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The influence of water quality on wetland-associated microbial communities

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Abstract

Within a wetland environment, bacteria in association with plant roots play a vital role in maintaining the health of freshwater ecosystems. In order to gain insight into the stability and processes occurring within natural and constructed wetland environments we need to develop a better understanding of the relationship between wetland plants, root-associated microbial communities and environmental factors. Human population growth and urbanization have resulted in greater contaminant loads (inorganic nutrients, fecal contamination etc.) entering our waterways. As such, we need a better understanding of how anthropogenic impacts influence the structure and function of the wetland-associated microbial communities that we rely on to maintain the integrity of our freshwater ecosystems. To meet this need we designed a series of experiments to investigate the hypothesis that wetland-associated microbial communities highly impacted by anthropogenic activities subjected to poor water quality inputs (high inorganic nutrient load) would differ from less impacted communities in terms of community structure, function, remedial capabilities and resilience. Furthermore, we hypothesized that plant species would play a role in how the associated microbial community would respond to these differences in water quality. To investigate these hypotheses we used a multi-faceted approach involving both *in situ* field-based studies (Grand River, ON) and *ex situ* lab-scale wetland mesocosm studies. We examined microbial communities in association with several different plant species (*Phalaris arundinacea, Iris versicolor, Potamogeton natans* and *Veronica spicata)* across field sampling locations (Grand River, ON). Lab-scale mesocosm studies involved sub-surface flow wetland mesocosms planted with either *P. arundinacea* or *V. anagallis-aquatica* receiving water from sites with contrasting water quality charactersitics. To ascertain the ability of the microbial communities associated with these mesocosm treatments to resist environmental perturbations, mesocosms were exposed to 5mg/L of inorganic phosphorus to simulate runoff from a rain event. We used PCR in combination with denaturing gradient gel electrophoresis (DGGE) to examine the structure of microbial communities in association with wetland plant roots and water-associated communities. Functional community characteristics were examined by obtaining community-level carbon source utilization patterns with Biolog^{TM} EcoPlates. We examined the influence of water quality and plant species on fecal contamination associated microbial pathogens by enumerating fecal coliforms as well as *Salmonella* spp., *Escherichia coli* and *Enterococcus* spp. specifically, from water and root-associated microbial communities using the membrane fecal coliform method and quantitative real-time PCR. The remediation potential of *ex situ* mesocosm-based microbial communities experiencing different water quality treatments in association with our study plant species were determined by quantifying inorganic nitrogen and phosphorus concentrations from mesocosm outflow water. From our field-based studies we found that the structure and function of microbial communities in association with wetland plant roots was affected by sampling location, however this effect was dependent on the plant species in question as well as the root-associated community type (rhizoplane or rhizosphere). Furthermore, plant species differed in their retention of microbial DNA from fecal contamination associated microorganisms. Our *ex situ* mesocosm-based wetland studies yielded comparable results. We found that the root-associated microbial communities from *P. arundinacea* and *V. anagallis-aquatica* were altered structurally and functionally by the different water quality treatments. However, functional characteristics of *P. arundinacea*-associated communities were affected by water quality treatment to a greater extent than those communities

associated with *V. anagallis-aquatica*. Furthermore, the influence of water quality treatment on microbial community structure and function differed by community type. Rhizoplane-associated microbial communities exhibited the most dramatic structural and functional changes when challenged with varying water quality treatments. Exposure to short-term phosphorus loading as 5 mg/L of inorganic phosphorus resulted in changes to microbial community structure and function in both plant species-associated microbial communities, most notably within the rhizoplane. Structural and functional community diversity was reduced following the inorganic phosphorus treatment for rhizoplane-associated microbial communities. Both mesocosm-based wetland communities performed equally well at removing inorganic nutrient loads from the various water quality sources.

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Introduction

1.1 What are Wetlands?

Wetlands are classified as ecosystems where the soil is saturated with water; they differ from upland ecosystems due to their high soil moisture levels and from aquatic ecosystems due to the presence of submerged aquatic vegetation and standing water of no more than 2 m in depth. This continuous presence of water is the factor that drives the type of vegetation that dominates the system and the animal communities that persist there (Cowardin *et al.*, 1979). Freshwater wetlands receive their water primarily from surface waters (e.g. runoff, streams, rivers, lakes, and human-generated wastewater discharges) but also from groundwater and precipitation to a lesser extent. Similarly, the output from wetlands can contribute to each of surface water, groundwater and evapotranspiration (Carter, 1986). Contaminants and undesirable substances can enter wetlands through any of the water sources previously mentioned, however, the highest inputs are generally from source waters altered by human activities such as municipal and industrial wastewater discharges and runoff from agricultural and urban settings (Johnston, 1991). Remediation of contaminated water entering a wetland can occur by three primary pathways: 1) physical retention of the contaminant within the wetland, 2) alteration of the contaminant via chemical reactions making it less harmful or 3) alteration of the contaminant by biological action (Sheoran and Sheoran, 2006).

1.2 The Structure of Microbial Wetland-Associated Communities

Wetland microbial communities can be discussed by considering three distinct habitat types: water communities, soil/sediment communities and plant-associated communities. Each of these habitat types will drive the selection of distinct microbial communities with unique community characteristics and capabilities.

1.2.1 Water Microbial Communities

Water-associated wetland microbial communities can be isolated from either surface water in a surface flow wetland system, or discharge effluent in a subsurface flow wetland system lacking aboveground standing water. Bacterial communities associated with water tend to be low in both total species diversity as well as overall numbers, as compared to communities associated with either soil or plants (Wetzel, 1975; Wassel and Mills, 1983). The structure and function of microbial communities associated with water vary due to changes in nutrient and organic matter availability (Gessner and Chauvet, 1994; Dodds *et al.*, 2000), temperature (Boyero *et al.*, 2011), system hydrology (Valett *et al.*, 1997) and land use (Mullholland *et al.*, 2008). Physicochemical properties such as O_2, oxidation reduction potential, pH and light are also significant in driving microbial community structure and function within these systems (Paerl and Pinckney, 1996). The most active water-associated microbial communities are typically isolated from detritus and microbial mat/biofilm communities at the sediment/water interface due to the higher concentration of nutrients and available surfaces for attachment (Paerl and Pinckney, 1996). Most of the information available on water-associated microbial communities originates from studies of lakes and free flowing water systems. Studies on wetland-associated water microbial communities in the literature are few and far between, and

largely focus on soil or sediment-associated communities. This could be due to the fact that soil/sediment communities are considered to be more significant in the remediation functionality of the wetland, and thus, more important to study. Wetland water with the exception of subsurface flow effluent, contains molecular oxygen which is a potent inhibitor of key metabolic nutrient transformations carried out by microbial assemblages (e.g. N_2 fixation, denitrification, sulfate reduction, methanogenesis, metal reduction), this combined with the lower total number of bacteria associated with water vs. soil could lead researchers to discount the importance of water-associated communities in wetland functions (Jorgensen, 1983; Fay, 1992; Gallon, 1992). However, studies of lake water-associated microbial communities have shown that these microorganisms can play a significant role in inorganic phosphorus removal via sequestration and alteration of available inorganic phosphorus into biologically unavailable refractory phosphorus-containing compounds (Gächter and Mares, 1985; Gächter *et al.*, 1988; Gächter and Meyer, 1993; Kulaev *et al.*, 2005).

1.2.2 Soil/Sediment Microbial Communities

Wetland soil or sediment microbial communities are known to play an important role in nutrient cycling and are especially valued within both natural and constructed wetlands due to their ability to remove excess inorganic nitrogen (Balser *et al.*, 2002; Boon, 2006; Reddy and DeLaune, 2008). Microbial processes occurring within wetland soils that are significant to inorganic nitrogen removal include nitrification, anaerobic ammonium oxidation (Anammox) and denitrification (Wallace and Austin, 2008; Lee *et al.*, 2009). Wetland soil microbial community structure and function have been shown to be driven primarily by the physicochemical properties of the soil. Physicochemical characteristics that have been shown to drive wetland soil community structure include carbon to nitrogen ratio (Peralta *et al.*, 2013),

total carbon and inorganic nitrogen soil concentrations (Ligi *et al.*, 2014), inorganic phosphorus concentrations (Wright and Reddy, 2000) calcium content (Ligi *et al.*, 2014) and pH (Peralta *et al.*, 2013; Ligi *et al.*, 2014). Natural and constructed wetlands with similar physicochemical properties have been shown to have similar microbial community structures with respect to species diversity, such that the natural vs. constructed nature of the wetland seems to be less important in determining community structure than the chemical properties of the soil (Peralta *et al.*, 2013). Similarly, the physical and chemical properties associated with soil or sediment are also a strong driver of vegetation development (Spieles, 2005). Characteristics such as bulk, density, moisture, organic matter content, organic carbon and soil type are drivers of wetland plant community development (Ballantine and Schneider, 2009; Ehrenfelt *et al.*, 2005). Plant community structure has been established as a key factor influencing wetland-associated microbial communities and can directly impact soil microbial communities by altering the physicochemical properties of the soil, particularly inorganic nutrient availability (Angeloni *et al.*, 2006).

