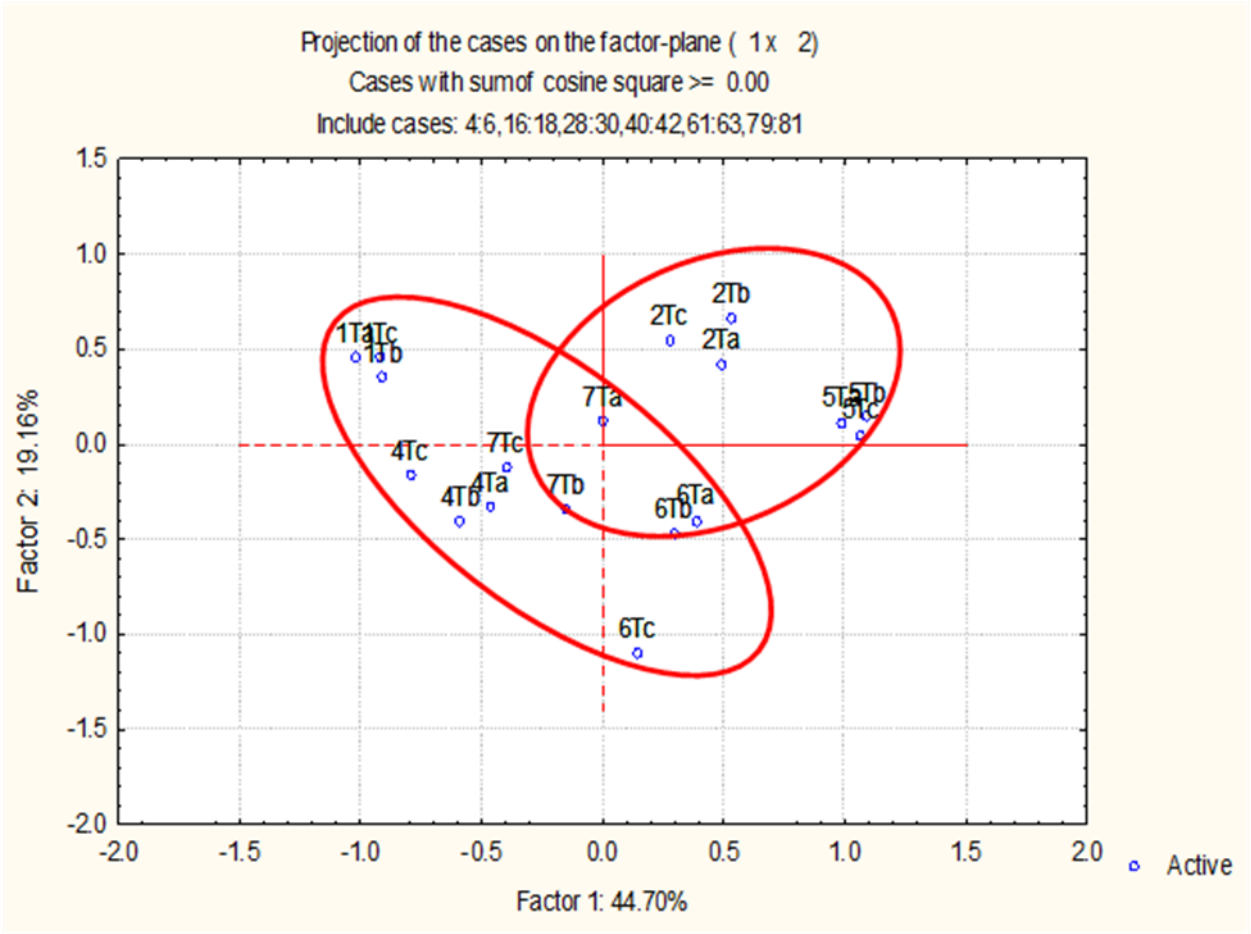


Figure 5.9: PCA ordination of the 10 cm depth (top-T) sample CSUPs gathered via BIOLOG^{TM} ECO plates for all mesocosms (1,4,6 - unplanted), (2,5,7 - planted). a, b, c - triplicate sample designations. Ovals represent manual groupings. Output generated using Statistica 8.1.

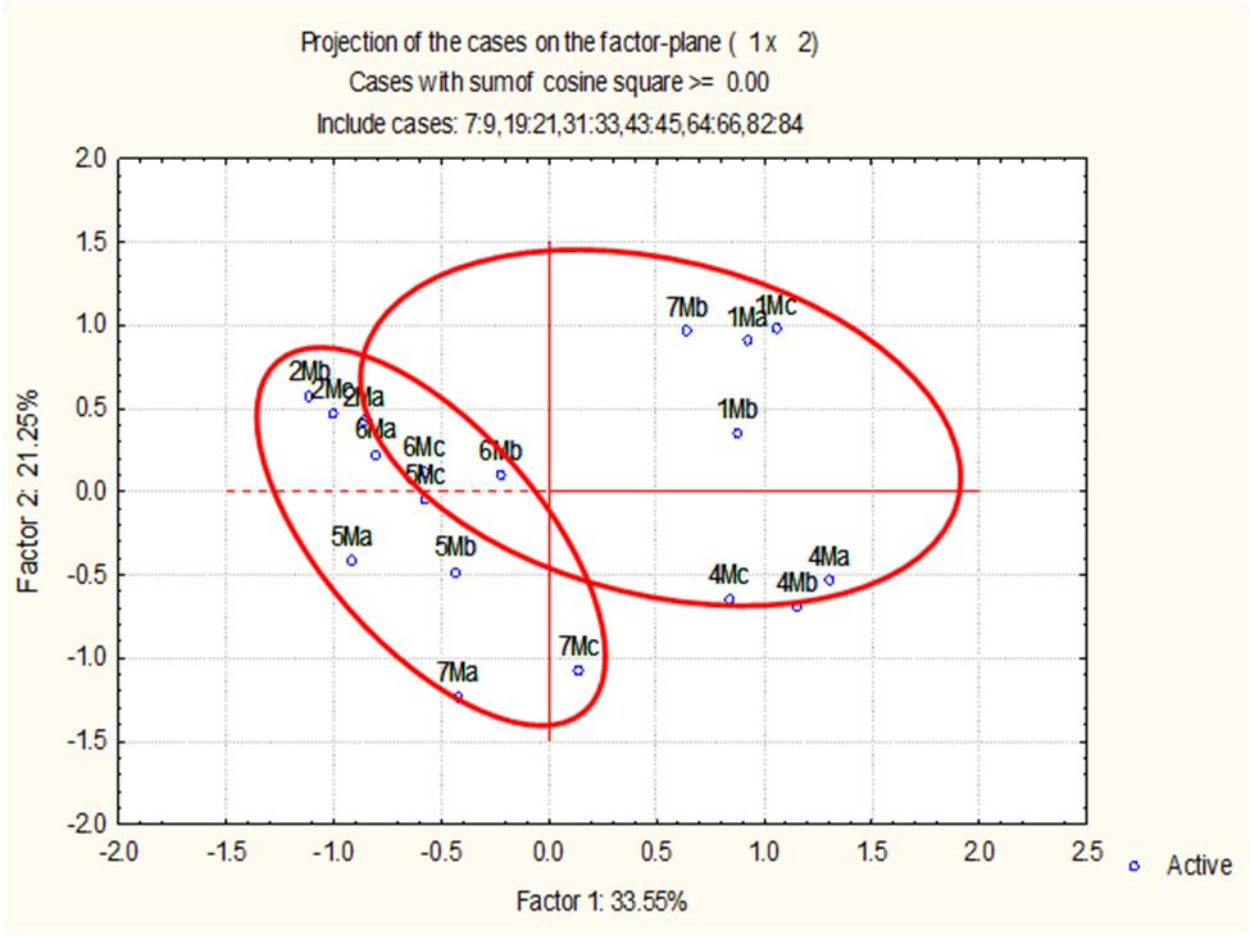


Figure 5.10: PCA ordination of the 30 cm depth (middle-M) sample CSUPs gathered via BIOLOG™ ECO plates for all mesocosms (1,4,6 - unplanted), (2,5,7 - planted). a, b, c - triplicate sample designations. Ovals represent manual groupings. Output generated using Statistica 8.1.

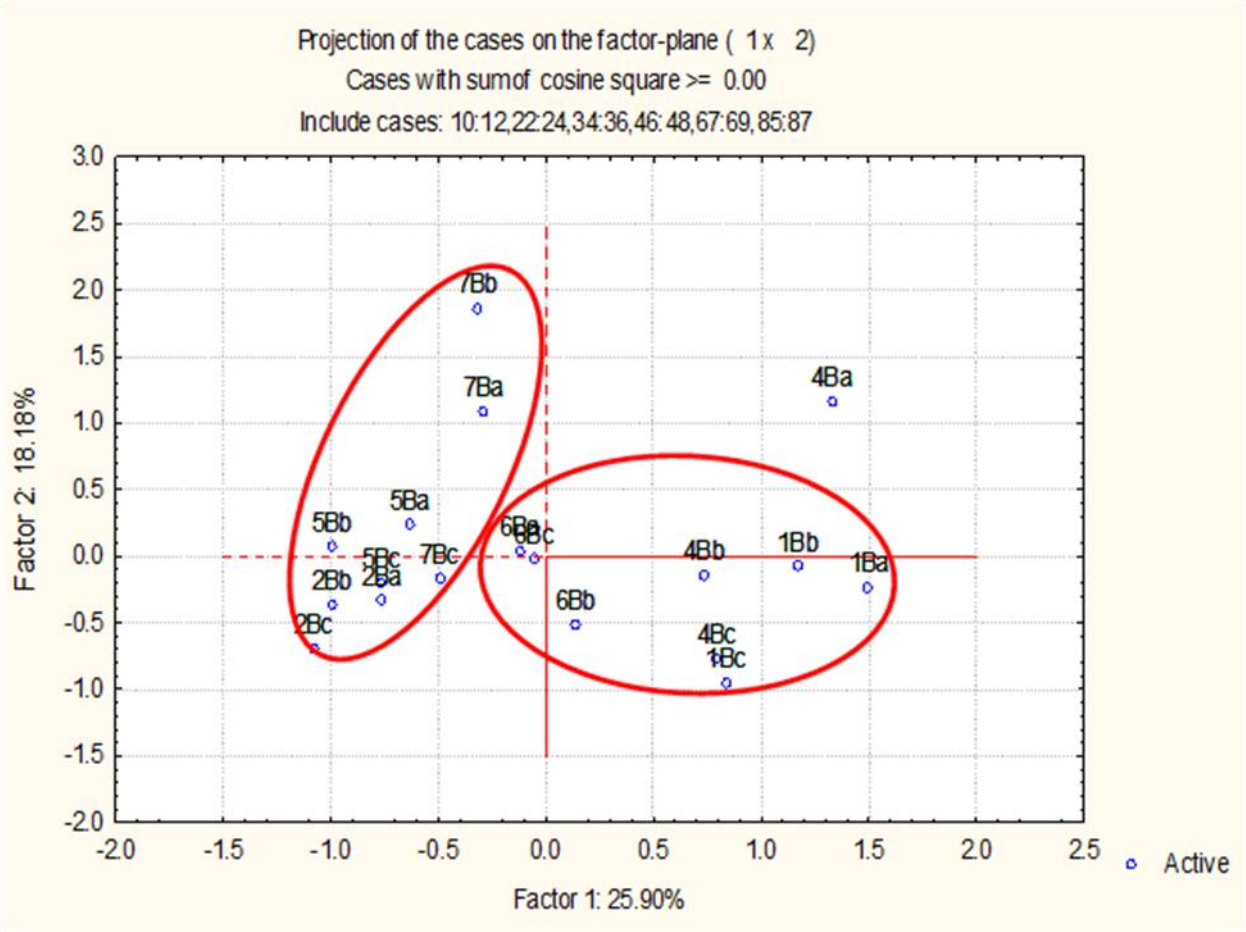


Figure 5.11: PCA ordination of the 60 cm depth (bottom-B) sample CSUPs gathered via BIOLOG™ ECO plates for all mesocosms (1,4,6 - unplanted), (2,5,7 - planted). a, b, c - triplicate sample designations. Ovals represent manual groupings. Output generated using Statistica 8.1.

Figure 5.12 presents the PCA ordination results for the interstitial water (IW), and the top (T), middle (M), and bottom (B) gravel samples for all mesocosms. This ordination contains a large amount of data, perhaps too much data to make multiple grouping statements, however one thing is clear, the interstitial water samples form a separate group from all of the gravel samples. This suggests that although the type of comparative information imparted by comparing either IW or gravel community samples is the same, the interstitial water samples are different from the gravel/sediment samples. Therefore, although the bacterial communities in the planted mesocosm media are different from the communities found in the unplanted mesocosm media, the difference between the interstitial water samples and the generalized

media samples for all mesocosms is more profound. These findings were expected as certain bacteria are found to prefer free water existence while other types of bacteria often prefer to live in an attached or "biofilm" environment (Faulwetter *et al*., 2009).

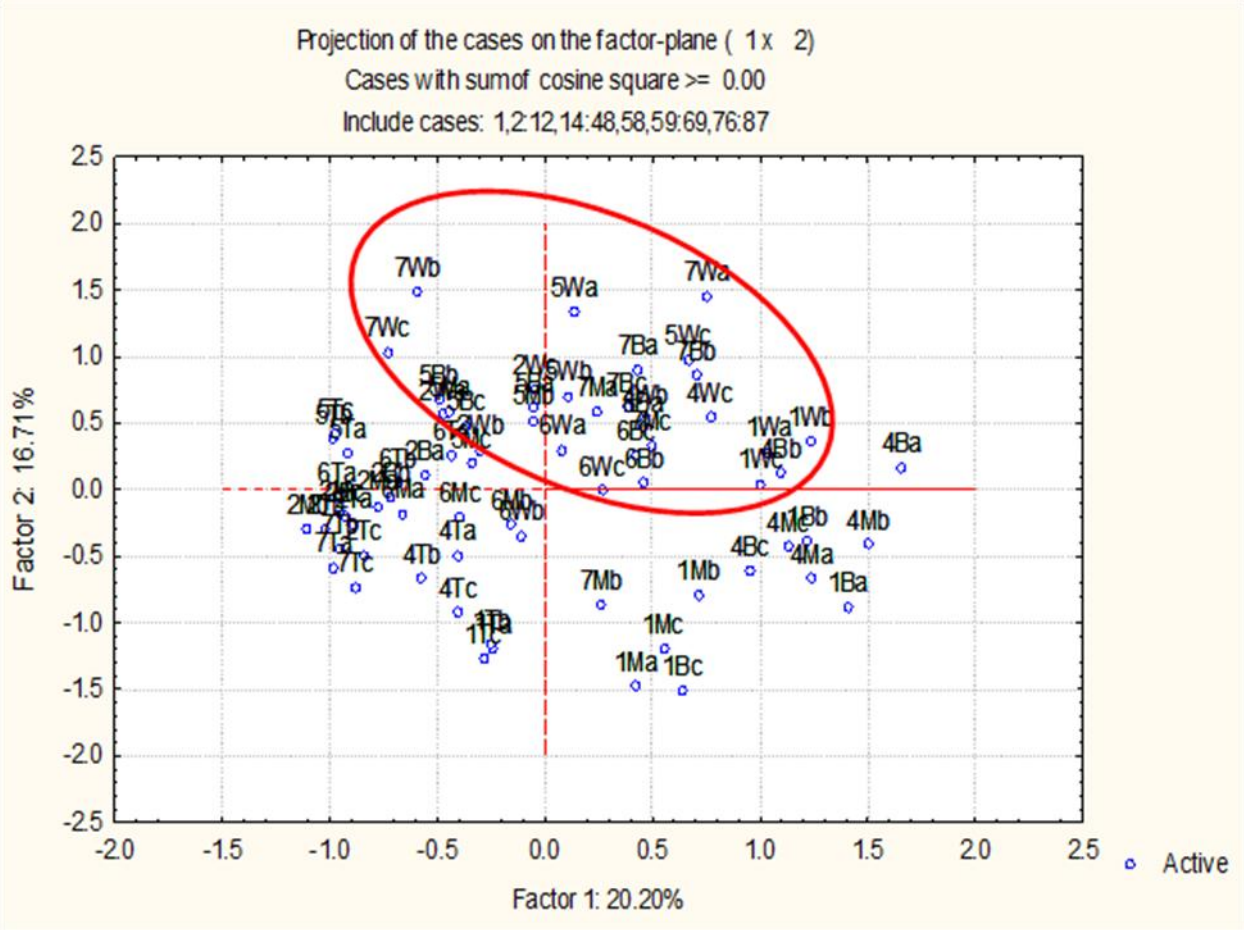


Figure 5.12: PCA ordination of the top (T), middle (M), bottom (B) and interstitial water (W) sample CSUPs for all mesocosms (1,4,6 - unplanted), (2,5,7 - planted). a, b, c - triplicate sample designations. Ovals represent manual groupings. Output generated using Statistica 8.1.

As previously discussed and as shown with the PCA ordinations, a definite difference in the bacterial communities can be seen in the mesocosm interstitial waters and at all depths based on plant presence. This study also looked to define if any spatial community differences occurred with depth in each mesocosm, therefore several unweighted pair-group average

(UPGMA) dendograms based on the Euclidean distances between CSUPs were generated. Figure 5.13 summarizes the similarities of the CSUP samples taken for mesocosm 1 (unplanted). Figure 5.13 shows the top gravel samples, to be most similar to the middle gravel samples, which are then clustered with the bottom samples. The sediment samples are then grouped with the interstitial water samples. This finding suggests that differences in the bacterial communities occur with depth; with the top samples being more similar to the middle samples (in comparison to the bottom samples). As well, the interstitial water samples are found to be clustered last with the larger media sample groupings, which supports the findings gleaned from Figure 5.12.