1.2.3 Plant-Associated Microbial Communities

Microbial communities can be associated with all surfaces of the plant including leaves, stems and roots. With respect to remediation purposes, the root-associated bacteria are considered to be the most relevant. The rhizosphere can be defined as the narrow region of soil surrounding plant roots that is influenced by the growth, respiration and root secretions of the plant, while the rhizoplane is the microbial biofilm directly attached to the root surface (Berendsen *et al.*, 2012). The root-associated microbial community is unique in that it contains a high abundance of microbial organisms; the rhizoplane and rhizosphere can contain as many as 10^{11} microbial cells per gram of root tissue and more than 30,000 prokaryotic species

(Egamberdivea *et al.*, 2008; Mendes *et al.*, 2011). The high abundance of bacterial cells present in root-associated microbial communities is primarily a function of the organic deposits made by plant roots into the surrounding environment (also called rhizodeposits) which act as a source of nutrients for the associated microbiota (Baudoin *et al.*, 2003; Philippot *et al.*, 2013). In general, the carbon content of bulk soil is relatively low such that the microbial community of the soil is starved for carbon (Garbeva *et al.*, 2011). Plants can actively secrete up to 40% of their photosynthates into the rhizosphere in the form of root exudates such as simple carbohydrates (sugars), carboxylic acids, and amino acids (Baudoin *et al.*, 2003; Bais *et al.*, 2006). Rhizodeposits may also come in the form of complex carbon compounds such as decaying root material from sloughed off border cells and mucilage (Philippot *et al.*, 2013). The result of this increased nutrient source located in the micro-environment surrounding plant roots, is that microbial abundance is much greater than in the surrounding bulk soil, a phenomenon known as the rhizosphere effect (Berendsen *et al.*, 2012).

It is widely accepted that root-associated microbial communities are actively shaped by the plant (Haichar *et al.*, 2008; Hartmann *et al.*, 2009; Berendsen et al, 2012; Philippot *et al.*, 2013). For this reason, these communities have a species-specific composition that is distinct from the surrounding bulk soil (Costa *et al.*, 2006). One of the primary forces driving the unique structure of these communities is the excretion of soluble carbon compounds by plant roots (Haichar *et al.*, 2008; Hartmann *et al.*, 2009). The composition of root exudates is plant species-specific and can have a strong influence on the microbial community structure (Costa *et al.*, 2006; Berendsen *et al.*, 2012). One way that root exudation profiles may shape the root-associated microbial community is with the prevalence of specific root exudate compounds. For example, a strong correlation was found between the ability of root-associated bacterial isolates

to grow on citric acid as their sole carbon source and the ability of these bacteria to colonize the rhizosphere of tomato (*Lycopersicon esculentum*), cucumber (*Cucumis sativus*) and sweet pepper (*Capsicum annuum*), three species of plants which predominantly exude organic acids including citric acid, succinic acid and malic acid (Kamilova *et al.*, 2006). Another way that plants may shape their root-associated communities is through the ratio of different types of root exudates secreted. For example, when the root exudation profiles of maize (*Zea mays*) plants were artificially modified by the exogenous application of artificial root exudates (carbohydrates (glucose, sucrose and fructose), carboxylic acids (citric acid, succinic acid, and lactic acid) and amino acids (alanine, serine and glutamic acid)) with contrasting ratios of carboxylic acids and amino acids, distinct microbial rhizosphere communities developed in each treatment (Baudoin *et al.*, 2003).

Plants may also actively control their root-associated microbial community composition through the secretion of secondary metabolites including antimicrobial compounds. For example, a group of heteroaromatic metabolites called benzoxazinoids are frequently released in large amounts from cereal roots where they act defensively against pathogenic bacteria, fungi and herbivorous insects (Niemeyer, 2009). One well studied example of this phenomenon is 2,4-dihydroxy-7-methoxy-2H-1,4-benzoxazin-3(4H)-one (DIMBOA) which is the primary benzoxazinoid compound secreted by maize (Neal *et al.*, 2012). The secretion of DIMBOA has been shown to significantly alter root-associated bacterial biomass and community structure in the rhizosphere (Chen *et al.*, 2010). DIMBOA can also act as a chemo-attractant for some species of plant-beneficial bacteria such as *Pseudomonas putida* which has been attributed with plant growth-promoting properties. These include its ability to solubilise essential nutrients (e.g. phosphorus) and to produce the plant hormone indole-acetic acid (IAA) which plays an

important role in plant growth and maturation (Mehnaz and Lazarovits 2006). Roots of maize producing DIMBOA have been shown to actively attract high numbers of *P. putida* which are able to effectively establish themselves within the root-associated microbial community. DIMBOA-deficient maize mutants are colonized by *P. putida* in significantly lower numbers (Neal *et al.*, 2012). Secondary metabolites that actively influence soil microorganisms and plant rhizosphere community structures are also known as allelochemicals (Whittaker and Feeny, 1971). Plant roots can secrete many different types of these compounds which act as chemo-attractants for different species of plant-beneficial bacteria (Whittaker and Feeny, 1971; Guo *et al.*, 2011). One group of allelochemicals that have been identified as important in shaping root-associated microbial communities are isoflavones. Isoflavones have been shown to alter the structure of bacterial and fungal root-associated communities when applied exogenously in physiologically relevant concentrations (Qu and Wang, 2008). Isoflavones are also known to act as chemo-attractants for some bacterial species (e.g. *Bradyrhizobium japonicum* and *Rhizobium meliloti*), increasing rhizosphere colonization by these microbes when secreted by plant roots (Caetano-Anollés *et al.*, 1988; Bias *et al.*, 2006).

Many bacterial species (plant-associated and otherwise) utilize diffusible molecules for cell-to-cell signalling to determine the density of cells belonging to their own species within a microenvironment. This type of signaling is called quorum sensing and is used to regulate certain genes that are normally expressed when cell density reaches a critical threshold, such as biofilm formation, adhesion, virulence factor expression, the production of antibiotics/exoenzymes and conjugal plasmid transfer (Teplitski *et al.*, 2000; Waters and Bassler, 2005). In Gram-negative bacteria these cell-to-cell signaling molecules are known as acyl-homoserine lactones (AHL) and several recent studies have found that many species of plants are able to secrete substances that

either interfere with bacterial AHL-regulated quorum sensing or mimic AHL molecules resulting in the induction of AHL stimulated gene expression. For example, a group of secondary metabolites, halogenated furanones, from the red alga *Delisea pulchra* are structurally similar to AHL molecules and have been shown to interfere with AHL mediated behaviors such as swarming motility in *Serratia liquefaciens* and carbapenem antibiotic synthesis and exoenzyme virulence factor production in *Erwinia carotovora* (Manefield *et al.*, 1999; Manefield *et al.*, 2001). Additionally, exudates from pea (*Pisum sativum*) seedlings were found to contain several different compounds that mimicked AHL molecules, some of which stimulated AHL-dependent swarming motility in *S. liquefaciens* and others which inhibited AHL-dependent violacein (antibiotic) synthesis and extracellular protease and chitinase production (Teplitski *et al.*, 2000). Furthermore, there are greater than 15 different compounds which have been identified from seedling exudates of barrel medic (*Medicago truncatula*) that stimulate or repress AHL-dependent behavioral responses in quorum sensing bacteria (Gao *et al.*, 2003).

Another way that plant roots create a unique habitat for microorganisms is through some of the active physiological processes that are naturally occurring within the root (e.g. respiration, nutrient uptake). These processes create a gradient of physicochemical conditions providing diverse microhabitats suitable for a wide range of bacterial species with contrasting physiological requirements (Vymazal *et al.*, 2007; Hartmann *et al.*, 2009). One of the most obvious chemical gradients occurring in the zone surrounding plant roots is oxygen, as oxygen is secreted from plant roots as a by-product of photosynthesis (Hartmann *et al.*, 2009). The concentration of oxygen in the rhizosphere can be as high as 36-66% of air saturation in species of wetland rush (*Juncus inflexus* and *Juncus articulatus*) with oxygen concentrations declining quickly with distance from the root (Blossfeld *et al.*, 2011). This chemical gradient can play an important role

in structuring the bacterial communities as it provides a habitat for both aerobic bacterial species and bacterially-mediated processes that are reliant on oxygen (e.g. nitrification) as well as anaerobic and micro-aerotolerant bacterial species that require oxygen-depleted microhabitats and anaerobic or low oxygen environments to carry out their metabolism (e.g. denitrification and non-symbiotic nitrogen fixation) (Weiss *et al.*, 2002; Vymazal *et al.*, 2007). The oxygenation of soils by plant roots can also have a dramatic effect on redox potentials, with microenvironments ranging from oxidizing conditions near the plant root surface to reducing conditions further from the oxygen secreting zone of the root system (Flessa, 1994; Hartmann *et al.*, 2009). A good example of the implications of this root-mediated redox gradient is the enhanced cycling of iron between its oxidized (Fe^{3+}) and reduced (Fe^{2+}) states, which is known to occur within the rhizosphere of wetland plants (Weiss *et al.*, 2004). Iron-oxidizing bacteria and iron-reducing bacteria are both abundant on the roots of wetland plants, and it has been demonstrated that Fe(II) oxidation and Fe(III) reduction are coupled within the root zone, promoting localized iron cycling within root-associated microenvironments (Weiss *et al.*, 2002; Weiss *et al.*, 2004). Additionally, plant roots are known to have a large impact on the pH of the soil surrounding their roots (Hartmann *et al.*, 2009). Plants secrete protons into the rhizosphere primarily in the form of un-dissociated acids such as carboxylic acids present in root exudates (Hinsinger *et al.*, 2003; Hartmann *et al.*, 2009). Additionally, the uptake of nutrients by the roots can lead to the release of dissociated protons into the rhizosphere to compensate for the acquisition of excess cations, as the process of nutrient acquisition by the roots is ultimately electroneutral (Sas *et al.*, 2001; Hinsinger *et al.*, 2003). Rhizospheric changes in pH can be dramatic, and differences between rhizosphere and bulk soil pH measurements of greater than 1 pH unit have been noted on numerous occasions (Hinsinger *et al.*, 2003; Blossfeld *et al.*, 2011). These pH differences

between the root surface and surrounding soil are dynamic rather than static (diurnal and seasonal variation), and can differ dramatically between plant species and even within the root architecture of a single plant (Blossfeld *et al.*, 2011). Rhizosphere pH changes can be important for the mobilization of inorganic nutrients which, in turn, will affect microbial processes such as nutrient cycling (Hartmann *et al.*, 2009; Weiss *et al.*, 2002).

1.3 Defining Water Quality

In order to effectively address the remediation of contaminated source waters and/or the treatment of various types of wastewater effluents before they re-enter the watershed, it is important to be able to define water quality in a meaningful way. A thorough description of what constitutes both good and poor water quality can be an important tool employed in the remediation of contaminated source waters. It provides us with a set of parameters that we can measure in order to quantitatively monitor the status of water bodies. The World Health Organization has defined the quality of an aquatic environment as a set of concentrations of inorganic and organic substances, the composition and state of the biological components of the aquatic ecosystem, and a description of both spatial and temporal variations in the water body that are affected by both internal and external factors (Chapman, 1996). Similarly, pollution of an aquatic environment is defined as any substance or energy that results in the impairment of water quality with respect to its anthropogenic uses (industrial, agricultural and municipal) or harm to human health or any living resources (Chapman, 1996). Additionally, the Joint Group of Experts on the Scientific Aspects of Marine Environmental Protection (GESAMP) states that pristine water quality is defined as the physical and chemical parameters that a body of water would have possessed before any type of anthropogenic impact had occurred, however, due to

atmospheric transport of contaminants, pristine waters are either very rare or non-existent in current times (GESAMP, 1988; Meybeck and Helmer, 1989).

With increasing human populations alongside expansions in industrialization, our requirements for water have increased along with our need for higher water quality. Drinking, personal hygiene, fisheries, agriculture, transportation, industrial production , cooling in fossil fuel and nuclear power plants, hydropower generation and recreation activities have all imposed pressures on a limited water supply, each requiring different levels of water quality for proper use (Chapman, 1996). Conversely, each of these activities also leads to the discharge of wastewater resulting in an impact on water quality. Changes to the landscape, including urbanization and deforestation, and the accidental releases of chemical substances *via* leaching or gaseous emissions can also have a negative impact on water quality and the health of aquatic environments (Chapman, 1996). Meybeck *et al.* (1989) have identified several of the most important water quality issues which have arisen from these anthropogenic impacts. These include increased levels of pathogenic organisms, suspended solids, decomposable organic matter, nutrient pollution, eutrophication, salinisation, trace elements, organic micropollutants, acidification and modification of hydrological regimes.

Specific parameters used in the assessment of water quality are based on these widespread issues and include measurements of parameters reflecting the relative impact of each of these issues on the source water being tested. In order to assess the presence and abundance of pathogenic organisms present in source water, microbiological indicators are used. These indicators include measurements of the number of fecal coliforms, total coliforms and pathogenic bacteria such as *Salmonella, Enterococcus,* and *Escherichia coli* in a given volume of water (typically 100 mL) (Jamieson *et al.*, 2002; Harwood *et al.*, 2005). The impact of suspended

solids on a particular water source is determined by measuring the total suspended solids (or sediments) (TSS), by filtering water through a designated pore size (0.45 μM is typical) to determine the dry mass of the excluded sediment (expressed as milligrams per liter) (Caux *et al.*, 1997). Suspended solids can also be assessed by the measurement of turbidity which is the level of clarity/transparency of water. When biotic and abiotic substances are suspended or dissolved in water they can cause light to be scattered and absorbed rather than transmitted. A turbidity meter measures this scattering of light due to the presence of suspended matter (Wetzel, 1975). Decomposable organic matter is measured as total organic carbon (TOC), chemical oxygen demand (COD) and biological oxygen demand (BOD) (Chapman, 1996). TOC is a measurement of the concentration of carbon present from organic compounds in water, COD is a measurement of the chemicals in a water source that can be oxidized and BOD is a measurement of the organic carbons present that can be oxidized by microorganisms (Aziz and Tebbutt, 1980). Nutrient loading in water systems is typically monitored by routinely assessing the concentrations of several chemical compounds related to inorganic nitrogen (nitrate, nitrite and ammonia) and phosphorus (phosphate) (Chapman, 1996). These compounds are of particular importance because of their potential to impact aquatic organisms negatively. These negative impacts may include direct toxicity (nitrite and ammonia specifically) or indirect toxicity resulting from *in situ* biological transformations of the primary compounds (Carpenter *et al.*, 1998). Furthermore, the presence of inorganic nitrogen and phosphorus in excess can also cause eutrophication which is detrimental to aquatica ecosystems (Carpenter *et al.*, 1998). Inorganic nitrogen and phosphorus compounds are routinely deposited into our water systems in large quantities as they originate from many different anthropogenic activities (Carpenter *et al.*, 1998). Eutrophication is the result of greater than normal growth of both algae and macrophytes, as such, the extent of

eutrophication in a water system is measured by determining the concentration of chlorophyll a in the water; chlorophyll a is a photosynthetic pigment required by plants and algae for oxygenic photosynthesis (Schalles *et al.*, 1998). Salinity of a water sample can be evaluated by determining the concentrations of major dissolved ions (sodium, potassium, calcium, magnesium, chloride, sulfate) or by determining the electrical conductivity (EC) of the water (Chapman, 1996). EC is a measurement of the charge-carrying ability of the water due to the presence of dissolved ions and can be converted to a concentration of total dissolved salts (TDS) with a calculation based on the relationship between these two measurements (Hutchinson, 1957; Williams and Sherwood, 1994). Monitoring trace elements typically entails measuring concentrations of trace metals as well as arsenic and selenium, while organic pollutants more broadly entails measurements of oil and hydrocarbon, surfactant, pesticide, phenol and organic solvent concentrations (Chapman, 1996). The level of acidification in a body of water is determined by measuring its pH (Chapman, 1996). Monitoring hydrological regimes involves measuring flow rates, water levels, river discharges and dissolved oxygen concentrations (Chapman, 1996).

Water quality assessments typically involve the measurement and evaluation of each of these different aspects of water quality. A subset of these parameters tailored to the specific requirements for the water in question, or the type of pollutants commonly being deposited in a given water body may also be used (Chapman, 1996).