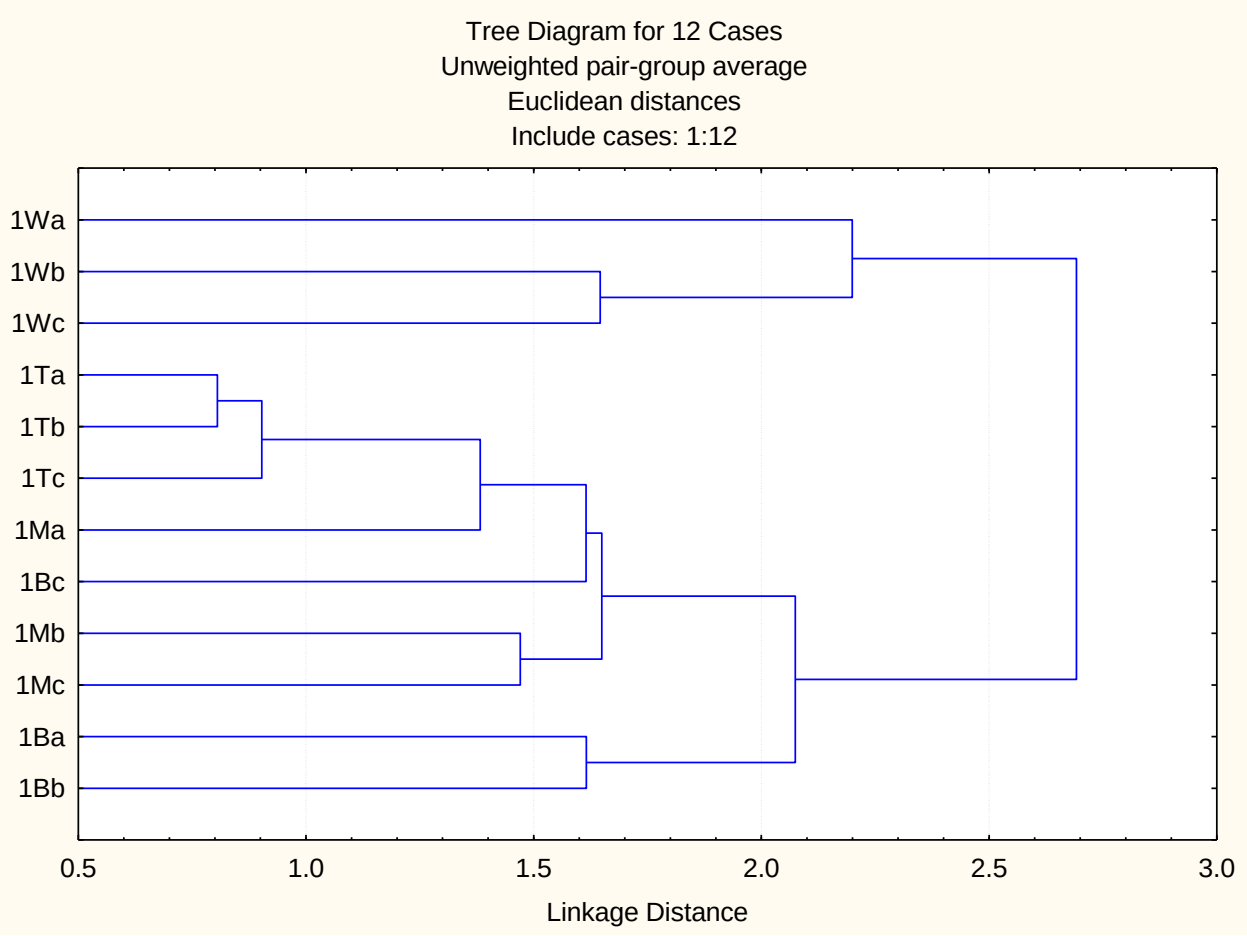


Figure 5.13: UPGMA clustering analysis dendogram for top (T), middle (M), bottom (B) and interstitial water (W) samples CSUPs for mesocosm 1 (unplanted). a, b, c - triplicate sample designations. Output generated using Statistica 8.1.

Figure 5.13 summarizes the vertical community gradient for an unplanted mesocosm, to see if the same type of relationship exists in a planted mesocosm Figure 5.14 is presented. Figure 5.14 summarizing the results for a planted mesocosm, mesocosm 2. The results summarized in Figure 5.14 are very similar to those found for the unplanted mesocosm summarized in Figure 5.13; the top gravel samples are found to be most similar to the middle gravel samples, which are then clustered with the bottom samples. The sediment samples are then lastly grouped with the interstitial water samples. This finding again suggests that differences in the bacterial communities occur with depth; with the top samples being more similar to the middle samples (in comparison to the bottom samples), and the same vertical relationship exists for both planted and unplanted mesocosms

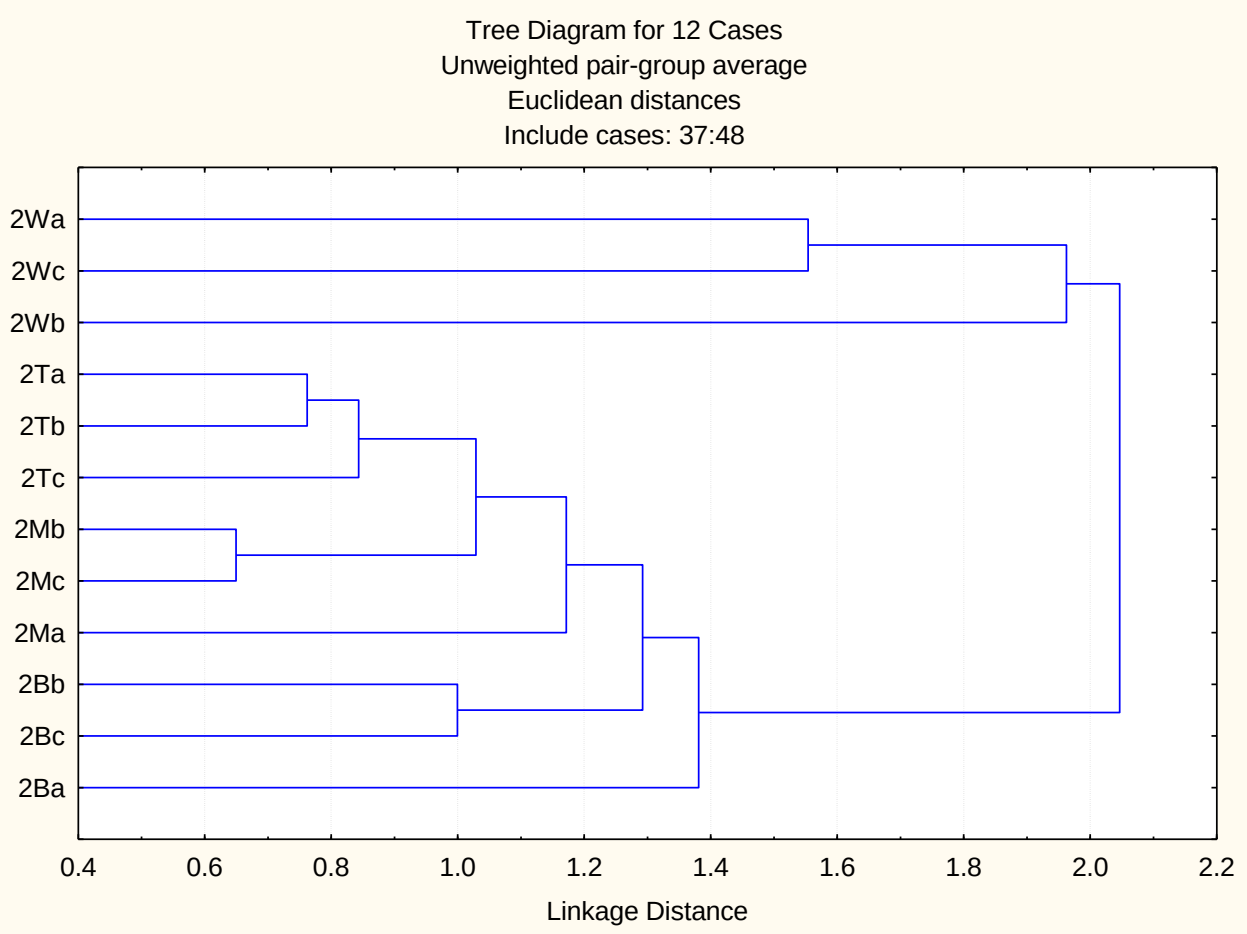


Figure 5.14: UPGMA clustering analysis dendogram for top (T), middle (M), bottom (B) and interstitial water (W) sample CSUPs for mesocosm 2 (planted). a, b, c - triplicate sample designations. Output generated using Statistica 8.1.

For the sake of brevity, dendograms for all mesocosms are not recounted here; however, it should be noted that the vertical community stratification summarized in Figures 5.13 and 5.14 is seen for all mesocosms studied.

Figure 5.15 summarizes the data in Figure 5.14, however also includes the root samples. Figure 5.15 shows the gravel and interstitial samples to be grouped in the same way as seen in Figure 5.14. The added root samples do not seem to follow any simplified trend. The root samples did not seem to correlate with their corresponding depth gravel samples, and the root samples did not seem to show any clear vertical gradient. The bottom root samples grouped well with the gravel samples, however were also shown to be similar to the top (T) root samples. One of the top (T) root samples and the middle (M) root samples were dispersed throughout the gravel sample groupings. This suggests that no clear vertical trend and no clear root-sample-to-gravel-sample correlation can be made for mesocosm 2.

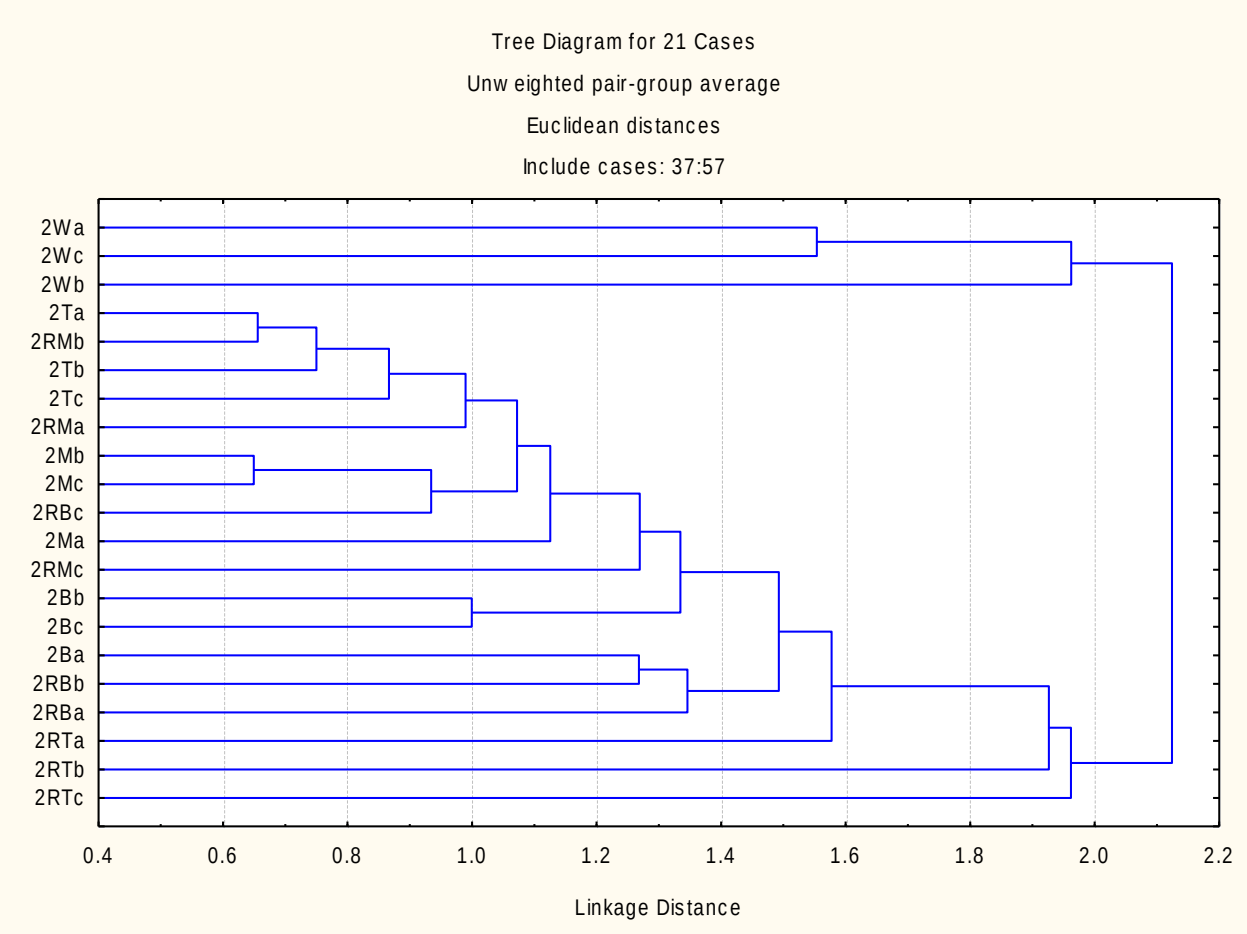


Figure 5.15: UPGMA clustering analysis dendogram for top (T), middle (M), bottom (B) and interstitial water (W) sample CSUPs for mesocosm 2 (planted) gravel and root (R) samples. a, b, c - triplicate sample designations. Output generated using Statistica 8.1.

Figure 5.16 presents a dendogram for only the root zone samples of mesocosm 2. Similar to Figure 5.15, no defined groupings based on sample depth could be found. This finding suggests that perhaps all of the root samples within mesocosm 2 were in fact quite individually defined due to localized environments created in the rhizospheric zone.

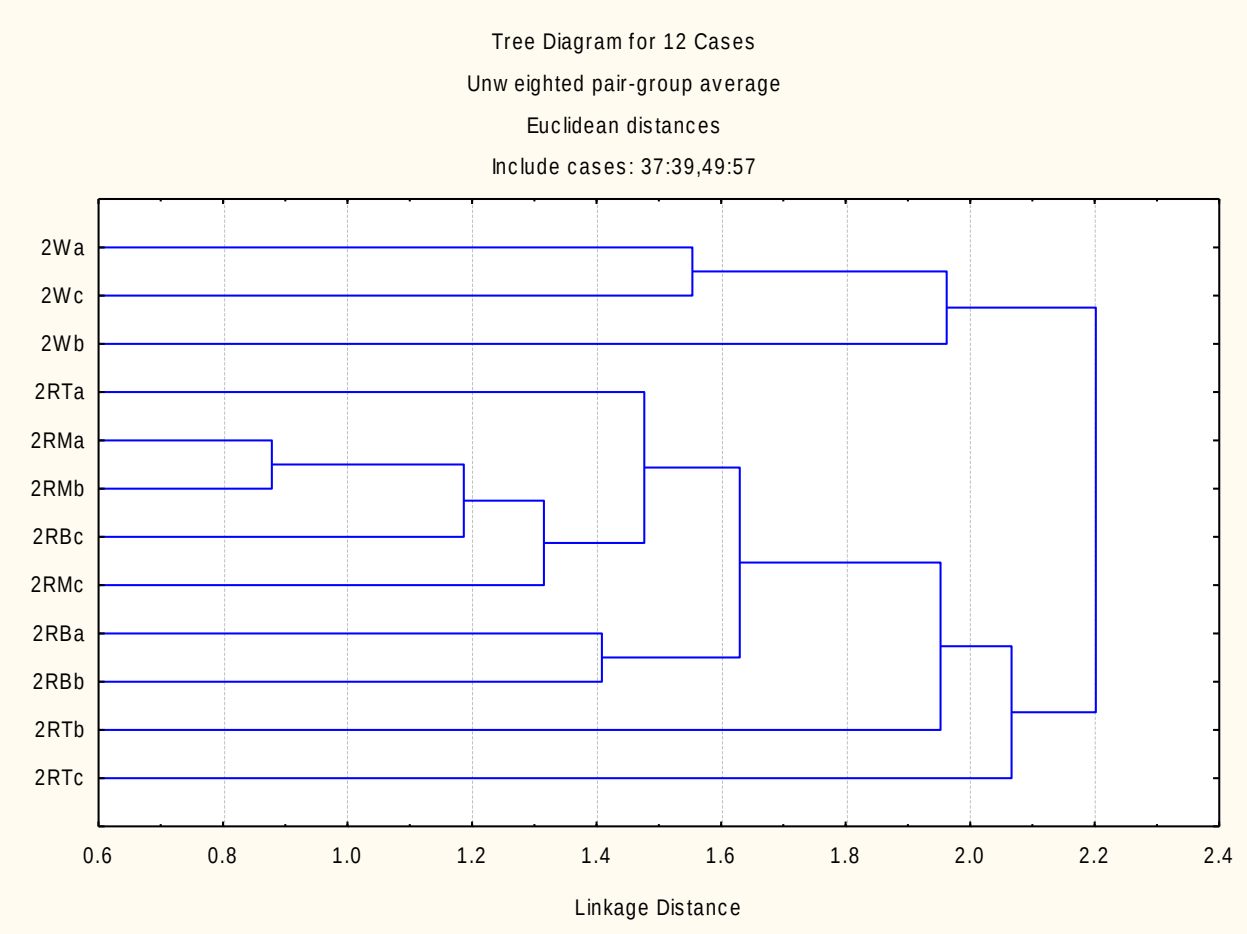


Figure 5.16: UPGMA clustering analysis dendogram for top (T), middle (M), bottom (B) and interstitial water (W) sample CSUPs for mesocosm 2 (planted) root (R) samples. a, b, c - triplicate sample designations. Output generated using Statistica 8.1.