1.4 The Effects of Various Types of Anthropogenic Impacts on Wetland Microbial Communities

1.4.1 Acid Mine Drainage and Coal Pile Runoff

The primary water quality concerns associated with acid mine drainage (AMD) and coal pile runoff (CPR) are related to high concentrations of toxic heavy metals (e.g. Fe, As, Mn, Zn), sulfates and acidic pH levels (Johnson, 2003; Hallberg and Johnson, 2005). These conditions result from the oxidation of pyrite (FeS_2), which occurs along with metal sulfides of commercial interest (Johnson, 2003). Heavy metals can be removed from AMD and CPR within wetlands via precipitation of insoluble oxyhydroxide compounds, a process that is enhanced by microbial Fe(II) oxidation and dissimilatory sulfate reduction (Kirby *et al.*, 1998; Hedin *et al.*, 1998). Treatment of AMD and CPR with constructed wetland technology has been shown to be a cost-effective and low maintenance alternative to chemical treatment methods traditionally used to treat this type of effluent and has been shown to be effective at removing heavy metals (specifically Fe) and increasing the pH of effluents (Lloyd *et al.*, 2004; Wieder, 1993).

The majority of studies that have been published surrounding the treatment of AMD and CPR using wetlands are focused around the efficiency of these systems at addressing water quality concerns (e.g. metal removal, sulfate removal, pH increase). Very few studies have examined the impact of the effluent on the wetland-associated microbial communities and their ability to remediate wastewater (Weber *et al.*, 2008). Among the few studies that have been published, several conclusions can be drawn as to how this effluent impacts wetland microbial communities.

Groups of microorganisms associated with effective remediation of AMD and CPR include acidophilic iron oxidizers (e.g. *Acidithiobacillus ferrooxidans, Leptospirillum ferrooxidans*), acidophilic sulfur oxidizers (e.g. *Halothiobacillus neapolitanus, Sulfirimonas* spp.), acidophilic heterotrophic bacteria (e.g. *Frateuria* spp., *Alcaligenes* spp, *Bordetella* spp.) and sulfate reducing bacteria (SRB) (e.g. *Thermodesulfovibrio* spp.) (Hallberg and Johnson, 2005; Logan *et al.*, 2005; Nicomrat *et al.*, 2008; Bomberg *et al.*, 2015). Studies have shown that the introduction of AMD and CPR to constructed wetlands results in a shift in community structure towards an increase in these groups of microorganisms along with that of methanogens in wetland sediment communities (Hallberg and Johnson, 2005; Logan *et al.*, 2005). Increases in acidophilic iron and sulfur oxidizers and heterotrophic acidophiles have also been shown to occur in water samples obtained from constructed treatment wetlands and wetland effluents (Hallberg and Johnson, 2005).

The function of microbial wetland communities exposed to AMD and CPR have also been studied using various methodological approaches. Weber *et al.* (2008) and found that exposure to AMD caused a change in the functional community profile of a wetland water-associated microbial community by increasing the diversity and evenness of carbon sources that could be used by the community (measured using Biolog^{TM} EcoPlates) and decreased richness and average well color development (average usage of all carbon sources). Collins *et al.*, (2004) also found that when plant-associated wetland microbial communities were exposed to CPR their functional profiles, measured as carbon utilization profiles (Biolog^{TM} EcoPlates), changed significantly. This was determined by obtaining samples from epiphytic surfaces of emergent vegetation in a series of surface flow constructed wetland systems receiving CPR. The effect of the CPR on the community was determined by observing differences in the functional profiles

from samples taken near the inlet vs. outlet of the constructed treatment wetland. The nature of the differences observed in the community functional profiles were not discussed in any detail, it was only stated that they differed significantly from one another. Similarly, another study done by Weber *et al.* (2010) found that microbial enzymatic activity, when measured indirectly by monitoring the transformation of fluoresceindiacetate (FDA) to fluorescein, was altered in interstitial water samples taken from constructed wetland mesocosms subjected to AMD, showing an overall decrease in community metabolic activity after exposure.

Exposure of wetland microbial communities to AMD has not been shown to decrease the effectiveness of wetland-associated microbial communities at remediating impacted waters. For example, when Logan *et al.* (2005) observed a change in the microbial community structure of water collected from a permeable reactive barrier system treated with AMD, noting an increase in SRB and methanogens within the community, this change was associated with an increase in heavy metal removal (Mn, Zn). Similarly, structural changes in the water and sediment wetland microbial communities reported by Hallberg and Johnson (2005) in a composite constructed wetland system receiving AMD was associated with a decrease in effluent iron concentrations and an increase in effluent pH.

The presence of plants in wetlands receiving AMD and CPR has been shown to stabilize microbial wetland-associated communities, reducing the impact of the AMD on microbial community functional profiles (Weber *et al.*, 2008). Weber *et al.* (2008) found that interstitial water communities obtained from unplanted mesocosms exhibited a more dramatic increases in carbon source usage diversity and evenness and a more dramatic decreases in average well color development and richness than constructed wetland mesocosms that contained plants (*Phragmites australis*). Weber *et al.* (2010) found that the presence of the wetland plant

Phragmites australis did not have an effect on the remediation (metal removal, pH increase) of AMD in constructed wetland mesocosms. Alternatively, Collins *et al.* (2004) found that planted wetland mesocosms had an effluent with higher pH and higher Mn concentrations than unplanted wetlands, although effluent iron concentrations were similar in planted and unplanted treatments.

1.4.2 Oil/Petroleum Hydrocarbons

Oil extraction, refining and shipping processes often result in spilled crude oil which contains toxic components that have been shown to negatively impact aquatic ecosystem diversity and function (Baldwin, 1922; Kauss *et al.*, 1973; Sanders *et al.*, 1980; Atlas *et al.*, 1991; Lin and Mendelssohn, 1996). Many spill sites are too inaccessible or fragile to allow for mechanical cleanup of spilled oil and rely heavily on microbial processes for remediation (Atlas and Bartha, 1992; Brooijmans *et al.*, 2009). There are several groups of wetland-associated microorganisms that have been shown to be effective at breaking down petroleum hydrocarbons and other toxic crude oil constituents such as SRBs, *Pseudomonas* spp., *Acinetobacter* spp. and *Streptomyces* spp. (Bachoon *et al.*, 2001; Brooijmans *et al.*, 2009; Huijie *et al.*, 2011; Beazley *et al.*, 2012). As such, constructed wetland technology can be a useful tool for the remediation of effluents containing crude oil (Nyman, 1999). An abundance of studies in the literature have examined the role of specific bacterial species involved in the metabolism of crude oil constituents, however very few studies have examined the in-depth impact of these effluents on overall wetland community structure and function, and how these two components interrelate.

It has been well established in the literature that exposure of microbial communities to crude oil results in a loss of microbial species diversity, but an increase in the abundance of resistant and hydrocarbon-degrading species (Atlas and Bartha, 1992; MacNaughton *et al.*, 1999;

Nyman, 1999; Yergeau *et al.*, 2012). Bachoon *et al.* (2001) found that the endogenous oil-degrading microorganisms in wetland microcosm sediments increased from approximately 0.1% of the total population to slightly less than 1% of the total population after exposure of the system to crude oil. This hydrocarbon-degrading bacterial population increase was determined to be attributed in some respect to an increase in the abundance of *Pseudomonas* spp. and *Streptomyces* spp., which increased in abundance post-oil exposure. When Beazley *et al.* (2012) examined the impact of the Deepwater Horizon oil spill on costal salt marsh sediments, they also reported an increase in the abundance and richness of previously described hydrocarbon-degrading microbial phyla (Proteobacteria, Bacteroidetes, and Actinobacteria). Alternatively, Yergeau *et al.* (2012) found that oil-impacted sediments from the Athabasca River had a distinct microbial community as compared to less impacted sediments collected further downstream from oil sands tailings ponds. Correlations between hydrocarbon concentrations and specific bacterial genera were also found, including positive correlations between *Schumannella* (Actinobacteria), *Hydrogenophaga* (Betaproteobacteria), *Azonexus* (Betaproteobacteria), *Salinimicrobium* (Bacteroidetes), *Achromobacter* (Betaproteobacteria), and *Gillisia* (Bacteroidetes) and the sediment content of total petroleum hydrocarbons, total straight-chain hydrocarbons, total aromatic hydrocarbons, naphthenic acids, or the sum of the U.S. Environmental Protection Agency 16 priority polycyclic aromatic hydrocarbons. Conversely, negative associations were found between the sediment concentrations of these crude oil constituents and the relative abundances of *Sorangium* (Deltaproteobacteria), *Hyalangium* (Deltaproteobacteria), *Rhodopila* (Alphaproteobacteria), and *Mesorhizobium* (Alphaproteobacteria) in the Athabasca River sediments. Hadwin *et al.*, (2006) found that bacterial community structure in freshwater wetland sediments exposed to oil sands processed

water was correlated with the napthenic acid concentration in the sediments as determined by cluster analysis of phospholipid fatty acid profiles. Furthermore, Huijie *et al.* (2011) found that microbial communities isolated from mangrove sediments exposed to crude oil constituents including mixed polycyclic aromatic hydrocarbons (PAH), phenanthrene (PHE), pyrene (PYR) and benzo[a]pyrene (BaP) exhibited distinct communities from each other as well as from untreated sediments, as determined by structural microbial community analysis using denaturing gradient gel electrophoresis (DGGE). The resulting communities post-treatment were also shown to be differential in their abilities to remediate crude oil. The sediment community grown in the presence of PHE was more successful at degrading PHE than the previously unexposed control community and was also the most successful at degrading mixed PAHs of all the communities. This indicates that both the composition of the crude oil and the concentration of the crude oil constituents exposed to a wetland microbial community will play a role in determining the wetland microbial community structure which, in turn, will affect the ability of that community to remediate crude oil contaminated effluents.