Figure 5.17 summarizes the results for all samples gathered for mesocosm 7 (planted). Mesocosm 7 did not have root mass in the lower depths (i.e. 35cm+) however did have considerable root mass in the top and middle sections of the bed media. Figure 5.17 is different from the results shown in Figure 5.15 and 5.16 for mesocosm 2, in that definite similarities between root samples and their corresponding depth gravel samples can be seen.

As previously discussed the results from Figure 5.2 showed variable organic detachment with depth, perhaps the fact that these mesocosms have been used for a number of research

endeavors, including pathogen removal and acid mine drainage treatment experiments, could partially account for the conflicting results seen for the root samples from these two planted mesocosms. Perhaps, the varied experimental programs and uses which each individual mesocosm underwent over their 5 year life time created the individualized profiles observed within each system.

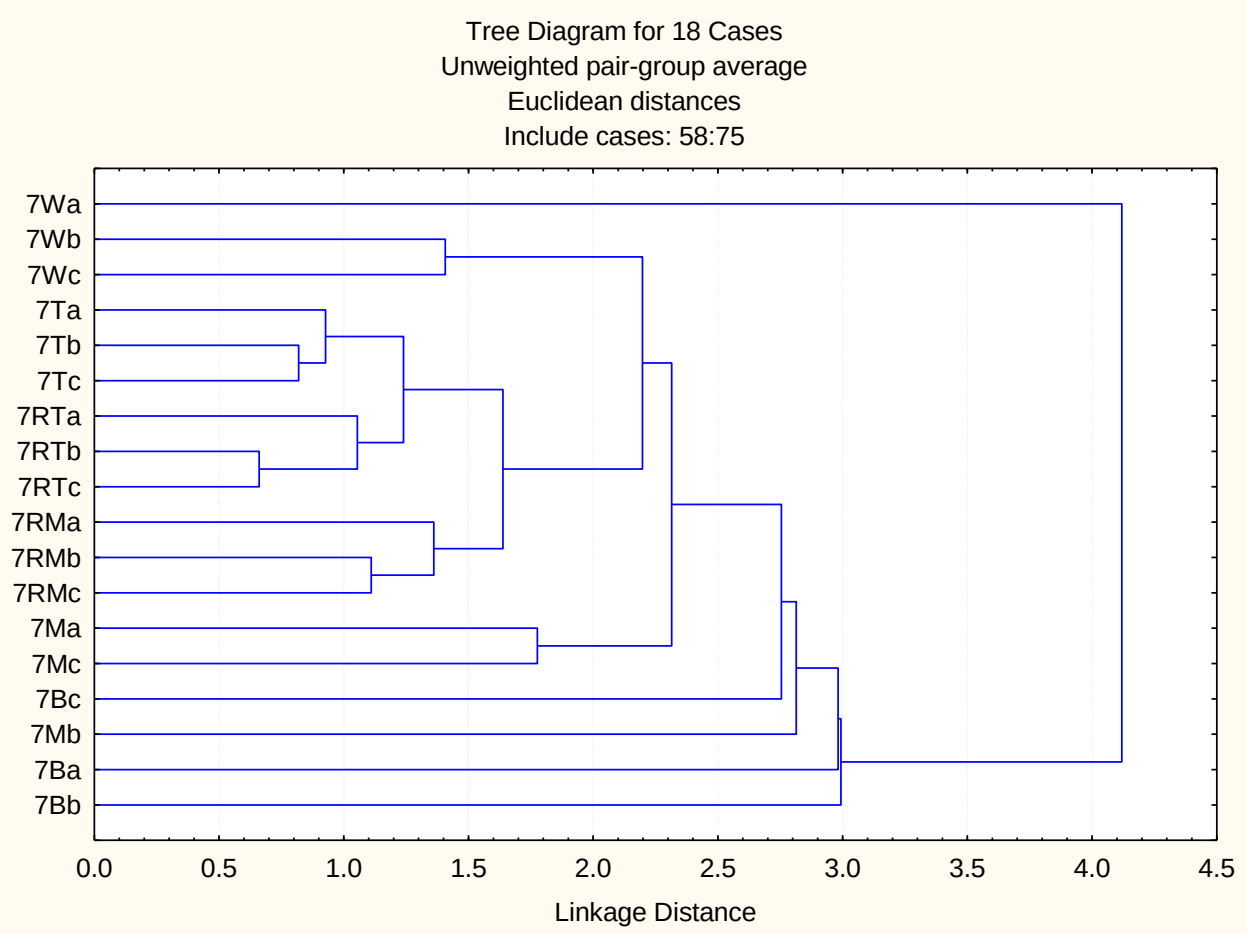


Figure 5.17: UPGMA clustering analysis dendogram for top (T), middle (M), bottom (B) and interstitial water (W) sample CSUPs for mesocosm 7 (planted) root (R) samples. a, b, c - triplicate sample designations. Output generated using Statistica 8.1.

5.5 Conclusions

No trend could be seen between organics detached from the gravel media with depth, suggesting the mesocosms systems to have been well developed with evenly distributed organic deposition and biofilm networks throughout the mesocosm systems. The amount of organics detached from the root samples increased with an increase in bed depth. This was likely due to an increase in the fibrous nature of the roots deeper in the bed.

A decrease in the substrate richness and diversity values was seen with increasing depth in the mesocosm. A decrease in the activities of the detached communities (measured as normalized AWCD) was also seen with increasing depth, suggesting the communities in the lower depths of the bed media to be less active than those near the mesocosm surface. Also, the activities of the root associated (rhizospheric) bacterial communities were much higher than the activities seen for the gravel associated bacterial communities. This may imply that the rhizospheric bacteria, although less abundant in the mesocosm, perhaps play a disproportionally large role in the removal and fate of water born contaminants.

It was shown that the information obtained on the general differences in mesocosm bacterial communities based on CSUPs is similar in the interstitial water and at all depths in the mesocosm. This suggests that when trying to decipher general community differences between mesocosm systems via the CLPP method, interstitial water samples may give the same type of information that would be obtained if one were to analyze the fixed microbial regime.

Not all planted mesocosms developed root systems throughout the entire bed depth. The fact that a general differences in the communities from planted and unplanted mesocosms was found at all depths, suggests that the presence of roots within at least part of the mesocosm system does not only have a localized effect on the attached bacterial population but on attached bacteria at all depths within the mesocosms.

Differences in the bacterial community structure, measured via the CLPP method, as a function of gravel depth was seen for all mesocosm systems. A vertical community-structure stratification was observed in all cases. A stratification was seen for the root samples in mesocosm 7, however no such trend was seen in mesocosm 2. Similarly the root sample communities within mesocosm 7 were shown to group well with their respective gravel depth samples; however, this trend was not seen in mesocosm 2. These conflicting results could perhaps be a consequence of the differing experimental histories of each mesocosm from their 5 year operational period.

Chapter 6: Bacterial Community Profiling and Hydrological Characterization of Constructed Wetland Mesocosms during Start-up

Overview

Understanding and explaining the large performance variations seen in similarly designed treatment wetland systems is a crucial step in moving constructed treatment wetland systems from its current description as an "alternative" treatment option to a more widely accepted technology. The objective of this study was to track bacterial community and hydrological changes in wetland mesocosms during an 8 month start-up period. Eight mesocosms were studied based on a duplicated 2^2 factorial design looking at the effect of plant presence and inoculum origin on mesocosm start-up dynamics. The mesocosms were characterized using standard methods for evapotranspiration (ET), porosity, dispersion coefficient, and overall microbial activity during the start-up period. The interstitial bacterial communities of the mesocosms were also characterized using BIOLOG^{TM} ECO plates via the CLPP method for average well colour development (AWCD), substrate richness, substrate evenness, substrate diversity, over-all community, and community similarities. It was found that mesocosm porosities decreased over time as a result of media related biofilm development. This biofilm development also contributed to a substantial increase in the dispersion coefficient (mixing properties) in the mesocosms over the entire start-up period. Dispersion coefficient values in planted systems reached values of ~50-55 cm^2/min where values in the unplanted systems reached values of ~30-35 cm^2/min. The general divergence trend in the mesocosm systems was quantified using a Euclidean-based divergence metric. All mesocosms showed a steep increase in community divergence until day 75, at which point a steady-state was reached. The interstitial communities were also characterized in terms of similarity based on the experimental design treatments. A four phase progression was seen that can be summarized as: a state of initial difference based on original bacterial sludge inoculum community profiles [day 0-6], a period where adjustments and shifts in the bacterial community occurred in all mesocosms [day 11-26], a time where all mesocosm interstitial CLPPs were quite similar [day

38-73], and a final shifting towards unplanted and planted mesocosm CLPP groupings [day 100-232]. The final convergence of similarly designed mesocosm systems observed in this study lends credence to the ideas behind the distinct experimental and environmental control created in the laboratory mesocosm wetland systems, suggesting these small-scale systems are appropriate for fundamental research endeavors.

6.1 Introduction

Although constructed treatment wetland systems have been proven as an effective water treatment technology, several challenges still plague the science behind this technology. Perhaps the most difficult challenge facing constructed wetland (CW) science is in understanding and explaining the large performance variations seen in similarly designed treatment wetland systems.

It is generally accepted that CWs contain a biological regime associated with the wetland substrate (Wynn and Liehr, 2001), and that microorganisms play a major role in ecosystem health and in the degradation of contaminants in the environment (Parkinson and Coleman, 1991; Aelion and Bradley, 1991). The role of the biological regime and the related mechanisms associated with contaminant treatment have been largely overlooked in favor of using hydrodynamic and simple first order removal rate models to describe constructed wetland-based treatment. The role and influence which the biological regime has on specific aspects in CWs has been given little to no attention. Furthermore, little is known regarding temporal bacterial community dynamics in CW systems. Bacterial community "seeding" cultures are often used to kick start pollutant degrading bacterial communities within CW systems, however little is known regarding their initial or long term development within CW systems. Seeding cultures are often gathered from waste water treatment plants and assumed to contain an appropriate seeding culture. For this and many other reasons, a greater effort into understanding microbial population density and diversity, both spatially and temporally, would be useful for design and optimization of CW systems (Faulwetter *et al.*, 2009).

One of the most frequently used models to describe CWs is the plug flow with dispersion (advection dispersion) equation (Werner and Kadlec, 2000). Contributing to the physical and hydrological processes in CWs are soils, microorganisms, plants and plant litter (Werner and Kadlec, 2000). Although microorganisms are quite often mentioned as a contributing factor to

the mixing characteristics in CWs, studies have not comparatively quantified the effect of both the microbiological and plant regimes on CW mixing properties. Mixing properties can become increasingly important as CW systems age. Preferential water flows can reduce the contact time seen by entering water pollutants, thus reducing the overall treatment performance of the CW system. If preferential flows can be mitigated and mixing promoted long term performance should be improved.

Both evaporation and transpiration in CWs can be extremely variable depending on the design of the system. Separate models have been developed to describe evaporative losses from the substrate (Liu *et al.*, 1997), and transpiration losses from plant leaves (Tuchscherer, 2003). The concurrent study of both microbiological and hydrological parameters in CWs has been given little attention. Heat generation and temperature have been found to be directly related to evaporation rates of water in mesocosm wetland systems (Weber, 2006), therefore heat generated by the microbial community in CWs should remain an ongoing area of study with respect to water balances.

The role of plants in CWs is a highly debated issue. Certain studies have shown that plants help to effectively remediate contaminated waters (House *et al.*, 1994), whereas other studies have shown plants to have no effect on treatment performance (Gray *et al.*, 2000). One aspect of subsurface CWs, which has been given some attention, is the rhizosphere or root zone. The rhizosphere can be described as containing a mixture of bacteria and plant root mass. The role of reeds in the management of water quality is fundamentally influenced by the periphyton communities on the underwater surfaces of aquatic macrophytes (Acs *et al.*, 2003; Albay & Akcaalan, 2003; Gross *et al.*, 2003; Lakatos *et al.*, 1991; Neely & Wetzel, 1995). The effect of this relationship between the biological and plant regimes on the water balance and hydrodynamics of CWs has not been quantitatively or concurrently studied.

As rhizospheric bacteria have been shown to affect plant growth, so have aquatic plants been shown to affect microbiological development in wetland media. Aquatic plants, such as *Phragmites australis* (the common reed), have the ability to transfer oxygen from their aerial tissues and release it into their rhizosphere (Karathanasis & Johnson 2003; Batty *et al.,* 2000). Plant root systems also provide mechanical support and perform many roles including the synthesis, accumulation, and secretion of compounds (Flores *et al*., 1999). The compounds secreted into the surrounding rhizosphere by roots are referred to as root exudates. Plants have been shown to exude 5-21% of all photosynthetically fixed carbon into the surrounding rhizosphere as root exudates (Walker *et al*., 2003; Marschner, 1995). Through this exudation of compounds, roots can often regulate the microbial community structure in the surrounding rhizosphere (Walker *et al*., 2003; Nardi *et al*., 2000).

Over the past 5 years there has been an increase in research focusing on the study of bacterial communities in wetland systems. With the advent of PCR-based methods and the ever growing use of community level physiological profiling (CLPP), bacterial community studies have become easier to design and perform. Although a number of static bacterial community studies have been performed [Hench *et al.*, (2004); Hallberg *et al*., (2005); Hadwin *et al.*, (2006); Sleytr *et al.* (2009)] there have been very few studies looking at temporal bacterial community dynamics in CW systems. Community variations can occur in many different fashions. Classic variations can be described as cyclic, directional, stochastic, or chaotic (Collins *et al*., 2000). Weber *et al.* (2008) used the CLPP method to track changes in mesocosm wetland interstitial water communities in response to acid mine drainage exposure, however apart from this single study very little work has been done to study the temporal trends with respect to bacterial community changes in CW mesocosms. This study looks to initiate work into uncovering this scientific void.

The objective of this study was to investigate the bacterial community and hydrological changes in wetland mesocosms during an 8 month start-up period. Eight mesocosms were studied

based on a duplicated 2^2 factorial design. In order to study the effect of plants on mesocosm start-up dynamics, 4 mesocosms were planted with *Phragmites australis*, with 4 mesocosms left unplanted. The study of bacterial culture inoculum differences on mesocosm start-up dynamics was undertaken using 4 mesocosms inoculated with bacterial sludge from a waste water treatment plant (WWTP) with the other 4 being inoculated with bacterial sludge from a dairy farm wastewater holding tank (DF). All 8 mesocosms were characterized using standard methods for evapotranspiration (ET), porosity, dispersion coefficient, and overall microbial activity. The interstitial bacterial communities of the 8 mesocosms were also characterized using a CLPP method based on BIOLOG^{TM} ECO plate data using average well colour development (AWCD), substrate richness, substrate evenness, substrate diversity, over-all community divergence from day 0 using a Euclidean distance measure, and community similarities based on experimental design treatments. With an understanding of how the different design treatments (i.e. plant effects, and inoculum origin effects) affect mesocosm start-up dynamics and ultimate development, perhaps a better understanding of the origin of the large performance variations in full scale treatment systems can be garnered. It is also hoped that this information may be useful for optimization and improved design of full scale wetland systems.

6.2 Materials and Methods

6.2.1 Experimental Design

A duplicated 2^2 factorial design was used to study the effect of plants (*Phragmites australis*), and inoculum origin on the development of wetland mesocosms during the start-up phase (Table 6.1). Inoculi were gathered from 1) the aerator and anaerobic digester of the Waterloo waste water treatment plant, these were mixed and referred to as the WWTP sample, and 2) sludge from a waste-water holding tank at a dairy farm (DF) outside of Ilderton, ON. Both inoculum types were of a mixed sludge/water type, and could be easily poured.

Table 6.1: Mesocosm Designs – based on a duplicated 2^2 factorial design

	Low level	High level
Plants	Absent	Present
Inoculum origin	WWTP	Dairy Farm

The mesocosms were of a similar design to those studied in Chapters 3, 4 and 5 with the major difference being the configuration of the water inlet. Whereas the water in the earlier studies (Chapter 5) entered from the top of the mesocosm, the water in the newly constructed mesocosms entered at about 65 cm from bottom of the mesocosm systems (approximately 5 cm below the water level). The mesocosms were constructed of the same material and were to the same dimensions as those in Chapters 3, 4 and 5.

Each mesocosm was comprised of schedule 80, polyvinylchloride (PVC) columns (90 cm by 25 cm Ø) filled to ~ 80 cm with pea gravel (80% limestone) and operated to 70 cm with water (Figure 6.1). Water was circulated with a 1/200 HP, 3200 rpm, March (Glenview, Illinois) series 1 (1A-MD 1/2) centrifugal pump. The water inlet was situated about 5 cm below the water level (Figure 6.1). Seeding was completed by adding fresh limestone in steps and pouring in ~160mL of inoculum at depths of 10 cm, ~40 cm and 65 cm. Plants (P*hragmites* australis) were collected from a local marsh, cultured in pots with peat moss and transferred to the mesocosms with a small amount of peat moss in the top section. Root depth was ~30 cm in all cases. The *Phragmites australis* used was of the native non-invasive type.

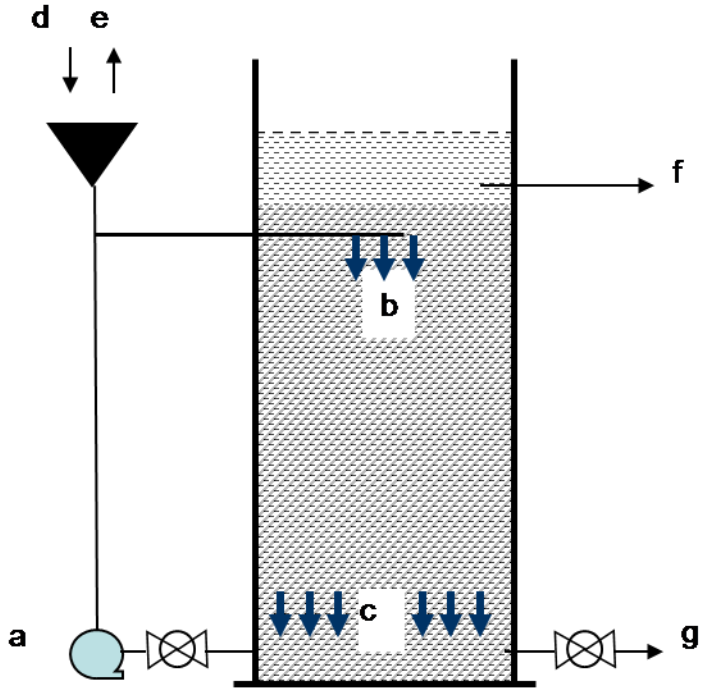


Figure 6.1: Mesocosm schematic: Water was circulated via a small centrifugal pump (a) in the mesocosm (b) and allowed to percolate through the pea gravel bed and collected at the bottom (c). An atmosphere exposed port served as an injection (d) and sampling (e) point. Drainage ports were located near the top to prevent overfilling (f), and near the bottom (g) for mesocosm drainage.

The mesocosms were completely drained once per week. After draining, the mesocosms were then refilled with mineral nutrient solution according to Hoagland's and Arnon (1938) as well as a simulated wastewater solution based on the descriptions of Droste (1996) and solutions used by Kargi and Karapinar (1995) and Wang *et al.* (2008). The nutrient solution was mixed in regular tap water and fed to the unit wetlands giving interstitial concentrations of 28.75 mg/L $NH_4H_2PO_4$; 151.5 mg/L KNO_3; 236 mg/L $Ca(NO_3)_2{\cdot}4H_2O$; 123.25 mg/L $MgSO_4{\cdot}7H_2O$; 9.175 mg/L FeNaEDTA; 0.715mg/L H_3BO_3 ; 0.4525mg/L $MnCl_2{\cdot}4H_2O$; 0.055mg/L $ZnSO_4{\cdot}7H_2O$; 0.0125mg/L $CuSO_4$ and 0.005mg/L $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$. The simulated wastewater consisted of ~1g/L molasses, 0.049 g/L urea, 0.0185 g/L $NH_4H_2PO_4$, yielding a glucose concentration of ~5.878g from the molasses, a COD of ~500mg/L and a COD:N:P ratio of ~100:5:1. Water was circulated at approximately 2.4 L/min, giving an average cyclic hydraulic retention time of approximately 4

min. All mesocosms were exposed to artificial illumination (14,000 lumens) with a 15 hr photoperiod. Plants were sprayed daily with water to reduce drying.

6.2.2 Community Level Physiological Profiling

The community level physiological profiling (CLPP) laboratory protocol was performed using the methods outlined in Chapter 2. Samples were gathered approximately every 3days for the first 2 weeks, every week for the following 3 weeks, bi-monthly for the next month, and monthly for the last 6 months. BIOLOG™ ECO plates were inoculated with 150 μL of mesocosm interstitial water and incubated at room temperature. Inoculi were controlled to an OD of ~0.19 (Weber *et al.,* 2009). The BIOLOG™ ECO plates were then read at an absorbance of 590 nm after an 84 hr incubation period.

6.2.3 Evapotranspiration

A study of the mesocosm water budget was performed spanning the 8 month start-up period. Water loss was measured by topping up the mesocosms until the overflow point was reached. Water loss was then evaluated as the amount of water needed to fill the mesocosm to the overflow point. Water loss was equated to be entirely due to evapotranspiration, as no other outputs existed.

6.2.4 Dispersion Coefficient

The internal hydraulic performance of aquatic systems can be quantified using inert, soluble chemical tracers (Dierberg *et al.*, 2005; Kadlec, 1994). NaBr tracer tests were conducted on the eight mesocosms over the 8 month period. Similar tracer studies have been previously and successfully applied on these mesocoms by Weber (2006), McHenry (2003) and Van Loon (2003). 2 mL aliquots of a 200g/L NaBr stock solution was injected into the mesocosms through the inlet port, a handheld conductivity meter was then inserted into the same port, measuring the conductivity of the circulating water. Conductivity readings were taken until stable values

were reached (typically 20-45 min). Data were then fit to a 1D advection dispersion equation as seen in Equation 1, using Aquasim v.1.0.0.1 (Eawag Institute, Switzerland, 1995).

1D advection dispersion equation

$$\frac{\partial C}{\partial t} = -v\frac{\partial C}{\partial x} + D\frac{\partial^2 C}{\partial x^2} \qquad (6.1)$$

Average flow rate and apparent cross sectional area were manually entered based on porosity and flow rates measured before starting the tracer test. The dispersion coefficient (D) was then determined using the parameter estimation function via both the simplex (Nelder and Mead, 1965) and secant methods (Ralston and Jennrich, 1978). Sample model and measured data are presented in Figure 6.2.

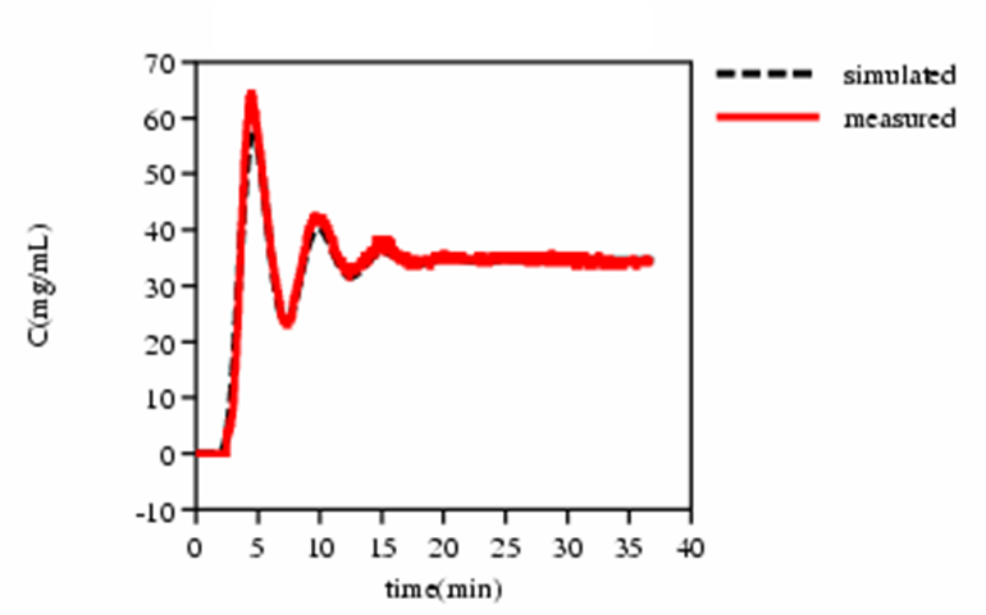


Figure 6.2: Br- tracer modelling – Aquasim 1.0.0.1 (1995) sample output

Mixing properties and the dispersion coefficient D can also be represented as a dimensionless wetland dispersion number (Kadlec and Knight, 1996):

$$W = \frac{D}{vL} = \frac{1}{Pe} \tag{6.2}$$

where: W = wetland dispersion number, dimensionless

v = velocity (cm/min)

L = length of reaction zone (cm)

Pe = Peclet number

Unfortunately a number of data sets spanning days 0 to 60 following initial start-up were lost due to file corruption; however, as later discussed, a sufficient amount of data was available so that the monitoring data was not compromised.

6.2.5 Porosity

Porosity can be defined as:

$$\phi = \frac{V_p}{V_m} \tag{6.3}$$

where: V_p is the non-solid volume (pores and liquid)

V_m is the total volume of material

Overall mesocosm porosity was calculated by determining the total volume of material (V_m) and the non-solid volume (V_p). V_m was calculated as the cylindrical volume of the mesocosm from the bottom to the overflow point. V_p was calculated by completely draining and then measuring the volume of water required to refill the column to the overflow point.

6.2.6 Microbiological Activity

The overall microbiological activity (MA) of the mesocosms was assessed using an indirect measurement of enzymatic activity associated with the fixed microbiological regime. In accordance with Schnurer and Rosswall (1982), the overall activity of the biomass in a complex sample can be correlated with the presence of esterases, lipases, and proteases, which catalyze the transformation of fluorescein-diacetate (FDA) to fluorescein (FL). This assay was used to assess the total biological activity in the mesocosm and has previously been applied to mesocosm biofilters (Weber *et al.* 2008; McHenry and Werker, 2005). One mL of a 5 mM FDA stock solution in acetone (Aldrich Chemical, Milwaukee, WI) was added to the mesocosm wetland. Samples of 2 mL were then drawn for a 1 hr period every 30 sec for the first 10 min, once every min for the following 20 min and every 2 min for the final 30 min. Fluorescein was then measured using a handheld fluorometer (Turner Designs, PicofluorTM, 490nm excitation, 520nm emission). Readings were normalized with respect to the maximum observable FL concentration based upon the FDA aliquot and mesocosm volume. The FDA utilization rate was determined for all time increments between 8 and 15 min. The final FDA utilization rate was calculated as the average of these 10 incremental slopes. Averaging of incremental utilization rates was performed to reduce error due to fluctuations in fluorescence readings. The final value was then multiplied by 1000 and for the purpose of this study and is called the microbiological activity (MA).

The MA as measured here has been shown to be attributed to the fixed biological regime of the mesocosm (Weber, 2006). MA measurements determined for interstitial water from the mesocosm units were found to be negligible; therefore, all MA measurements were interpreted

to be representative of the fixed microbiological regime in the mesocosms. Interstitial microbial activity can be associated with the measured AWCD calculated using the BIOLOG™ ECO plates.

6.3 Data Analysis

6.3.1 Community Level Physiological Profiling

Analysis of the community level physiological profiling (CLPP) data was performed using the methods outlined in Chapter 2. Absorbance readings (590nm) at 84 hrs were identified as the metric of choice for further CLPP data analysis. BIOLOG™ ECO plates (Biolog Inc., Hayward CA., USA) consist of 96 wells. The wells contain 31 different carbon sources, and a blank in triplicate. One plate was used for each time point (15 time points in all) for each mesocosm requiring a total of 120 plates over the 8 month period. For each plate 3 replicate carbon source utilization patterns (CSUPs) were collected giving a total of 360 objects (data sets). Each data set represents 31 variables piece giving a total of 11160 data points for analysis.

The absorbance values from 84 hr were initially standardized by first correcting by the corresponding blank value and then dividing by the average well colour development (AWCD) for that time point. Assessment of normality, homoscedasticity and linear correlations in the entire data set according to Weber *et al.* (2007), yielded a recommended natural logarithmic transformation for subsequent PCA. At each step some points were designated as obvious outliers and removed in order to analyze the remaining data. A maximum of 4 of the 32 points were removed for any single analysis. Outliers occurred due to excessively high readings for blanks yielding negative responses for all wells. Negative well responses were coded as zeros during data treatment, and therefore the outlier data sets were simply removed from the analysis rather than keeping the discussed 0 response plates. PCA was completed using Statistica 8.1.

6.3.2 Community Divergence Measure

The reason for the use of this new measure in interpreting CLPP data is to greatly decrease the complexity of the CLPP data set and make interpretation based on a single simple metric. The Euclidean distance measure was used in this study as a measure of dissimilarity of the CSUPs gathered for any one mesocosm at any point over the monitoring period. The Euclidean distance can be calculated in n dimensions, where in theis study n=31 (31 differnet carbon source utilization responses). Given the two points:

$$P = (p_1, p_2, ..., p_n) \tag{6.4}$$

$$Q = (q_1, q_2, ..., q_n) \tag{6.5}$$

The Euclidean distance can be calculated as:

$$\sqrt{(p_1 - q_1)^2 + (p_2 - q_2)^2 + ... + (p_n - q_n)^2} \tag{6.6}$$

The reference point used for all Euclidean distance calculations was the original CSUP of any single mesocosm gathered at day 0. Therefore 8 separate Euclidean distance trends (one for each mesocosm) were calculated to fully represent the divergence of the interstitial bacterial communities in the mesocosm systems. For an in-depth description on the development of this metric refer back to Chapter 3, however note that for this study the Euclidean distances were not normalized to fall within the values of 0 and 1. Rather, as all of the mesocosms started from a similar point in this study, use of the non-normalized Euclidean distance measures was maintained.

6.3.3 Substrate Related Diversity Indices

As first suggested by Zak *et al.* (1994), BIOLOG™ plates can also be used in a more traditional ecological sense, to calculate diversity indices based on carbon source utilization patterns (CSUPs). The Shannon index or what is often called "diversity" is a common ecological metric used to track and understand shifts in communities over space and time. Using the CSUP gathered from a single BIOLOG™ plate, substrate diversity (H) can be calculated as:

$$H = -\sum p_i \ln(p_i) \tag{6.7}$$

where:

H - substrate diversity

p_i - ratio of the activity of a particular substrate to the sums of activities of all substrates

activity - chosen metric for analysis (absorbance value (590nm) at 84 hrs)

Two other parameters associated with substrate diversity which can be calculated using CSUPs are substrate richness (S) and substrate evenness (E). Substrate richness is a measure of the number of different substrates utilized by a microbial population. Substrate evenness is defined as the equitability of activities across all utilized substrates; substrate richness is calculated as the number of wells with a corrected absorbance greater than 0.25. Substrate evenness is calculated as:

$$E = H / H_{\max} \tag{6.8}$$

Substrate diversity, *evenness* and richness we calculated using the average response from the three replicates on any one plate to give a single result for each plate. Recent examples of studies utilizing the Shannon index calculated from CSUPs gathered using BIOLOG™ plates include He *et al.* (2008), Farnet *et al.* (2008), and Weber *et al.* (2008).

6.3.4 Time course Data

Most of the data collected for this study is represented as time course data. The average of 4 mesocosms for either planed/unplanted or WWTP/DF curves are shown in the different plots. Error bars are only shown if a significant difference between points was observed. This was not the case for most trends and therefore error bars are omitted in most cases for ease of visualization and interpretation. Separate repeated measures analysis of variance (ANOVA) tests were completed for each time course data set. Analysis was completed using JMPIN 4.0.2, 2000 (SAS Institute Inc.).

6.4 Results and Discussion

6.4.1 Hydraulic Parameters

Figure 6.3 shows the evapotranspiration (ET) data collected over the 8 month start-up period. In general, although the ET rates did fluctuate, no significant increase or decrease in evapotranspiration rates can be seen over the 8 month period. Using a repeated measures ANOVA analysis, differences between ET rates for planted/unplanted and WWTP/DF were non-significant at the 95% confidence level (see Table 6.2). However, visually it can be seen that perhaps the planted mesocosm ET rates were higher than the unplanted mesocosm ET rates after day 100 (Figure 6.3A). This would be expected as the plants add a transpiration rate to the base evaporation rate of all mesocosms.

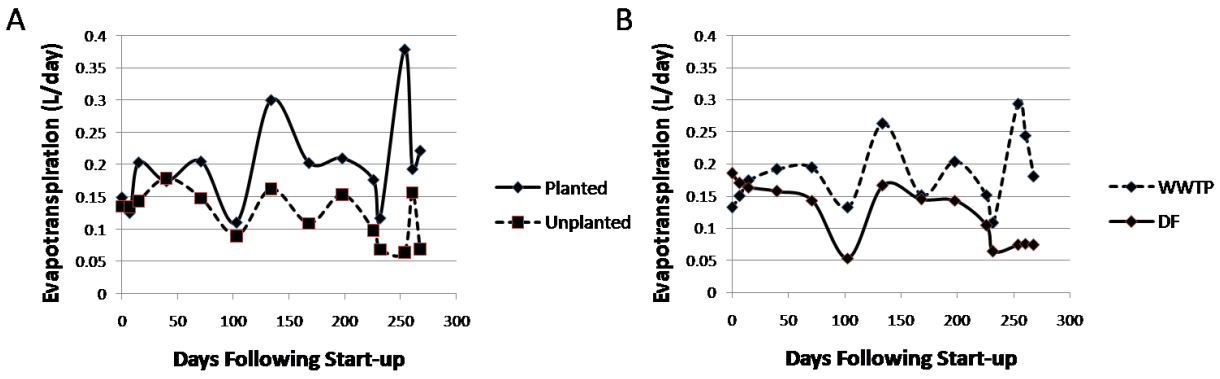


Figure 6.3: Evapotranspiration data (L/day) collected over the 8 month monitoring period for A) planted and unplanted; B) waste water treatment plant (WWTP) inoculum and dairy farm (DF) inoculum.

Table 6.2: Summary of p-values for the respective mesocosm treatments generated via a repeated measures analysis of variance. Significant results (at the 95% confidence level) are highlighted in grey. Analysis completed using JMPIN 4.0.2, 2000 (SAS Institute Inc.).

p-values	plants	inoculum	plants*inoculum
evapotranspiration	0.3776	0.1811	0.8426
porosity	0.1891	0.0042	0.0081
dispersion coefficient	0.0053	0.8281	0.7977
microbial activity	0.1227	0.0586	0.4513
AWCD	0.6056	0.4738	0.4109
richness	0.8523	0.3617	0.6672
evenness	0.3850	0.3456	0.9684
diversity	0.3350	0.2011	0.6492
divergence	0.5737	0.2824	0.9064

Using a repeated measures ANOVA analysis no significant difference, at the 95% confidence level, in the ET rates of the WWTP and DF mesocosms was observed (see Table 6.2); although through visual inspection it appears that the ET rates in the WWTP mesocosm systems were slightly higher than the ET rates of the DF systems.

Significant variability in the overall ET rates over the 8 month course was also observed. This is likely due to changing temperatures as, over the 8 month start-up period, uncontrollable temperature fluctuations occurred within the laboratory where the mesocosms were housed (20-25°C).