The effects of crude oil exposure on the metabolic functions of wetland-associated microbial communities have also been examined, using both carbon source utilization patterns and metabolic activity measurements of microbial carbon emission rates (CO_2 and CH_4). Hadwin *et al.* (2006) found that when wetland sediments were exposed to varying concentrations of napthenic acids there was no significant difference in the carbon source utilization profiles generated by the wetland sediment microbial communities in each treatment. However, cluster diagrams created from DGGE data indicated that naphthenic acid content was an influential factor on wetland sediment bacterial community structure, indicating a discourse between the effects of crude oil exposure on community structure compared to community function.

Conversely, when Nyman (1999) exposed microcosms containing costal marsh sediments to two different types of crude oil (Arabian Crude and Louisiana Crude), it was reported that microbial carbon respiration increased compared to no oil treatments, and this increase was beyond what would have been expected from the amount of carbon added to the system from the oil. These findings indicate that microbial metabolic activity was enhanced by the addition of the crude oil to the system. This is contradictory to findings made by Hadwin *et al.* (2006), however these two studies used different methods to measure microbial metabolic activity and also studied different systems (sediments wetlands in the Athabasca oil sands vs. costal marsh sediment microcosms) which could account for the discontinuity in the findings.

Only one study examined the role of wetland plants altering the effects of crude oil on wetland-associated microbial communities. Nyman (1999) studied two groups of costal marsh sediments dominated by two different plant species, *Panicum hemitomon* Shult. and *Sagittaria lancifolia* L. It was reported that microbial carbon emissions differed between the sediments collected from the two different sites, with metabolic activity being 1.4 times faster in *P. hemitomon* sediments than *S. lancifolia* sediments, however both sediment microbial communities responded to the addition of crude oil similarly (increasing C emission rates). However, after crude oil addition, the percent of carbon emissions resulting from methane was higher in the *P. hemitomon* sediments than in the *S. lancifolia* sediments.

1.4.3 Agricultural Runoff

Agricultural runoff contains extremely elevated levels of inorganic nutrients, primarily nitrogenous compounds (nitrate, nitrite, ammonium) and phosphates which can lead to eutrophication of natural water systems (Ghafari *et al.,* 2008; Shaw *et al.,*2009). Microbial

processes, specifically nitrification-denitrification and anammox are extremely effective at removing excess inorganic nitrogen from influent water within wetlands (Faulwetter *et al.*, 2009). The role of microbial processes in the removal of inorganic phosphorus from wetlands is less well studied, but there is some evidence indicating that bacteria found in aquatic freshwater environments (water and sediments), are capable of assimilating large amounts of inorganic phosphorus under conditions of excess, and converting it into refractory phosphorus- containing organic compounds that are not biologically available (Gächter and Mares, 1985; Gächter *et al.*, 1988; Gächter and Meyer, 1993). Thus, wetlands present a viable option for the treatment of agricultural runoff, and have been shown to be effective at improving the water quality of various types of agricultural effluents (Vymazal *et al.*, 2009). The majority of the studies that have been completed in this area have examined the efficiency of these constructed treatment wetland systems at remediating agricultural effluents. Relatively few studies have looked at the effects of these effluents on the microbial communities in the wetlands receiving these effluents. Among the studies that have been published, some conclusions can be drawn as to the effects of agricultural runoff on the structure and function of wetland-associated microbial communities.

The effect of elevated levels of inorganic nutrients on the structural profiles of wetland-associated microbial communities is ambiguous. Ahn *et al.* (2007) treated wetland microcosms with either high or low concentrations of phosphorus, simulating P-loading that would be experienced by wetland communities receiving agricultural runoff. A difference in the sediment microbial communities under high (2.4 mg P/L) and low phosphorus (0.5 mg P/L) loading conditions was observed. Low-P treatments were associated with higher species diversity and richness than high-P treatments, which had slightly higher measures for species evenness. Increasing P-concentration resulted in the loss of microbial community members belonging to

the groups *Acidimicrobium, Methylomonas, Propionibacterium, Rhodopila, Saccharopolyspora*, Tar. *Marianensis, Thiobacillus* and *Mycobacterium* and resulted in an increase in the abundance of an unknown environmental clone (T78). They also found that these changes in community structure were associated with an increase in the removal of soluble reactive phosphorus (SRP) from the system in the high-P treatment, which showed a decrease in water SRP concentrations over a 1 month period while the low-P treatment exhibited no significant change in SRP concentration, or a moderate increase. Similarly, when Rich and Myrold (2004) examined the structure of wetland soil bacterial communities (agricultural field, riparian zone and creek bed) being exposed to high N load fertilizer runoff, they found a significant difference in the community structure of microbial denitrifiers at each location using the analysis of terminal restriction fragment length polymorphisms (TRFLPs). However, these community differences could also be attributed to other factors than N loading from fertilizer runoff, as plant community composition and soil chemistry was not equivalent among the sites. Cao *et al.* (2008) found that salt marsh sediment bacterial community structure was significantly correlated with the dissolved organic carbon content of the marsh sediments as determined by analysis using TRFLPs. However, Mentzer *et al.* (2006) did not see a significant change in the soil microbial community structure within prairie wetland microcosms subjected to either high or low nutrient treatments. Mentzer *et al*. (2006) used phospholipid fatty acid analysis (PFLA) to track changes in the community structure of wetland soil bacterial and fungal communities treated with fertilizer, ¼ strength fertilizer and no fertilizer. No significant change in the bacterial community structure was observed. However, the different nutrient loading treatments did have a significant effect on mycorrhizal and saprophytic fungal communities. Although the majority of the studies published in the literature have found that conditions mimicking that of agricultural runoff did

result in structural changes to wetland microbial communities, there is some disagreement among the findings. The studies in question used very different types of wetland systems and different methods of measuring structural changes to microbial communities, which could explain some of the confounding results. While only one study examined structural community changes in relation to microbial effluent remediation, that study found that structural community changes were accompanied by an increase in the ability of that community to remove soluble reactive phosphorus from the water (Ahn *et al.*, 2007).

Zhao *et al.* (2010) examined the effects of C:N:P ratios on the metabolism of wetland rhizosphere microbial communities in planted and unplanted pilot scale sub-surface flow constructed wetlands. The study consisted of 6 treatments, three treatments varied total organic carbon levels while maintaining stable N and P levels and three treatments varied total nitrogen levels while maintaining constant organic carbon and phosphorus levels. Microbial activity was monitored using Biolog^{TM} EcoPlates to measure carbon source utilization patterns. Total microbial activity, as measured by the average well color development (AWCD) in the EcoPlates was higher in the planted wetlands as compared to the unplanted wetlands but did not differ among C:N:P treatments. Furthermore, the diversity of carbon sources used again differed between planted and unplanted systems. However, among C:N:P treatments differences in metabolic diversity were only observed for unplanted systems where increasing organic carbon or nitrogen caused a reduction in diversity. This trend has been observed previously with other types of wetland systems and effluents, and indicates that the presence of plants may have a stabilizing effect on wetland-associated microbial communities, negating some of the effects effluents may have on the community structure and metabolic functionality of wetland microbial communities. For example, Ahn *et al.* (2007) found that the presence of plants in wetland

microcosms receiving high and low phosphorus loading treatments exhibited a less dramatic shift in microbial sediment community structure than in unplanted microcosms. Additionally, planted microcosms exhibited higher measures of species diversity and richness than unplanted microcosms in both treatments.