It was observed that the overall porosity in all mesocosm systems decreased over the 8 month start-up period (Figure 6.4). Using a repeated measures ANOVA analysis significant differences at the 95% confidence level were seen between WWTP and DF treatments (see Table 6.2). It is observed that the porosities of the mesocosms receiving the DF inoculums are lower than those in the mesocosms receiving the WWTP inoculums. This may be due to the differing adaptive natures of the differing seeding cultures, with species found in the DF inoculum developing a biofilm environment more quickly than the WWTP inoculums. This may be useful during the initial start-up phase, however may also lead to clogging in the future. Further investigation and tracking would be needed to assess this possibility. Table 6.2 also reveals an interaction between the plants and the inoculums type confirming the presence of significant interaction between resident bacteria and the plant root zone in CW systems.

The consistent decrease in porosity in all mesocosms was expected. The decrease in porosity can be explained by an increase in developed biofilm volume and associated biomass and organic material in the bulk and pore space of the mesocosm media (Kadlec *et al*., 2008). As the bacterial communities in the original inoculum begin to develop and attach to the bed media much of the void volume in the mesocosm systems becomes filled. Weber (2006) reported mesocosm systems of similar size and design (flow rates, pea gravel substrate, etc.) with porosities as low as 0.2895 in fully developed mesocosm systems. The porosities seen in Figure 6.4 seem to be approaching relatively similar levels. The porosity of clean sand or gravel in full scale systems is often found to be in the range of 0.3-0.45 (Kadlec *et al*., 2008).

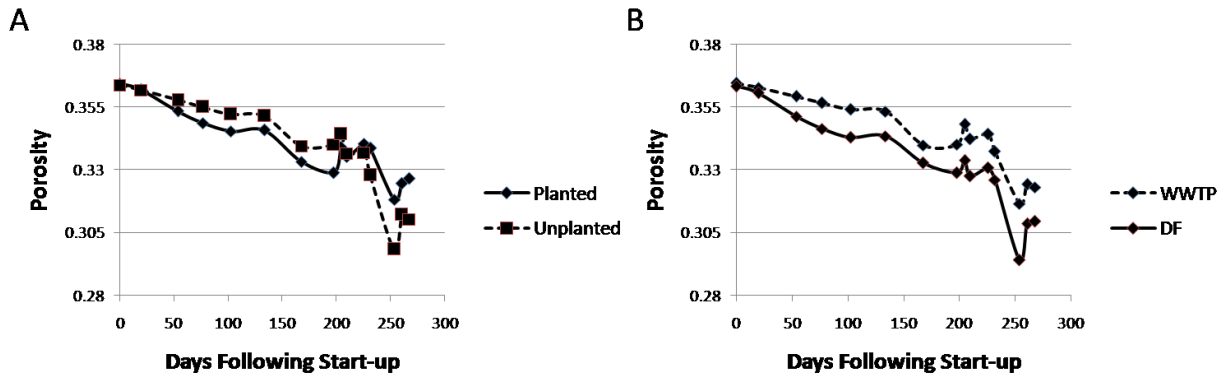


Figure 6.4: Porosity data collected over the 8 month monitoring period for A) planted and unplanted; B) waste water treatment plant (WWTP) inoculum and dairy farm (DF) inoculum.

Figure 6.5 provides the dispersion coefficient values for the mesocosms over the monitoring period. As expected, the dispersion coefficient values increased over time. This is again likely due to the increased biomass volume, and decrease in porosity within the mesocosms which, in effect, help promote mixing (measured as a dispersion coefficient). No significant differences between WWTP and DF design treatments (Figure 6.5B) were observed (see Table 6.2) although a distinct difference in the dispersion coefficients between planted and unplanted mesocosm systems was seen (Figure 6.5A, Table 6.2). Although the amount of organic matter being deposited in each mesocosm seems relatively similar from the porosity trends summarized in Figure 6.4, the addition of plants to the mesocosm design appears to promote an increase in the dispersion coefficient after ~day 100. This can be explained by the development of the plant root zone in the wetland media, which adds a complicated physical network of roots into the wetland media.

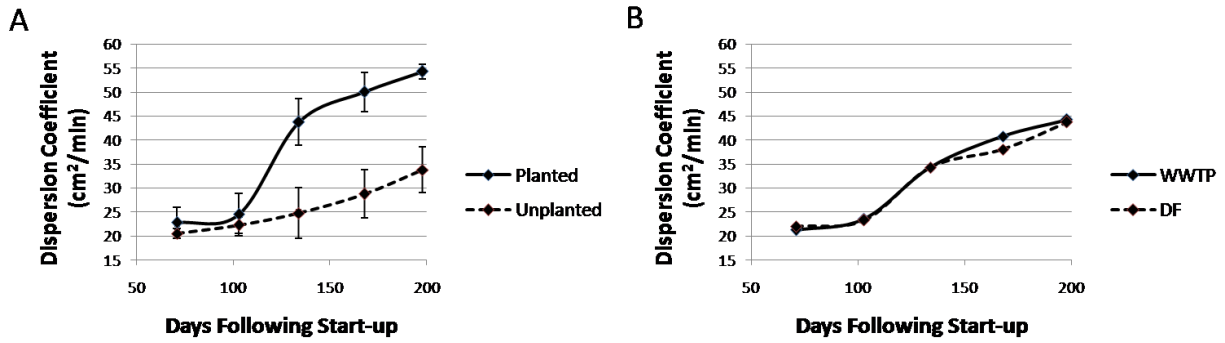


Figure 6.5: Dispersion coefficient (cm^2/min) data collected over the 8 month monitoring period for A) planted and unplanted; B) waste water treatment plant (WWTP) inoculum and dairy farm (DF) inoculum.

Kadlec and Knight (1996) reported HSSF dispersion number of 0.3 ± 0.03. For comparison purposes the results found here can be converted into dimensionless wetland dispersion numbers giving 0.33 ± 0.12. The mixing characteristics found here are comparable to full scale operation values. A larger breadth of values is reported here as a number of different wetland designs were characterized over a period of initial development.

6.4.2 Microbiological Parameters

Figure 6.6 summarizes the microbiological activity data for the monitoring period. As can be seen the general trend is for the MA to increase over time. As previously discussed, this initial start-up period is a time where the bacterial communities are adapting and attaching to the mesocosm media. Using a repeated measures ANOVA analysis no significant differences in the MA for the experimental-design treatments was observed at the 95% confidence level, however visual inspection does suggest that the MA in the planted mesocosms was slightly higher than in the unplanted mesocosm systems (Figure 6.6A). Although this observation is not statistically significant perhaps the plant-root/substrate interaction zone referred to as the rhizosphere is creating an environment allowing for accelerated bacterial community development.

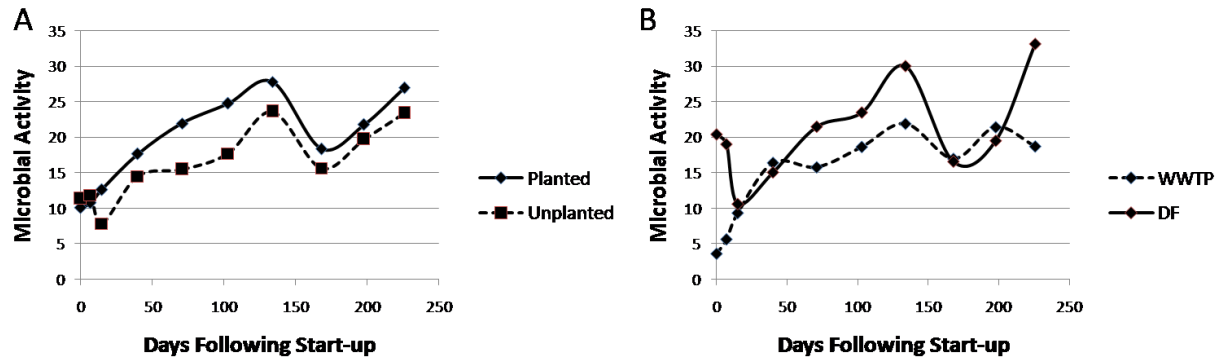


Figure 6.6: Microbial activity (MA) data collected over the 8 month monitoring period for A) planted and unplanted; B) waste water treatment plant (WWTP) inoculum and dairy farm (DF) inoculum.

Similarly, visual observation would suggest that the DF inoculated mesocosm systems have an increased MA in comparison to the WWTP mesocosms (Figure 6.6B). The p-value for this effect was actually quite small (0.0586, see Table 6.2) reinforcing this suggested observation. It can be seen that on day 0 when the inoculum was new to the mesocosm the DF bacterial communities showed much higher MAs. It could be that the visual observational trend showing the DF MAs as larger than the WWTP MAs may be due to the differing starting bacterial populations. Perhaps the original community from the DF has an innately higher activity potential than the WWTP community therefore propagating to a higher MA in the mesocosms over time. The initial decrease in MA for the DF mesocosms may be due to a reduced ability to adapt to the new environment.

In addition to tracking the overall MA of the bacterial community in the mesocosm systems BIOLOG^{TM} ECO plates were also used to characterize the mesocosm interstitial communities. As described in Section 6.3.3 a number of standard ecological parameters can be calculated using the BIOLOG^{TM} ECO plate data. Figures (6.7-6.10) summarize these parameters over the monitoring period.

Figure 6.7 summarizes the AWCD determined for the mesocosm interstitial water over the monitoring period. Similar to many of the hydrological parameters previously summarized, no significant difference could be seen between the AWCD values for any of the experimental-design treatments (see Table 6.2); however a general trend could be seen. The AWCD in all mesocosms decreased until about day 100, where a pseudo steady-state value was reached. This initial decrease is likely due to initial die-off of bacterial species which could not adapt to the mesocosm conditions, and a general migration of bacteria from the free-water to the biofilm.

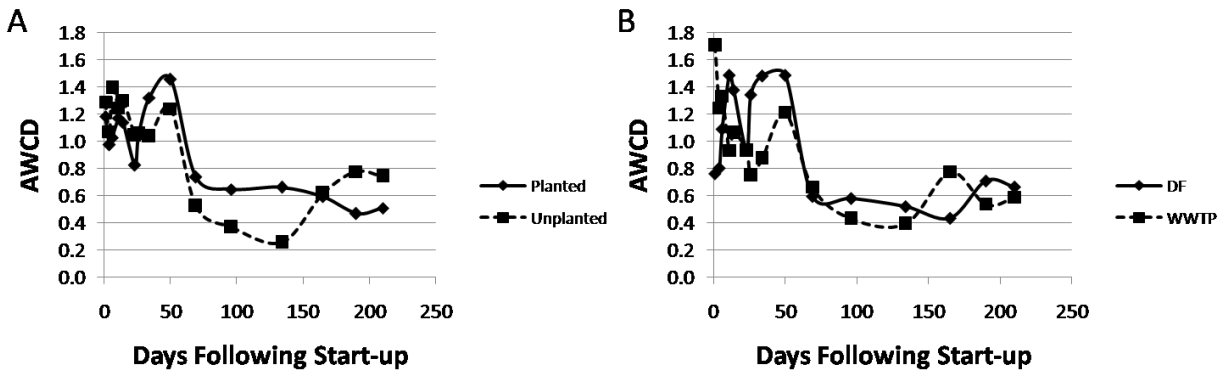


Figure 6.7: Average well colour development (AWCD) data collected over the 8 month monitoring period for A) planted and unplanted; B) waste water treatment plant (WWTP) inoculum and dairy farm (DF) inoculum.

Figure 6.8 summarizes the substrate richness found for the CLPPs of the mesocosm interstitial waters over the monitoring period. Similar to the AWCD results no significant difference could be seen between the richness values for any of the experimental-design treatments (see Table 6.2), however a general trend could be seen. The richness in all mesocosms decreased until about day 100, where a pseudo steady-state value was reached. Substrate richness is a measure of the number of species in a sample.

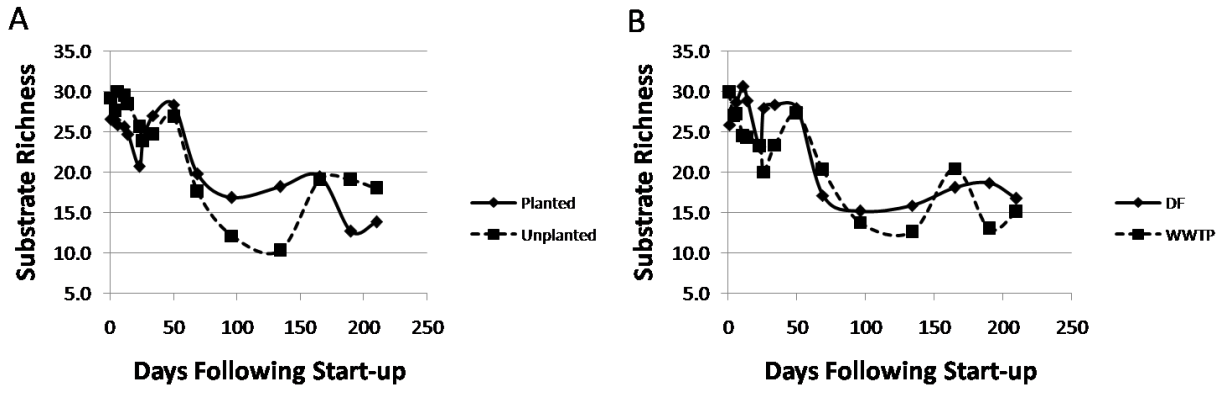


Figure 6.8: Substrate Richness data collected over the 8 month monitoring period for A) planted and unplanted; B) waste water treatment plant (WWTP) inoculum and dairy farm (DF) inoculum,.

Figure 6.9 summarizes the substrate evenness for the CLPPs of the mesocosm interstitial waters over the monitoring period. Similar to the AWCD and richness results no significant difference could be seen between the evenness values for any of the experimental-design treatments (see Table 6.2), however a general trend could be seen. The evenness in all mesocosms decreased until about day 100, where a pseudo steady-state value was reached. This result can be explained as the interstitial bacterial community becoming more consistent and adapted after an initial acclimation period.

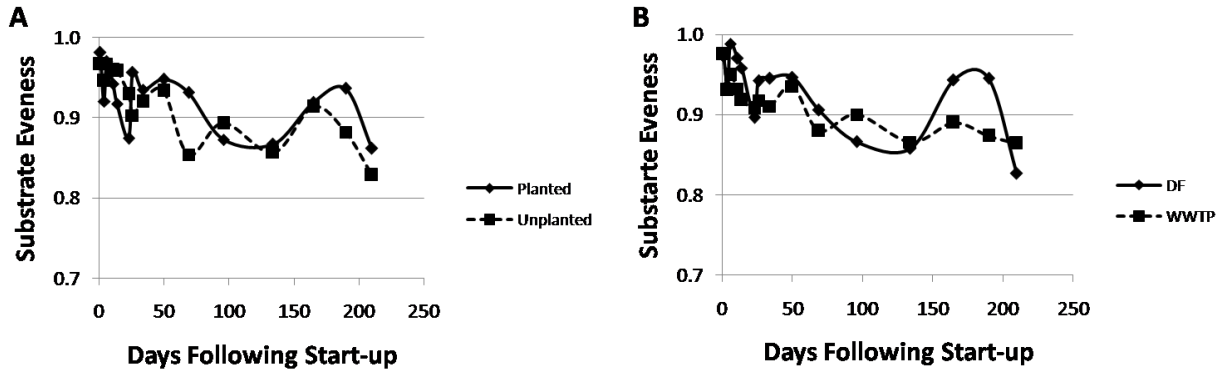


Figure 6.9: Substrate evenness data collected over the 8 month monitoring period for A) planted and unplanted; B) waste water treatment plant (WWTP) inoculum and dairy farm (DF) inoculum.

Figure 6.10 summarizes the substrate diversity for the CLPPs for the mesocosm interstitial water over the monitoring period. Similar to the AWCD, richness and *evenness* results no significant difference could be seen between the diversity values for any of the experimental-design treatments (see Table 6.2), although a general trend was evident. The diversity in all mesocosms decreased until about day 100, where upon a pseudo steady-state value was reached. Similar to the AWCD and richness results, this initial decrease is likely due to initial die-off of bacterial species which cannot survive under the new conditions in the mesocosm and movement of bacteria from the free-water into the fixed biofilm.

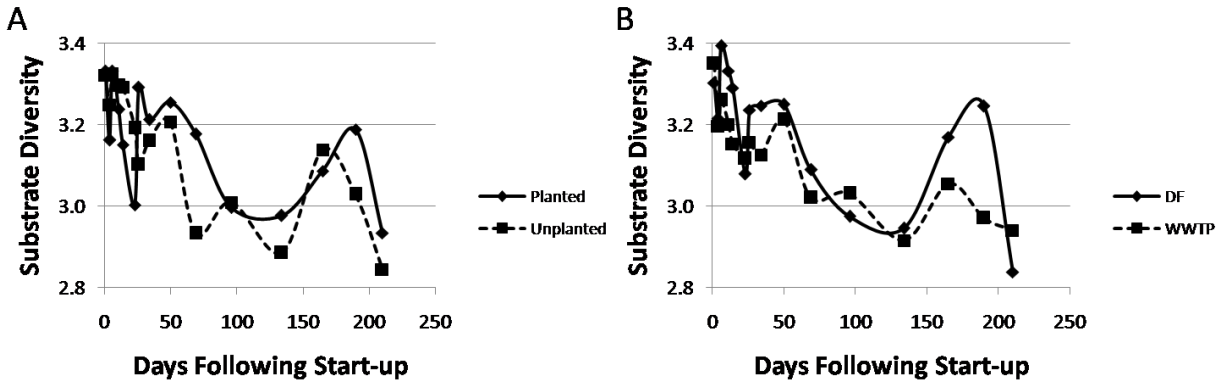


Figure 6.10: Substrate diversity data collected over the 8 month monitoring period for A) planted and unplanted; B) waste water treatment plant (WWTP) inoculum and dairy farm (DF) inoculum.

BIOLOG^{TM} ECO plates were also used to characterize the divergence trends in the mesocosm interstitial bacterial communities. From Figure 6.11 it can be seen that community divergence occurs from the start (day 0). Community divergence is measured as the Euclidean distance between the CSUPs gathered as described in section 6.3.2, with reference to the day 0 CSUP. See Chapter 3 for an in-depth description of this community divergence metric.

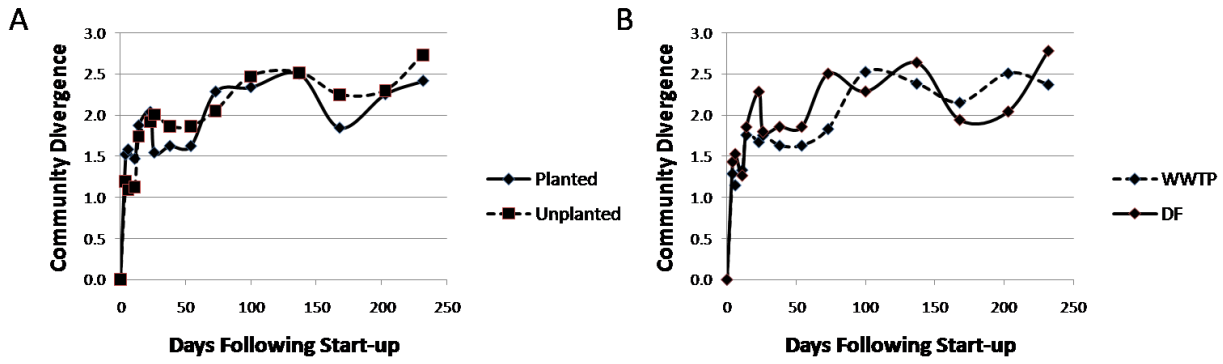


Figure 6.11: Community divergence calculated as the Euclidean distance of each respective monitoring day CSUP with respect to the day 0 CSUP, over the 8 month monitoring period for A) planted and unplanted; B) waste water treatment plant (WWTP) inoculum and dairy farm (DF) inoculum.

As can be seen in Figure 6.11, no significant differences in the divergence trends between experimental-design treatments was observed (see Table 6.2 for statistical results). The general divergence trend is as expected; the communities rapidly diverge from day 0 to about day 50. This is likely due to some of the bacterial species associated with the different inoculi not surviving in the new mesocosm environment or on the nutrients and simulated waste-water provided. After approximately 75-100 days the community divergence appears to reach a steady-state. This is likely due to the bacterial communities establishing a more hospitable atmosphere in and around the wetland media to form a stable biofilm. By day 75 it is likely that the bacterial species from the original inoculum were well adapted to growing in the "new" mesocosm environment. This finding is in agreement with Truu *et al.* (2009) who also found that it can take up to ~100 days for bacterial communities to stabilize in CW systems.

The similar divergence trends seen by the different experimental-design treatments was interesting. It was expected that the different treatments would have an effect on the divergence trends, however as it seems from the data, the experimental conditions (i.e. well controlled laboratory environment), similar wetland media, same feed water composition, flow rates and temperatures etc., likely helped drive and reduce differences in all divergence trends.

CLPP characterization of the interstitial waters was conducted over the entire monitoring period. Each day that a CLPP was performed can be represented by a PCA ordination for that particular mesocosm. PCA ordinations show similarities of differing objects, in this case CSUPs from the BIOLOG^{TM} ECO plates, on a 2D plane. Information extracted from PCA ordinations can be extensive with the most common use being to classify different CSUPs into differing groups based on their proximity on the PCA ordination. For this study 15 different ordinations could be shown to represent each day, however as many of these plots show similar results, and for the sake of brevity, only a select number of PCA ordinations generated throughout the monitoring period will be shown. An attempt to summarize all of the PCA ordinations into a single table will be given following the PCA ordination discussion.

Figure 6.12 shows the PCA ordination for day 0 of the monitoring period. What can be seen are two relatively distinct groupings. The CSUPs representing the interstitial communities from the WWTP mesocosms are found as a group on the right side of the chart, with the DF mesocosm CSUPs on the left. This result was expected and highlights the fact that the inoculi came from different sources.

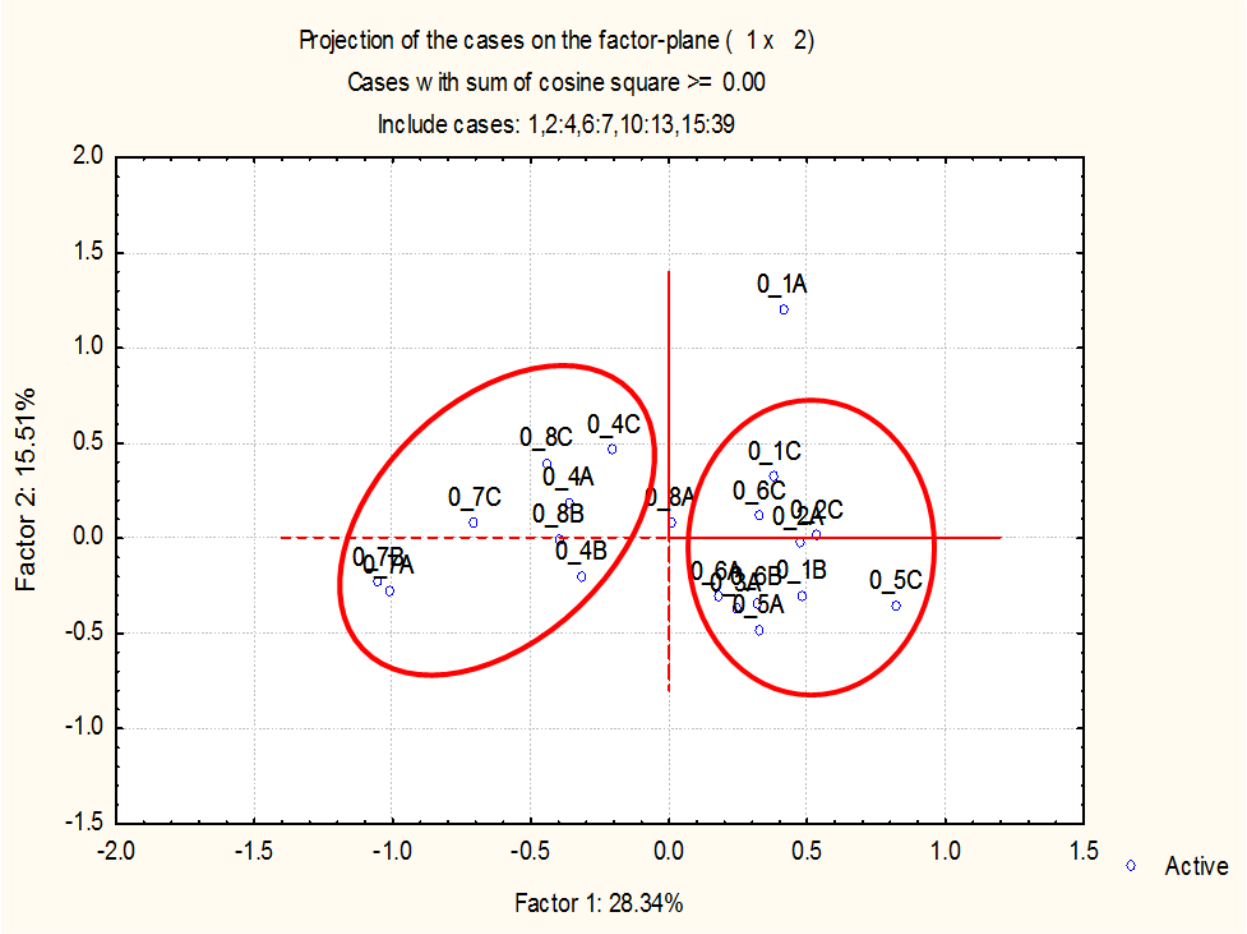


Figure 6.12: Day 0 PCA ordination based on the CSUPs for planted WWTP inoculated mesocosms (1 and 2), planted DF inoculated mesocosms (3 and 4), unplanted WWTP inoculated mesocosms (5 and 6), and unplanted DF inoculated mesocosms (7 and 8). Each mesocosm CSUP (object) is shown in triplicate (A, B, C). Output generated using Statistica 8.1.

Figure 6.13 shows the PCA ordination for day 4 of the monitoring period. Here the CSUPs representing the interstitial communities from the WWTP mesocosms are again found on the right side of the chart, however are broken up into 2 separate groups. These sub groups represent the planted and unplanted WWTP interstitial communities. The DF mesocosm CSUPs can be found on the left side of Figure 6.13. These results were as expected, as the mesocosm interstitial communities did not have a large amount of time to adjust or adapt to the new environment and therefore can be grouped much the same as seen on day 0. It was however interesting to see a community shift due to the presence of plants in the WWTP mesocosms

and supports the idea that different inoculi may be more suited and may more rapidly adapted to producing a CW that has early treatment potential.

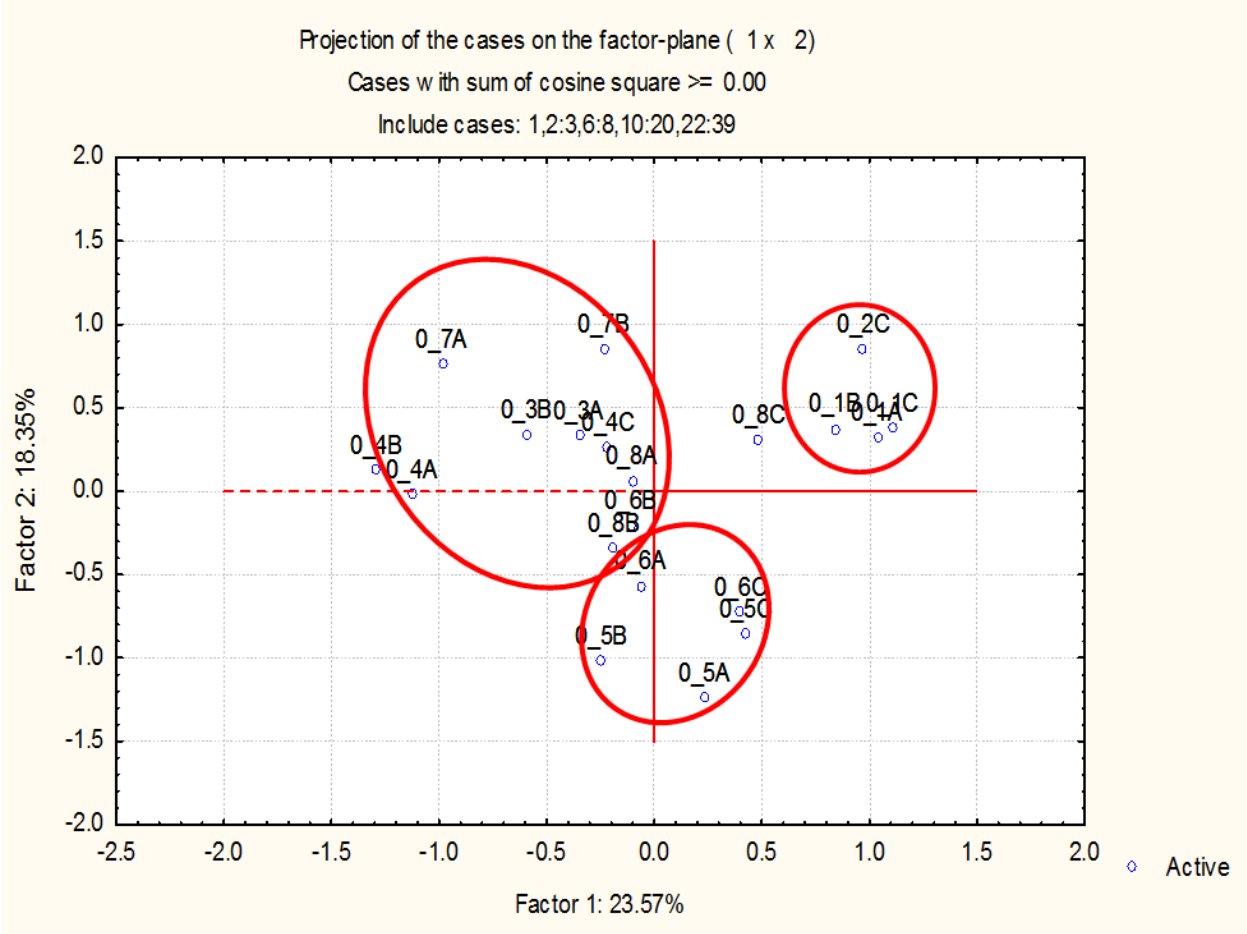


Figure 6.13: Day 4 PCA ordination based on the CSUPs collected via BIOLOG™ ECO plates, for planted WWTP inoculated mesocosms (1 and 2), planted DF inoculated mesocosms (3 and 4), unplanted WWTP inoculated mesocosms (5 and 6), and unplanted DF inoculated mesocosms (7 and 8). Each mesocosm CSUP (object) is shown in triplicate (A, B, C). Output generated using Statistica 8.1.

Figure 6.14 shows the PCA ordination for day 38 of the monitoring period. No defined group in this ordination presents itself. This is likely due to the development of similar communities composed of similar species for all mesocosm system inoculi. Although the inoculi came from different sources, the sources were similar in a number of respects (i.e. mammalian sewage

treatment systems). Perhaps the types of species that were able to survive and flourish in the mesocosms are found in both inoculi, therefore accounting for the similarity in CSUPs after an initial adjustment period. Nitrifiers, denitrifiers, and aerobic carbon degrading bacterial strains commonly exist in microbially-mediated sewage treatment facilities, and are likely found in both inoculi used here (Curtis and Sloan, 2006).

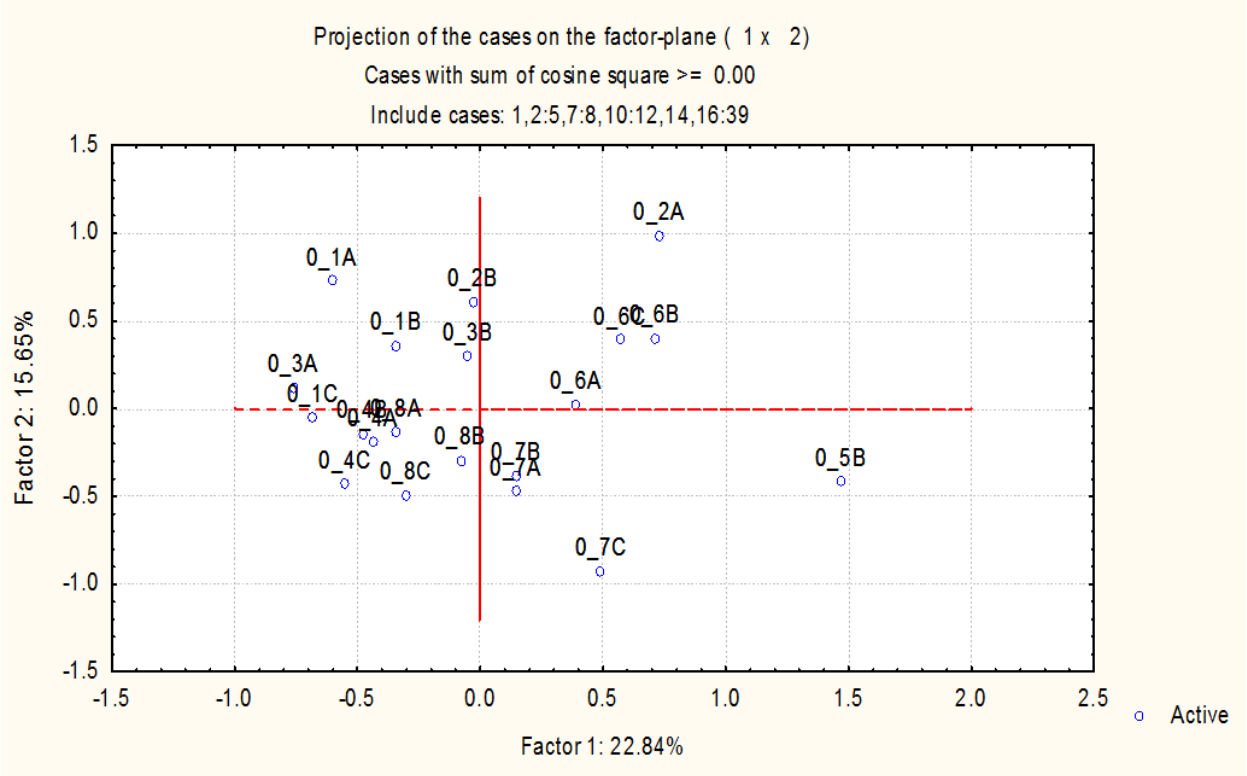


Figure 6.14: Day 38 PCA ordination based on the CSUPs for planted WWTP inoculated mesocosms (1 and 2), planted DF inoculated mesocosms (3 and 4), unplanted WWTP inoculated mesocosms (5 and 6), and unplanted DF inoculated mesocosms (7 and 8). Each mesocosm CSUP (object) is shown in triplicate (A, B, C). Output generated using Statistica 8.1.

Figure 6.15 shows the PCA ordination for day 168 of the monitoring period. Although this ordination is not definitively clear it can be seen that the planted mesocosm objects are mostly found on the left side of the ordination, with the unplanted systems found on the right. There is some overlap within the centre of the ordination though, and therefore discussion should be

taken with caution. With this said, this result was expected. *Phragmites australis* is known to secrete both oxygen and carbohydrates into the rhizospheric zone of wetland systems (Karathanasis & Johnson 2003; Walker *et al*., 2003; Nardi *et al*., 2000). This added carbohydrate source and slightly increased local dissolved oxygen is likely to have allowed for a distinct shift in the resident bacterial communities in the mesocosm systems. These results are in agreement with Weber *et al.* (2008) who found interstitial bacterial communities in similarly designed planted and unplanted mesocosm systems to be distinctly different.