Several other studies measured changes in microbial metabolic profiles by looking at specific microbial enzymatic activities. Mentzer *et al.* (2006) found that wetland sediment microbial communities exhibited increased extracellular activity (specifically β-glucosidase, acid phosphatase, chitobase, phenol oxidase and peroxidase) when subjected to increased inorganic nutrient loads in the form of fertilizers. Similarly, Cao *et al.* (2008) found that the activity of microbial denitrifying enzymes within salt marsh sediments differed significantly depending on the dissolved organic carbon content of the sediments. Alternatively, Rich and Myrold (2004) did not find a significant correlation between inorganic nitrogen loading in microbial sediment wetland communities receiving agricultural runoff and denitrification enzyme activity. As in the studies examining the effects of agricultural runoff on wetland microbial community structure, the findings on changes to microbial community functional profiles also indicates conflicting results, likely for similar reasons.

1.4.4 Urban-Associated Impacts: Runoff and Wastewater Effluent

Urban impacts on water quality typically occur as increases in nutrient loads (dissolved organic carbon, inorganic nitrogen and phosphate), salinity (runoff from road systems) and pathogenic microorganisms associated with effluent from waste water treatment plants (WWTPs) (Meybeck *et al.*, 1989). The methods employed in wetlands to remediate water inputs with high nutrient loads have been discussed previously, but what is of particular importance to

the remediation of urban wastewater effluents is the ability of wetland-associated microbial communities to reduce pathogen loads. Typical human fecal matter can contain a large number and variety of different pathogenic microorganisms causing disease in humans ranging from relativity non-serious gastroenteritis to life-threatening illnesses such as dysentery, cholera and typhoid fever (Chapman, 1996). Mechanisms of pathogen removal or reduction in wetlands include: 1) increased oxygenation in the rhizosphere (Curtis *et al.*, 1992; Vymazal, 2005), 2) competition with resident microflora (Cooley *et al.*, 2003; Cooley *et al.*, 2006), 3) physical filtration through plant root systems (Kansiime and Nalubega,1999; Kansiime and van Bruggen, 2000; Karathanasis *et al.*, 2003), 4) retention and integration of pathogenic bacteria into rhizosphere communities (Kansiime and Nalubega,1999; Kansiime and van Bruggen, 2000) and 5) secretion of antimicrobial compounds by plants and microorganisms in the plant rhizosphere (Gopal and Goel, 1993; Axelrood *et al.*, 1996; Neori *et al.*, 2000; Fett, 2006).

The impact of urban wastewater effluents on the structure of wetland-associated microbial communities has been investigated by a small number of papers, with conflicting findings being reported. For example, Wu *et al.* (2010) found that WWTP effluent containing fecal matter-impacted water column bacterial community compositions in costal urban watersheds, resulting in a higher ratio of Bacilli, Bacteroidetes, and Clostridia to α-Proteobacteria in sites receiving higher WWTP effluent inputs. Ravit *et al.* (2003) conducted a study where the structure of two wetland sediment-associated microbial communities in brackish marshes dominated by *Phragmites australis* and *Spartina altemiflora* were compared using PFLA, when one marsh was undisturbed and one marsh was highly impacted by anthropogenic activity. The study reported that the microbial community structure in the sediment microorganisms associated with each plant species differed between the two sites. The

undisturbed site exhibited a community structure with a greater species richness and diversity than the site highly impacted by anthropogenic inputs. Alternatively, Perryman *et al.* (2011) examined the effects of urban storm water runoff and septic tank density on sediment microbial communities within stream ecosystems. Denitrifying community composition, as measured by TRFLPs of nosZ genes, was shown to be strongly influenced by the amount of storm water runoff being received by the site but not by the septic tank density surrounding the site. The more densely urbanized sites receiving greater amounts of storm water runoff exhibited higher sediment concentrations of cations, reactive phosphorus and lower organic carbon content while sites with higher septic tank density exhibited higher concentrations of nitrate and total sediment nitrogen. As well, Truu *et al.* (2009) examined soil microbial communities associated with *Salix* sp. being irrigated with secondary-treated wastewater over a three year period and did not observe any significant differences in the soil microbial community diversity, as measured by 16S DGGE analysis, compared to control plots not receiving effluent, despite the fact that the irrigated plots had significantly higher concentrations of total nitrogen, phosphorus and potassium.

Findings surrounding the impact of urban effluents on the functional profiles of wetland-associated microbial communities are similarly conflicting. For example, Ravit *et al.*, (2003) found that sediment microbial enzyme activity in β-glucosidase, acid phosphatase, chitobase, phenoxidase and peroxidise was higher in wetland sediments that were not impacted by urban activities compared to similar wetlands receiving high urban wastewater effluent loads. Similarly, Truu *et al.* (2009) found that increased activity of microbial alkaline phosphatase and N-mineralization were associated with soil plots receiving secondary-treated wastewater effluent compared to untreated controls. However, in the same study when carbon utilization profiles

were examined using Biolog^{TM} EcoPlates, no significant differences were observed between the treated and untreated plots. In the case of these two studies, it becomes clear that the methodology being used to measure microbial functional changes and metabolic activities can determine the study outcome, with one method identifying differences among the treatments while the other did not detect any changes. This indicates that in order to get a more complete understanding of changes occurring in a microbial community at the functional level, multiple methods of measuring metabolic activity may be required.

The removal efficiency of pathogenic microorganisms from various types of wastewater effluents using constructed treatment wetlands with emergent macrophytes has been evaluated. Reductions in indicator species have been shown to be between 90 and 99% which is comparable with other treatment processes traditionally used to reduce pathogen load in contaminated wastewater (Miescier and Cabelli, 1982; Wolverton, 1989; Watson *et al.*, 1990). Studies comparing the effectiveness of pathogen removal in both planted and unplanted constructed wetlands of the same type have shown that planted wetlands are significantly more effective at reducing pathogen load in wastewater (Rivera *et al.*, 1995; Soto *et al.*, 1999; Warren *et al.*, 2000; Karathanasis *et al.*, 2003). Additionally, the species of plants used in the constructed wetland is also an important factor to consider when examining constructed wetland parameters influencing pathogen removal efficiency. Karathanasis *et al.* (2003) showed that when comparing constructed wetland systems planted with cattails (*Typha latifolia*), fescue (*Festuca arundinacea*) and mixed plant species (yellow flag iris (*Iris pseudacorus*), canna lilies (*Canna* x. *generalis*), day lilies (*Hemerocallis fulva*), hibiscus (*Hibiscus moscheutos*), soft-stem bulrush (*Scirpus validus*), and mint (*Mentha spicata*)), the polyculture and cattail-planted constructed wetlands were more efficient than the fescue at removing fecal coliforms and fecal streptococci

from untreated domestic wastewater. This study also suggested an increase in removal efficiency with increased root biomass. Similarly, when fecal coliform removal in duckweed (*Spirodela polyrhiza*), water lettuce (*Pistia stratiotes*) and algal-based stabilization ponds were compared, differences were observed between the treatments with the algal-based system removing significantly more pathogens than either of the duckweed and water lettuce ponds, with duckweed removing pathogens more efficiently than water lettuce (Awuah *et al.*, 2004).

1.4.5 Underlying Themes

By examining the effects of different types of effluents and water quality impacts on microbial wetland-associated communities, several general conclusions can be drawn. Firstly, the literature available agrees that the exposure of wetland microbial communities to wastewater typically leads to an altered microbial community structure in the majority of reports (e.g. Ravit *et al.*, 2003; Hallberg and Johnson, 2005; Ahn *et al.*, 2007; Cao *et al.*, 2008; Wu *et al.*, 2010). However, these structural changes may or may not be reflected in the functional profile of the community. This could be due to a few factors, for example, it is possible that changes on the functional level are occurring, however they are not being captured due to the methodology being used in that study. Alternatively, in methods such as carbon source utilization profiles using Biolog^{TM} EcoPlates (community level physiological profiling-CLPP), the change in the structure of the microbial community may be occurring at a level that is not being captured by the total community functional profile that is being shown with CLPP. The relative abundance of the microorganisms being enriched, or inhibited, by a given effluent may be too low relative to the total microbial community being captured during CLPP. Furthermore, the changes being made to the wetland-associated microbial community structure by exposure to a given effluent tends to enhance the ability of that community to remediate wastewater, and is not in any way

detrimental. As well, the changes being observed in microbial community structural and functional profiles tend to be more dramatic upon exposure to industrial effluents (AMD and crude oil), compared to urban or agricultural. This may be due to the nature of the water quality changes occurring in each different type of effluent. For example, the elevated concentrations of heavy metals being seen in AMD are far beyond what most organisms would ever experience in a natural unimpacted system. Similarly, most microbial communities would not typically be exposed to crude oil constituents, while high levels of inorganic nutrient loads and pathogenic microorganisms are more likely to occur in natural communities with changes in seasons, flooding and runoff. Additionally, with the exception of extremely high levels of nitrite and salts far beyond what is typical of WWTP effluent and field runoff, these types of contaminants are not as toxic to the organisms (Carpenter *et al.*, 1998). As such, the changes we are seeing in the communities are less dramatic in the second instance.