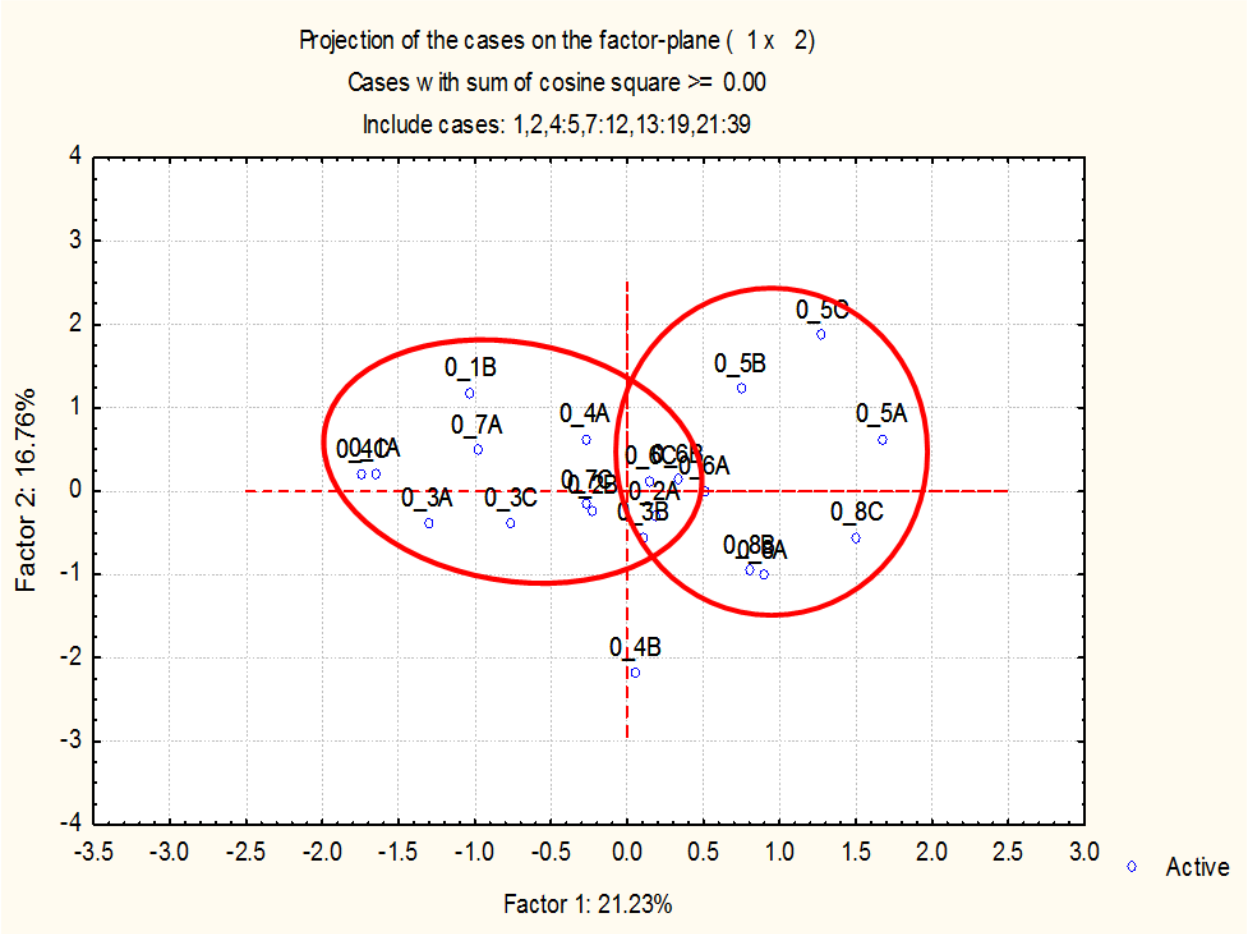


Figure 6.15: Day 168 PCA ordination based on the CSUPs collected via BIOLOG™ ECO plates, for planted WWTP inoculated mesocosms (1 and 2), planted DF inoculated mesocosms (3 and 4), unplanted WWTP inoculated mesocosms (5 and 6), and unplanted DF inoculated mesocosms (7 and 8). Each mesocosm CSUP (object) is shown in triplicate (A, B, C). Output generated using Statistica 8.1.

Figure 6.16 shows the PCA ordination for day 232 of the monitoring period. This ordination is quite similar to Figure 6.15, with perhaps a clearer separation of groups. In general the planted mesocosm systems can be found on the right side of the ordination, with the unplanted systems found on the left. However, there is one exception, and that is for mesocosm 5 (unplanted, WWTP) which is found to group well with the planted mesocosms. Therefore, although the planted and unplanted systems seem to be distinctly different for the most part at day 232, there may perhaps still be some on-going community-development/divergence occurring in the systems during this time.

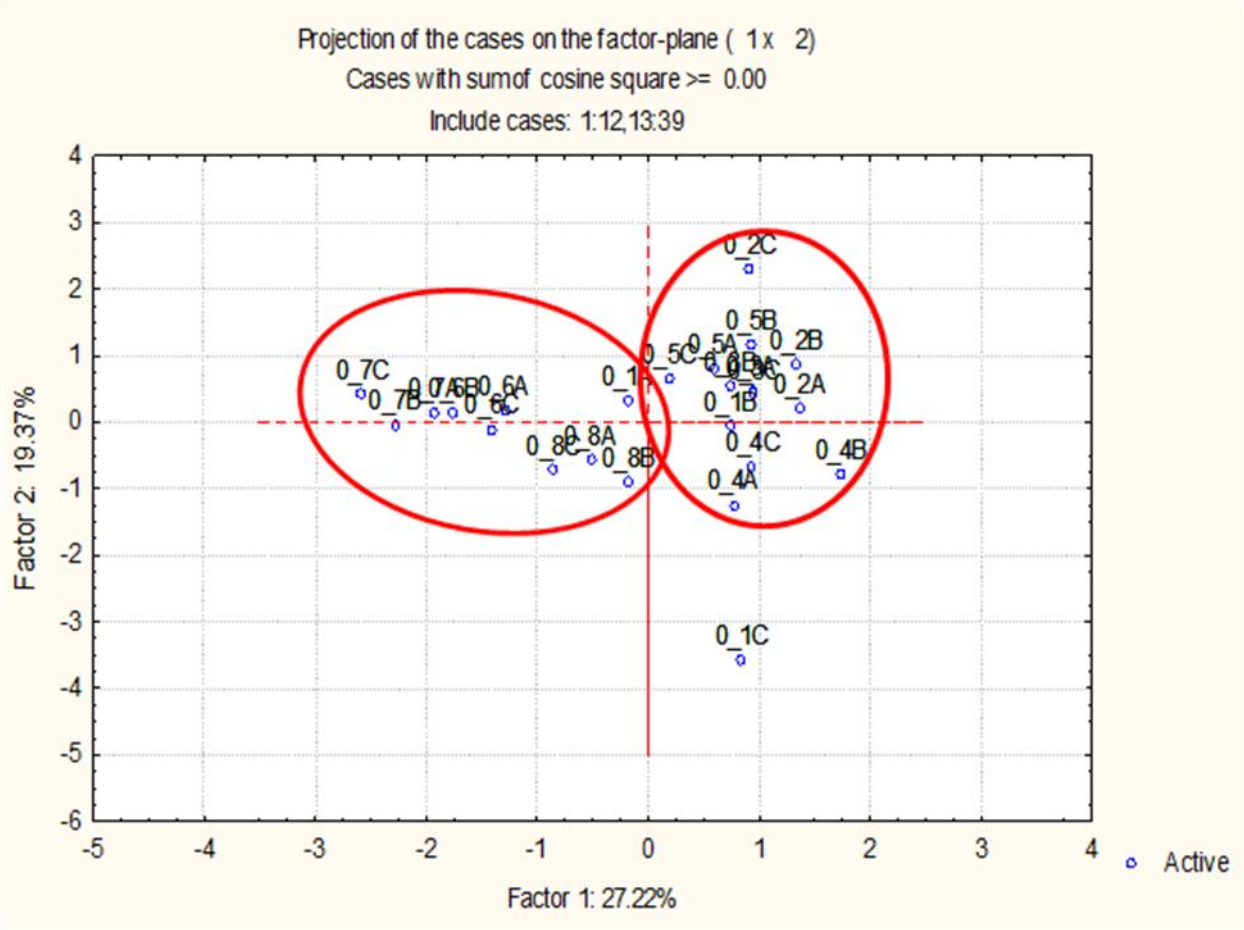


Figure 6.16: Day 232 PCA ordination based on the CSUPs collected via BIOLOG™ ECO plates, for planted WWTP inoculated mesocosms (1 and 2), planted DF inoculated mesocosms (3 and 4), unplanted WWTP inoculated mesocosms (5 and 6), and unplanted DF inoculated mesocosms (7 and 8). Each mesocosm CSUP (object) is shown in triplicate (A, B, C). Output generated using Statistica 8.1.

Table 6.3 summarizes the PCA groupings from the 15 separate analyses completed over the monitoring period. It can be quite challenging to summarize mega-datasets such as this, especially when trying to summarize 15 different 2D ordinations into a singular tabular format, therefore there are limitations with regards to comparing the data as presented in the table. Grouping patterns are to be only compared for each specific day (row). For example even though a similar symbol is used to describe mesocosms from different days, M5 at day 0 is not in any way similar to, and was not at all compared to M1 at day 4. Comparison of patterns in either the vertical (column) direction or any diagonal directions cannot be done using this summary table.

Table 6.3: Summary of PCA groupings for the CSUPs gathered for the interstitial mesocosm bacterial communities. [+++] Grouping 1, [///] Grouping 2, [---] Grouping 3 or undecipherable grouping.

	Planted	Planted	Planted	Planted	UP	UP	UP	UP
	WWTP	WWTP	DF	DF	WWTP	WWTP	DF	DF
Day	M1	M2	M3	M4	M5	M6	M7	M8
0	+++++++	+++++++	//////////	//////////	+++++++	+++++++	//////////	//////////
4	+++++++	+++++++	//////////	//////////	----------	----------	//////////	//////////
6	+++++++	----------	//////////	//////////	+++++++	+++++++	//////////	//////////
11	----------	----------	//////////	//////////	+++++++	+++++++	//////////	//////////
14	+++++++	----------	//////////	//////////	+++++++	+++++++	//////////	//////////
23	----------	//////////	+++++++	----------	//////////	+++++++	//////////	//////////
26	+++++++	//////////	//////////	//////////	+++++++	+++++++	//////////	//////////
38	----------	----------	----------	----------	----------	----------	----------	----------
54	----------	----------	----------	----------	----------	----------	----------	----------
73	----------	----------	----------	----------	----------	----------	----------	----------
100	+++++++	----------	//////////	+++++++	----------	//////////	//////////	//////////
137	+++++++	+++++++	+++++++	+++++++	----------	//////////	//////////	//////////
168	+++++++	+++++++	+++++++	+++++++	//////////	//////////	//////////	//////////
203	+++++++	+++++++	+++++++	+++++++	+++++++	//////////	//////////	//////////
232	+++++++	+++++++	+++++++	+++++++	+++++++	//////////	//////////	//////////

Table 6.3 can be broken up into 4 different sections. First, the groupings from day 0-6 can be said to be largely based on inoculum differences. Second, the time period of day 11-26 seems to be an adjustment period where the loose groupings between WWTP and DF can be seen, however many shifts do occur within this period, making those distinct groupings difficult to

decipher using any single PCA plot. Third, the time period between days 38-73 appears to be a time where no distinct groupings can be made based on the experimental design treatments. This is a time after the initial adjustment period where the bacterial communities in all of the mesocosm systems seem to be quite similar. Lastly, the time period from 100-232 days appears to be a time where the mesocosm interstitial CLPPs can be grouped into planted and unplanted community groupings. This progression can be summarized as moving from a state of initial difference based on input community profiles, to a period where adjustments and shifts occur in all mesocosms, to a time where all mesocosm interstitial CLPPs are quite similar, to a final shift towards unplanted and planted mesocosm CLPP groupings. This progression is summarized in Figure 6.17.

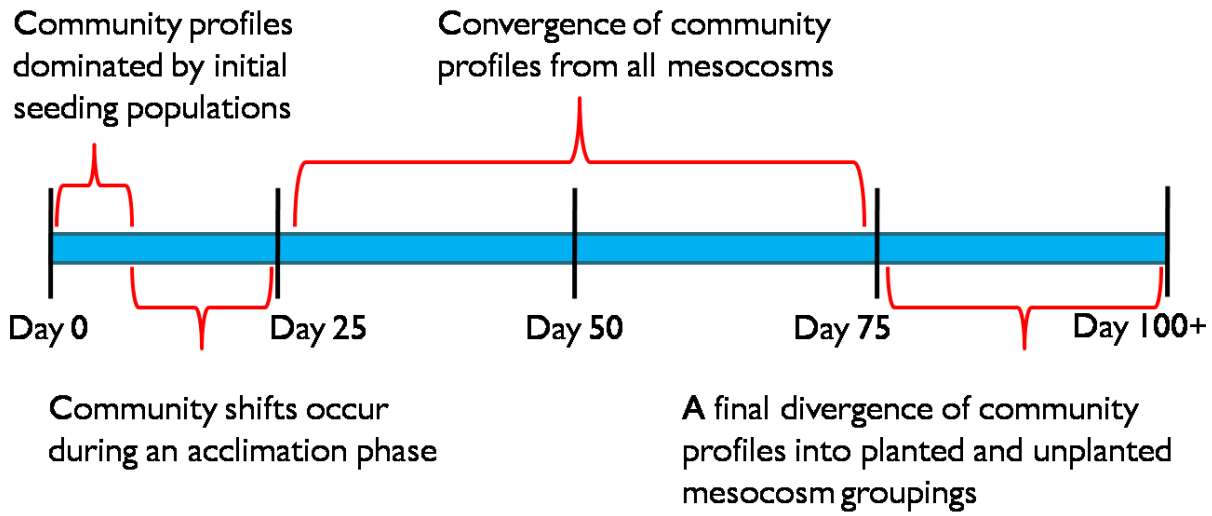


Figure 6.17: Progression of the bacterial community groupings measured over the 8 month start period.

Different types of community divergences have been previously observed in similar small scale bioreactor systems. Nadarajah (2007) saw chaotic development in small scale bioreactor systems, where Weber *et al.* (2008) saw convergence of similarly designed mesocosm wetland systems based on the presence or absence of plants. The results found here support those of Weber *et al.* (2008), and can be described as intuitive from an ecological perspective.

6.5 Conclusions

A number of trends could be observed in the mesocosm systems over the start-up period. A decrease in the porosity of all mesocosms was seen. This was likely the result of the development of a biofilm. This hypothesis was supported by an observed increase in overall mesocosm microbial activity. This biofilm development contributed to a substantial increase in the dispersion coefficient (mixing properties) in the mesocosms over the entire start-up period. Differences in the dispersion coefficient values in the mesocosms could be explained by the presence/absence of plants. Dispersion coefficient values in planted systems reached values of ~50-55 cm^2/min where dispersion coefficients in the unplanted systems reached values of ~30-35 cm^2/min.

A general divergence trend in the mesocosms was observed based on a Euclidean divergence metric developed in Chapter 3. All mesocosms showed a steep increase in community divergence until day 75-100, at which point a steady-state was reached. The interstitial communities were also characterized in terms of similarity based on the experimental design treatments. Four phases were identified during mesocosm development that can be summarized as:

1) A state of initial difference based on inoculum community profiles [day 0-6]

2) A period where adjustments and shifts occur in all mesocosms [day 11-26]

3) A time where all mesocosm interstitial CLPPs are quite similar [day 38-73]

4) A final shift towards unplanted and planted mesocosm CLPP groupings [day 100-232]

A number of different types of community divergences have been previously observed in similar small scale bioreactor systems. The results found here can be described as intuitive from an ecological stand point and have relevance with respect to CW optimization and engineering.

Chapter 7 – Principle Outcomes and Recommendations

7.1 Principle Outcomes

The overall objective of this work was to study the temporal and spatial dynamics of the bacterial community in wetland mesocosms. The specific sub-objectives were:

A) Develop and apply the CLPP method using BIOLOG^{TM} Eco plates for the characterization of bacterial communities in constructed wetland (CW) mesocosms
B) Develop a metric for tracking community divergence using CLPP data
C) Develop a method for the detachment of viable bacteria from wetland gravel
D) Investigate fixed film bacterial communities associated with both wetland mesocosm gravel media and root systems at varying subsurface depths
E) Monitor changes in the interstitial bacterial communities in wetland mesocosms following the simultaneous start-up of multiple systems
F) Appraise the significance of plants and seeding community origin on bacterial community development in wetland mesocosms

Figure 7.1 summarizes the research presented in this thesis.