There is also a reoccurring trend in the literature with respect to the influence of plant presence on the response of microbial communities to changes in water quality conditions. The presence of plants imposes a stabilizing effect of microbial community structure and function, lessening the changes being observed upon exposure to a given effluent (Ahn *et al.*, 2007; Weber *et al.*, 2008; Weber *et al.*, 2010). Furthermore, plant presence has consistently been shown to improve contaminant removal efficiencies in treatment wetlands exposed to impacted effluents with the exception of the removal of some heavy metals from AMD (Collins *et al.*, 2004; Vymazal *et al.*, 2009).

1.5 Future Research Needs

Overall, there is very little research available surrounding the impacts of water quality on wetland-associated microbial communities. Of the studies that have been done, most focus solely on the sediment-associated microbial community (e.g. Ravit *et al.,* 2003; Rich and Myrold, 2004; Mentzer *et al.,* 2006; Cao *et al.,* 2008*;*Truu *et al.,* 2009; Perryman *et al.,* 2011). Very few studies examine the impact of these effluents specifically on root-associated or water-associated microorganisms. These communities (sediment, water, rhizosphere and rhizoplane) differ dramatically in structure and function. Considering the established importance of root-associated microbial communities in the remedial functions of wetlands, more effort needs to be put forth in the examination of how these communities may be impacted by changes in water quality. Ideally, all of these microbial communities should be examined together, as each one has an important role to play in how a wetland functions. Furthermore, many studies focus on constructed treatment wetlands, while relatively few studies have examined the impacts of these anthropogenic effluents on natural wetland systems. Natural wetland systems are extremely important in protecting the integrity of our aquatic ecosystems, so more work needs to be done in this area. Riparian zones in particular have not been studied in much detail, and they are often the only obstacle between runoff from urban and agricultural land and our waterways (Osborne and Kovacic, 1993).

1.6 Research Hypotheses and Objectives

To further our understanding of how changes to water quality can affect plant-bacteria community dynamics and their capacity to remediate contaminated water, I will be testing two

hypotheses. In order to test these hypotheses I will meet several objectives which are identified by the letters a-c corresponding with each hypothesis.

1) Natural and constructed wetland systems highly impacted by anthropogenic activities will differ from less affected systems in community composition, function, remediation capabilities (ability to remove contaminants and pollutants from water) and response to environmental changes.
    a. Use an *in situ* field-based approach to examine similarities and differences among the microbial communities associated with a wetland plant (*P. arundinacea*) at sampling locations with contrasting water quality characteristics from a structural and functional perspective (Chapter 3).
    b. Use an *ex situ* mesocosm-based approach to look at structural and functional differences between microbial communities associated with wetland plants treated with water from a low water quality sampling location and from a high water quality sampling location using lab-scale constructed wetland mesocosms (Chapter 5). The mesocosm-based approach will reduce some of the variability associated with field-based research and allow us to test the ability of the different wetland communities to remove contaminants.
    c. Use an *ex situ* mesocosm-based approach to examine how wetland-associated communities adapted to either high or low water quality conditions will respond to environmental perturbations by simulating a rain event with associated run-off . This will be achieved by loading inorganic phosphorus into the mesocosms. Changes to community structure, function and remedial capabilities will be monitored after the phosphorus loading event (Chapter 6).

2) The microbial community composition of wetland plants will differ among species. Different plant species will harbour unique microbial communities that vary in their community compositions, functionality, remediation capabilities and in their response to perturbations in water quality.

    a. Use an *in situ* field-based approach to compare community structural and functional characteristics between different plant species (*I. versicolor, P. natans, V. spicata*) at field locations with contrasting water quality characteristics (Chapter 4).

    b. Use an *ex situ* lab-based approach to compare the community structure, function and remediation capabilities of wetland-associated microbial communities in mesocosms planted with different plant species (*P. arundinacea* and *V. anagallis-aquatica*) receiving contrasting water quality treatments (Chapter 5).

    c. Compare the ability of high and low water quality-treated microbial communities associated with either *P. arundinacea* or *V. anagallis-aquatica* to resist perturbations in water quality by loading mesocosms with phosphorus (Chapter 6).

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Chapter 2

General Materials and Methods

2.1 Sample Collection and Preparation

Bacterial communities were assessed from river water, rhizosphere soil and rhizoplane samples in field-based studies (Chapter 3 and 4), while interstitial water, inflow water, rhizosphere and rhizoplane material was assessed from mesocosm-based studies (Chapter 5 and 6). River water samples and inflow water samples were collected aseptically in sterilized glass bottles from the middle of the riverbed 30 cm below the water's surface. Interstitial water samples were collected by placing a sterile glass bottle beneath the outflow port of each mesocosm and collecting the water as it drained from the tank. Please note that for the majority of water samples (river water and mesocosm inflow water) one experimental replicate is reflected in the data. This is because there is only one central river, and in the case of the mesocosms, only one inflow bucket per water source treatment feeding all mesocosms at a given time. We felt that one sample would be reflective of the water conditions without significantly increasing the amount of samples being processed, which was also time sensitive. Therefore, when data represents averages and standard error reflects variation in experimental replicates only, these measurements will lack error bars. Rhizosphere soil samples were obtained by removing plant root systems and shaking roots to collect the attached soil. Samples were stored in Whirl-paks® (The Aristotle Corporation, Stamford, Connecticut USA) for transport to the lab. Rhizoplane samples were collected by removing root material from the base of the plant using sterile forceps and nitrile gloves. Root samples were stored in Whirl-paks® for transport to the lab where they were washed 3x in sterile sodium-free phosphate buffer before being weighed

into 3 g parcels and transferred to 297 mL of sterile buffer in glass bottles. Rhizoplane biofilm material was then obtained from washed roots using a Branson sonicator (Branson 2800 Ultrasonic cleaner, Branson Ultrasonics, Richmond, Virginia USA), with sonication for 1 min, with a 1 min rest period, followed by another minute of sonication (Miyasaka *et al.*, 1991; Bulgarelli *et al.*, 2012).

2.2 Structural Community Characterization

Prior to the extraction of bacterial DNA, water and rhizoplane samples were filtered (250 mL) under sterile conditions using a 0.22 µm polycarbonate filter (Millipore^{TM}, Bedford, Massachusetts USA). The filters where then shredded aseptically using sterile scalpel blades and the filter pieces were placed into the bead tubes used for DNA extraction provided as part of the PowerSoil^{TM} DNA isolation kit (Mo-Bio Laboratories, Carlsbad, California USA). Soil samples (rhizosphere) were added to the bead tubes as described in the PowerSoil^{TM} DNA isolation kit protocol. DNA extraction was then performed as described in the DNA isolation kit protocol and extracted DNA was stored at -80°C until further processing occurred 7-14 days later.

Structural community analysis was performed using denaturing gradient gel electrophoresis (DGGE). This establishes a molecular fingerprint of the bacterial community by separating same-sized PCR products by their guanine-cytosine content which determines how readily they are denatured (Muyzer and Smalla, 1998). Extracted DNA was subjected to PCR using the primers 357f (5′-CCTACGGGAGGCAGCAG-3′) with a GC clamp attached to the 5' end (5′-CGCCCGCCGCGCCCCGCG-CCCGTCCCGCCGCCCCCGCCCG-3′) and 518r (5′-ATTACCGCGGCTGCTGG-3′) (Sigma Aldrich, Oakville, Ontario CA) which targets the V3 region of the 16S ribosomal DNA in bacteria (Ogino *et al.*, 2001). Due to accumulation of PCR

inhibitors within experimental mesocosms (Chapter 5 and 6), SsoAdvanced™ Universal Inhibitor-Tolerant SYBR® Green Supermix (Bio-Rad Laboratories Inc., Hercules, California USA) was used for all PCR reactions related to mesocosm-based samples. The mastermix for this 50µL PCR reaction was prepared using 25 µL of SsoAdvanced™ Universal Inhibitor-Tolerant SYBR® Green Supermix, 0.5 µM of each primer, 15 µL of Milli-Q (Millipore) water and 5 µL of DNA template. The reaction was run on a Bio-Rad™ i-cycler iQ (Bio-Rad Laboratories Inc., Hercules, California USA) under the following conditions: initial denaturation for 3 min at 98°C, followed by 20 cycles of 98°C for 15s, 65 °C for 1 min and 60°C for 1 min each. The annealing temperature was decreased by 1°C every 2 cycles to a temperature of 56°C on the 20th cycle. This was followed by 10 additional cycles of 98°C for 15s, 55°C for 1 min and 60°C for 1 min. The PCR product (5µL) was then run on a 1.5% (W:V) agarose gel in 1x TAE buffer at 100 V for 45 min to verify the success of the reaction. Gels were stained using ethidium bromide and imaged on a Bio-Rad™ GelDoc™ XR (Bio-Rad Laboratories Inc., Hercules, California USA). Successful reactions resulted in the presence of a 233 bp band and no PCR products visible in the negative lane. The negative lane was loaded with PCR product obtained from a reaction run with 5 µL of Milli-Q water in place of DNA template.