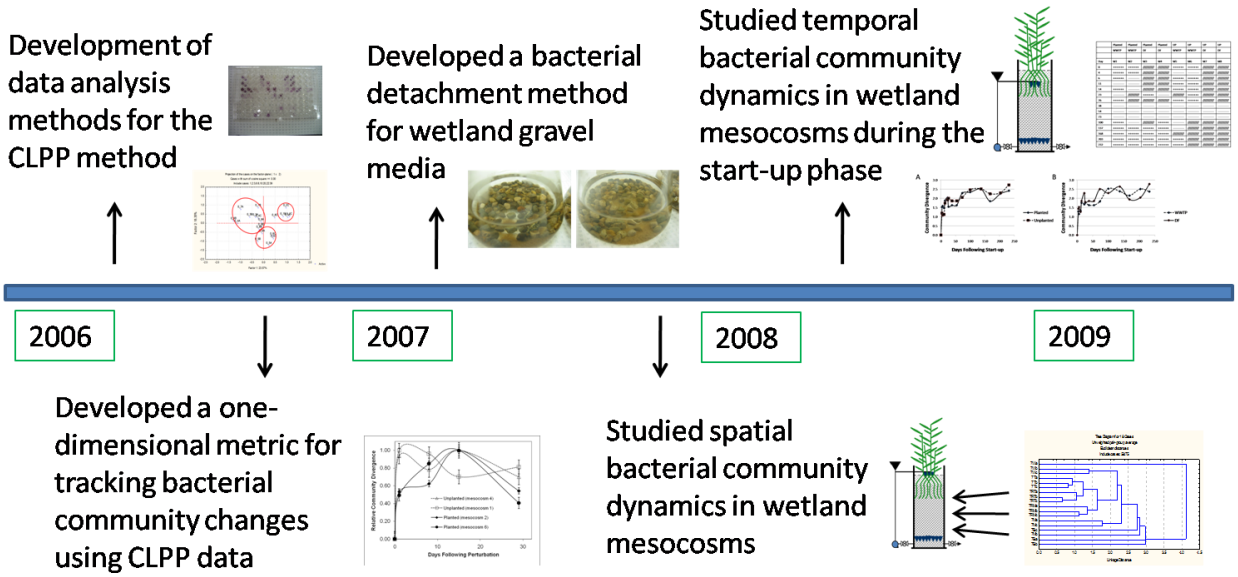


Figure 7.1: Research timeline.

7.1.1 Community Level Physiological Profiling – (Thesis Objective A)

Chapter 2 outlined the developed CLPP method. Although the CLPP method can be considered a technique which offers an easily applied protocol yielding information regarding mixed microbial community function and functional adaptations over space and time, the data analysis involved can be quite challenging. Much of the effort devoted in this thesis was directed towards multivariate methods of data analysis in order to interpret the CLPP data gathered using BIOLOG™ ECO plates. Being able to bring together a standardized CLPP laboratory protocol and data analysis methodology was a significant cornerstone and resulted in a textbook chapter contribution to *Methods in Molecular Biology: Bioremediation* [in press].

7.1.2 One-Dimensional Metric for Tracking Bacterial Community Divergence using Sole Carbon Source Utilization Patterns – (Thesis Objectives A & B)

Chapter 3 outlines the use of the CLPP method for tracking bacterial community divergence in constructed wetland mesocosms in response to a perturbation. Two commonly employed data analysis methods, 1) PCA and 2) guild analysis were used to reduce the dimensionality of the

CLPP data for subsequent interpretation. These two methods proved useful in tracking community divergence in the interstitial water of CW mesocosms, proving the CLPP method to be feasible and useful in this context. The main goal and significant outcome from this chapter was in the development of a new one-dimensional metric which can be used to track community divergence using the CLPP BIOLOG^{TM} ECO plate method. In comparison to the PCA and guild analysis methods, this one-dimensional divergence metric was shown to accurately describe community divergence in the mesocosm systems. The one-dimensional divergence metric cannot by definition yield information regarding community similarities or differences in a static state, however proved suitable for tracking community divergence over time. The community divergence method as presented is easy to use and does not require a background in multivariate statistics. It is suggested that this community divergence metric could be considered a useful addition to the toolbox for scientists and engineers interested in bacterial community divergence. Chapter 3 which describes the development and use of this proposed divergence metric has been submitted to the *Journal of Microbiological Methods* [June, 2009].

7.1.3 Method for the Detachment of Viable Bacteria from Wetland Gravel - (Thesis Objective C)

One of the major objectives of this thesis (Objective D) was the use of the CLPP method to characterize of the biofilm-associated bacterial communities in wetland mesocosms. Using the CLPP method to characterize biofilm-associated bacterial communities CW has not been previously done; as such an appropriate biofilm detachment protocol needed to be developed. As the CLPP method is a metabolic-based community characterization method, viable bacteria are required for analysis. The need for viable cells discounted a number of previously developed biofilm and bacterial detachment methodologies including scraping, swabbing, sonication, blending, and digestions. Variations of a simple shaking technique were evaluated for their effectiveness in the detachment of viable bacterial communities from biofilm surrounding constructed wetland mesocosm pea gravel media. Suspensions from the different shaking technique protocols were characterized for organics, inorganics, viable bacteria,

community level physiological profile (CLPP) and several BIOLOG™ ECO plate substrate related diversity indices. Using these metrics for the evaluation of the different protocols the most effective protocol was found to include the use of phosphate buffer solution with mechanical shaking and the addition of various enzymes to the detachment solution.

7.1.4 Bacterial Community Stratification in the Substrate of Constructed Wetland Mesocosms – (Thesis Objective D)

Bacterial community samples associated with the pea gravel bed media were taken from the top (10 cm depth), middle (30 cm depth), bottom (60cm depth) of all mesocosms, profiled using the CLPP method, and compared. Interstitial water communities were also profiled and compared to the pea gravel-associated bacterial communities from all depths. The use of both planted and unplanted mesocosms enabled the comparison of the respective bacterial communities. Root associated (rhizospheric) bacterial communities were also gathered from varying depths within the planted mesocosms and compared to the media and interstitial community profiles.

Organic deposition was found to be homogeneous throughout the mesocosms. Bacterial communities closer to the surface of the mesocosms were found to have slightly higher activities. Rhizospheric communities were found to be significantly more active than their gravel media associated counterparts, suggesting that rhizospheric bacteria, although less abundant in the mesocosm systems, perhaps play a larger role in the removal and fate of water born contaminants.

A decrease in the substrate richness and diversity values was seen with increasing mesocosm depth. This result coupled with the similar trend seen for activity suggests that the bacterial communities in the lower depths of the mesocosm systems are perhaps less healthy and robust than their bacterial counterparts closer to the bed surface. This may have implications in terms

of contaminant treatment. With the lower depth bacterial communities being less active, less healthy, and less robust it is likely that the bacterial communities closer to the bed surface will play a greater role in long term water treatment operation.

Differences in the bacterial community structure as a function of gravel depth were seen for all mesocosm systems. Vertical community stratification was observed in all cases.

It was shown that the general differences in mesocosm bacterial communities at all depths in the mesocosm systems were similar to the differences interstitial water communities. This suggests that when trying to decipher general community differences between mesocosms interstitial water samples are an accurate reflection of these differences.

Not all planted mesocosm systems had developed root systems throughout the entire bed depth. The fact that differences in the communities from planted and unplanted mesocosms were found at all depths suggests that the presence of roots within at least part of the mesocosm system does not only have a localized effect on the attached bacterial population but on attached bacteria from all depths within the mesocosms.

7.1.5 Bacterial Community Profiling and Hydrological Characterization of Constructed Wetland Mesocosms during Start-up - (Thesis Objectives E & F)

Eight newly created CW mesocosm systems were characterized using standard methods for evapotranspiration (ET), porosity, dispersivity, and overall microbial activity during an 8 month start-up period. The interstitial bacterial communities of the 8 mesocosms were also characterized using BIOLOG^{TM} ECO plates via the CLPP method for average well colour development (AWCD), substrate richness, substrate evenness, substrate diversity, over-all

community, and community similarities based on experimental design treatments (plant presence, and seeding inoculum origin).

It was found that mesocosm porosities decreased over time as a result of media related biofilm development. This biofilm development also contributed to a substantial increase in the dispersion coefficient (mixing properties) in the mesocosms over the entire start-up period. Dispersion coefficient values within planted systems reached values of ~50-55 cm^2/min where values within the unplanted systems reached values of ~30-35 cm^2/min.

The general divergence trend in the mesocosm systems was quantified using the Euclidean divergence metric developed in Chapter 3. All mesocosms showed a steep increase in community divergence until day 75-100, at which point a steady-state was reached. The interstitial communities were also characterized in terms of similarity based on the experimental design treatments. A four phase progression for all mesocosm was observed that can be summarized as: 1) a state of initial difference based on original bacterial sludge inoculum community profiles [day 0-6], to 2) a period where adjustments and shifts within the bacterial community occur within all mesocosms [day 11-26], to 3) a time where all mesocosm interstitial CLPPs are quite similar [day 38-73], to 4) a final shifting towards unplanted and planted mesocosm CLPP groupings [day 100-232].

7.2 Recommendations

The studies described herein all dealt with understanding fundamental microbiological processes or distributions within constructed wetland mesocosms. The CLPP method proved useful in obtaining information regarding both spatial and temporal bacterial community dynamics. Results also showed that plants have a significant effect on the bacterial communities found both within the rhizosphere region and in the general CW bed media. Three

main recommendations are made here, all of which aim at bringing the field of CW engineering to a platform of greater confidence with respect to design and control.

Recommendations for future work include:

1) Application of the methods developed here to the pilot and/or full scale setting. Understanding bacterial community dynamics in full scale systems would perhaps allow for increased performance predictions and enhanced performance control, making CW treatment systems a more viable and reliable water treatment technology.

2) Correlation of A) the differences in resident CW bacterial communities, and B) general bacterial community characteristics such as community diversity and activity, with CW treatment performance. This can initially be accomplished at the lab scale using mesocosm systems, however over time should also be extended to large scale systems.

3) Further validation and connectivity of the mesocosm approach to both pilot scale and large scale CW treatment systems. This can be accomplished through comparison of standard characteristics such as evapotranspiration, porosity, and dispersivity; however should also be expanded to microbiological parameters such as CLPP comparisons, community diversity, community evenness, community richness, and overall microbial activities. The use of mechanistic models could also be employed in the validation process through concurrent model calibrations to both large scale and mesocosm scale treatment systems, use of microbial activity and some type of community divergence metric could be included into future models. Comparison of calibrated parameters could then give an idea of how well the mesocosm systems represent full scale systems, and in what ways some of the mechanistic treatment processes are biased at the mesocosm scale.

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Chapter 1

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Appendices

Appendix A – Abbreviations

A - wetland area [m^2]

ALD - anoxic limestone drain

AMD - acid mine drainage

AWCD - average well colour development

BOD - biological oxygen demand

B - treatment coefficient/intercept

C - concentration [#/m^3]

C* - background concentration

C_0 - effluent concentration

CCorA - canonical correlations analysis

CFU - colony forming units

C_i - influent concentration

CLPP - community level physiological profiling

COD - chemical oxygen demand

CSTR - completely stirred tank reactor

CSUP - carbon source utilization pattern

CW - constructed wetland

DA - discriminant analysis

DCA - detrended correspondence analysis

DF - dairy farm

DGGE - denaturing gradient gel electrophoresis

DNA - Deoxyribonucleic acid

DO - dissolved oxygen

DOC - dissolved organic carbon

DON - dissolved organic nitrogen

E - substrate evenness

EPS - extracellular polymer substances

FDA - fluorescein diacetate

FISH - fluorescent in situ hybridization

FL - fluorescein

FWS - free water surface

h - water depth [m]

H - substrate diversity

HLR - hydraulic loading rate [mass/time]

HRT - hydraulic retention time

HSSF - horizontal subsurface flow

k - first-order rate constant

K - half saturation constant

k/q - Da (Damkőhler number)

k_1 - first-order, zero background aerial rate constant [m/d]

k_{20} - reaction rate coefficient at 20ºC

k_T - reaction rate coefficient at temperature (T)

k_{v1} - volume-based, first-order decay rate [1/d]

LB - Luria-Bertani

MA - microbiological activity

NMDS - non metric dimensional scaling

NOM - natural organic matter

Pe - peclet number, dimensionless

PC - principal component

PCA - principal component analysis

PCoA - principle coordinates analysis

PCR - polymerase chain reaction

PGPR - plant growth promoting bacteria

POC - particulate organic carbon

PON - particulate organic nitrogen

PVC - polyvinyl chloride

q - hydraulic loading rate

R - death rate [#/day]

RDA - redundancy analysis

rRNA - ribosomal ribonucleic acid

S - substrate richness

STP - sewage treatment plant

T - temperature (°C)

TC - total coliform

THM - trihalomethane

TN - total nitrogen

TOC - total organic carbon

TP - total phosphorus

TRFLP - terminal restriction fragment length polymorphism

TSS - total suspended solids

u - growth rate

u_{max} - maximum growth rate

UPGMA - unweighted pair group method using arithmetic mean

UV - ultra violet

v - velocity (cm/min)

V - wetland water volume [m^3]

VF - vertical flow

W - wetland dispersion number, dimensionless

WWTP - waste water treatment plant

ε - volume fraction of water

θ - temperature factor

τ - nominal detention time [days]

Appendix B – Waterloo Region Tap Water Characteristics

Microbiological Parameters	Process	MAC or IMAC	# of Samples	# of Detectable Results	Sampling Date(s)	Range	Exceedence	Typical Source of Contaminant
Free Chlorine Residual (mg/L)	SUPPLY	4	67+Online	66+Online	01-Jan-02 to 31-Mar-02	0.00-1.742	No	Recommended level of at least 0.2 mg/L of free chlorine to maintain microbiological quality in the distribution system. An adverse result occurs if free chlorine <0.05 mg/L and total chlorine <0.25 mg/L.
Total Chlorine Residual (mg/L)	SUPPLY	3	13	13	03-Jan-02 to 13-Mar-02	0.74-1.63	No	Recommended level of at least 1 mg/L of total chlorine to maintain microbiological quality in the distribution system. An adverse result occurs if free chlorine <0.05 mg/L and total chlorine <0.25 mg/L.
Total Coliforms (Present/Absent)	SUPPLY		10	0	03-Jan-02 to 13-Mar-02	P/A	No	Presence of Total Coliforms indicates possible presence of fecal matter. Indicator of adverse water quality if detected in treated water.
E. Coli (Present/Absent)	SUPPLY		10	0	03-Jan-02 to 13-Mar-02	P/A	No	Presence of E. Coli indicates fecal contamination. Indicator of adverse water quality if detected in treated water.
Heterotrophic Plate Counts (counts/1 mL)	SUPPLY	500	3	1	16-Jan-02 to 07-Mar-02	0-4	No	HPC testing is used to monitor disinfection efficiency at water treatment plants and to measure water quality deterioration in distribution systems and in reservoirs.
Turbidity (NTU)	SUPPLY	1	56+Online	56+Online	01-Jan-02 to 31-Mar-02	0.00-0.40	No	Indicates presence of particles in water due to treatment difficulties.
Inorganic Parameters (Table C)	**Process**	**MAC or IMAC**	**# of Samples**	**# of Detectable Results**	**Sampling Date(s)**	**Range**	**Exceedence**	**Typical Source of Contaminant**
Barium (mg/L)	SUPPLY	1	1	1	14-Nov-01	0.096	No	Natural component of water at this level.
Fluoride (mg/L)	SUPPLY	1.5	67	67	27-Nov-01 to 08-Feb-02	0.26-1.1	No	Added to prevent tooth decay. Natural component of water at this level.
Iron (mg/L)	SUPPLY	0.3	1	1	14-Nov-01	0.026	No	Natural component of water at this level.
Manganese (mg/L)	SUPPLY	0.05	1	1	14-Nov-01	0.029	No	Natural component of water at this level.
Nitrate (mg/L)	SUPPLY	10	1	1	14-Nov-01	0.62	No	Natural component of water at this level.
Sodium (mg/L)	SUPPLY	20	1	1	14-Nov-01	8	No	The aesthetic objective is 200 mg/L. However, MOH requires report of sodium at 20 mg/L or higher, since sodium may be a problem for those with sodium restricted diets.
Zinc (mg/L)	SUPPLY	5	1	1	14-Nov-01	0.005	No	May be present due to corrosion taking place in galvanized pipes.
Volatile Organics (Table B)	**Process**	**MAC or IMAC**	**# of Samples**	**# of Detectable Results**	**Sampling Date(s)**	**Range**	**Exceedence**	**Typical Source of Contaminant**
Trihalomethanes (mg/L)	SUPPLY	0.1	4	4	06-Dec-00 to 01-Nov-01	0.0073	No	Byproduct of chlorination. MAC is based on a running annual average of four quarterly samples and compared to the average of the most recent four detects.
Pesticides and PCBs (Table D)	**Process**	**MAC or IMAC**	**# of Samples**	**# of Detectable Results**	**Sampling Date(s)**	**Range**	**Exceedence**	**Typical Source of Contaminant**
Table D of ODWPR	SUPPLY		1	0	01-Nov-01	0	No	
Aesthetic Objective/Operational Guideline	**Process**	**MAC or IMAC**	**# of Samples**	**# of Detectable Results**	**Sampling Date(s)**	**Range**	**Exceedence**	**Typical Source of Contaminant**
Alkalinity (mg/L)	SUPPLY	500	1	1	14-Nov-01	240	No	Alkalinity is a measure of resistance of the water to the effects of acids added to water.
Chloride (mg/L)	SUPPLY	250	1	1	14-Nov-01	14	No	Widely distributed in nature, generally as the sodium (NaCl), potassium (KCl) and calcium ($CaCl_2$) salts.
Colour True (TCU)	SUPPLY	5	1	1	14-Nov-01	6	Yes	Faint yellow/brown colour is often caused by organic materials created by the decay of vegetation or by iron and manganese compounds produced by processes occurring in natural sediments or in aquifers.
Hardness (Grains)	SUPPLY	5.85	1	1	14-Nov-01	20.88	Yes	Caused by dissolved calcium and magnesium, and is expressed as the equivalent quantity of calcium carbonate.
Hardness (mg/L as $CaCO_3$)	SUPPLY	100	1	1	14-Nov-01	357	Yes	Caused by dissolved calcium and magnesium, and is expressed as the equivalent quantity of calcium carbonate.
pH	SUPPLY	8.5	1	1	14-Nov-01	7.6	No	Indicates the acidity of a water sample. High pH may result from corrosion of specific types of pipes.
Sulphate (mg/L)	SUPPLY	500	1	1	14-Nov-01	55	No	High levels may be associated with calcium, which is a major component of scale in boilers and heat exchangers.

Data taken from the regional municipality of Waterloo website: http://chd.region.waterloo.on.ca/WEB/Region.nsf/0/57F555069B5A60AA85256C06005F8596/$file/Table%2027.pdf?openelement (last accessed August 20, 2009)

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