The DGGE was carried out through adaptation of the protocol described by Green *et al.* (2009). An 8% (W:V) polyacrylamide gel was prepared with a linear denaturing gradient from 40 to 65% with 100% denaturant defined as 7M urea and 40% (V:V) formamide. A CBS Scientific™ DGGE-2401 machine (CBS Scientific Inc., Del Mar, California USA) was used to run the gels for 17 hours at 70V and 60°C. The polyacrylamide gel was loaded with 20 µL of PCR product per lane. The middle and outside lanes of the gel were loaded with 15 µL of a DGGE ladder comprised of a 50:50 (v:v) mixture of PCR product obtained from the DNA of

microorganisms grown in pure-culture under lab conditions and 1M Tris HCl (pH 8). The DNA ladder contained an equal mixture of PCR product obtained from the following eleven microorganisms: *Flavobacterium* spp. (ATCC® 51823), *Aeromonas hydrophilia* (ATCC® 49140), *Bacillus cereus* (Ward's Science Plus), *Alcaligenes faecalis* (ATCC® 33950), *Bacillus megaterium* (ATCC® 10778), *Pseudomonas aeruginosa* (Ward's Science Plus), *Streptomyces griseus* (ATCC® 10137), *Nitrosomonas europaea* (ATCC® 25978), *Pseudomonas chlororaphis* (ATCC® 13985), *Desulfotomaculum nigrificans* (ATCC® 19998), and *Clostridium perfringens* (NCTC® 8237). The ladder allows for warping and smiling of gels to be corrected using the appropriate software and acts as a standard to adjust for any differences between gels with respect to conformation or staining.

DGGE gels were stained using a 1X solution of SYBR Gold (Invitrogen, Burlington, Ontario CA) in 1X TAE buffer for approximately 90 min while shaking at 60 rpm. Gels were then transferred to a Bio-Rad™ GelDoc™ XR for imaging using Quantity One® 1-D analysis software (Bio-Rad Laboratories Inc., Hercules, California USA).

2.3 Functional Community Characterization: Community-Level Physiological Profiling

The Biolog™ EcoPlate consists of 31 different carbon sources which have demonstrated relevance to the study of microbial communities from natural environments (Insam, 1997). The EcoPlate contains each carbon source and an empty well (blank) in triplicate within a 96 well plate. These plates are an important culture-based tool for the characterization and differentiation of microbial communities under various environmental conditions (e.g. Garland, 1997; Guckert *et al.*,1996). Biolog™ EcoPlates are preferred over other methods used to monitor changes in

microbial communities across a wide degree of environmental conditions (e.g. phospholipid fatty-acid analysis) due to their highly sensitive nature (Firestone *et al.,* 1998).

The Biolog™ EcoPlate protocol used for this experiment was adapted from Weber and Legge (2010). Water and rhizoplane samples were prepared as previously described under the sample collection heading. Soil samples underwent further preparation before inoculation with the addition of 1 g of soil into a sterile flask containing 99 mL of sodium-free phosphate buffer which was shaken at 250 rpm for 30 min in a New Brunswick™ Scientific C76 water bath shaker (Edison, New Jersey USA) held at room temperature (21±2 °C) (Aguirre de Cárcer *et al.,* 2007; Mula-Michel and Williams, 2013). A multichannel pipette was used to inoculate 150 µL of sample into each of the Biolog™ EcoPlate wells. The plates were read at 590 nm using an xMark™ Microplate Absorbance Spectrophotometer (Bio-Rad Laboratories Inc., Hercules, California USA) before being stored in the dark at room temperature. Subsequent readings were taken at 24, 48, 72 and 96 hours.

2.4 Water Chemistry

Water chemistry was determined for both inflow and outflow water samples using a DR/890 HACH® Spectrophotometer and a DR3900 HACH® VIS Spectrophotometer (London, Ontario CA). Concentrations of ammonia, nitrate, nitrite and total reactive phosphorus were obtained using HACH® methods 8155, 8171, 8507 and 10209 respectively. Ammonia analysis was performed using the HACH® Permachem® reagents ammonia salicylate (cat. 2395266) and ammonia cyanurate (cat. 2653199). Nitrate and nitrite quantification was performed using the HACH® Permachem® reagents NitraVer®5 (cat. 2106169) and Nitriver®3 (cat. 2107169), respectively. Total reactive phosphorus concentrations were determined using HACH® Phosphorus TNTplus® vials (TNT843) for reactive and total phosphorus quantification by the

ascorbic acid method. Sample readings were obtained in triplicate and 1 mg/L standard solutions of each of the compounds previously mentioned were run through the spectrophotometers after every 3rd sampling event to verify equipment accuracy.

2.5 Culture-Based Enumeration of Fecal Coliforms and Heterotrophic Microorganisms

Water samples were subjected to the membrane filtration fecal coliform method (m-FC) which is the standard indicator of fecal contamination in water and is used as a water-quality standard (Hufham, 1974; Jamieson *et al.,* 2002; Harwood *et al.,* 2005). The method used in this experiment was taken from Standard Methods for the Examination of Water and Wastewater (1998). Water samples in volumes of 10 µL, 100 µL, 1 mL, 10 mL and 100 mL were aseptically filtered through 0.45 µM celluloses filters (Millipore™, Bedford, Massachusetts USA) and transferred onto BD Difco™ membrane fecal coliform agar(Franklin Lakes, New Jersey USA). The culture media was prepared without the addition of rosalic acid as fungal growth was not an issue with our samples, and thus was not required. Plates were incubated at 44.5 °C for 48 hours before being counted. Samples obtained from field studies only were also plated onto R2A Agar (BD Difco™ Franklin Lakes, New Jersey USA) to establish heterotrophic plate counts, and were incubated at room temperature for 120 hours.

2.6 Molecular Methods for Detection of Pathogenic Microorganisms Associated with Fecal Contamination

The absence of microbial growth on solid media is not necessarily indicative of whether an organism is ecologically present and metabolically active (McMahon *et al.*, 2007). Real-time quantitative PCR (qPCR) was used to enumerate the copy number of specific genes associated with key pathogenic microorganisms of interest within field-collected and mesocosm-collected

samples (Chapters 3,4 and 5). The following enteric organisms were chosen for quantification from water, rhizosphere and rhizoplane samples: *Escherichia coli*, *Enterococcus* spp. and *Salmonella* spp. These organisms were selected for quantification due to the fact that they are indicators of fecal contamination and thus indicators of water-quality, and are among the most significant human pathogens originating from fecal contamination in freshwater systems (Cabral, 2010).

DNA extracted from mesocosm samples were subjected to qPCR performed with a Bio-Rad™ i-cycler iQ Real-Time PCR System (Bio-Rad Laboratories Inc., Hercules, California USA). SsoAdvanced™ Universal Inhibitor-Tolerant SYBR® Green Supermix (Bio-Rad Laboratories Inc., Hercules, California USA) was used for these reactions. Enumeration of *E. coli* was performed using primers for *uidA* as stated in Table 2.1. The reaction was run in 25 µL volumes with 12.5 µL of Supermix, 0.5 µL of each primer, 7.5 µL of milli-Q H_2O and 4 µL of DNA template per reaction, with running conditions as follows: 98°C for 3 min (1x), 98 °C for 15 s followed by 60°C for 1 min (x40). Enumeration of *Enterococcus* spp. was performed using primers developed by Matsuda *et al.* (2009) for 16S ribosomal DNA as stated in Table 2.1. The reaction was run in 30 µL volumes with 15 µL of Supermix, 1.8 µL of each primer, 14 µL of Milli-Q H_2O and 10 µL of DNA template per reaction, with running conditions as follows: 98°C for 3 min (1x), 98 °C for 15 s, 55°C for 30s and 60°C for 30s (x40). Enumeration of *Salmonella* spp. was performed using primers for *invA* as stated in Table 2.1. The reaction was run in 25 µL volumes with 13 µL of Supermix, 1 µL of each primer and 10 µL of DNA template per reaction, with running conditions as follows: 98°C for 3 min (1x), 98 °C for 15 s, 60°C for 1 min (x50) and 60°C for 10 min (x1).

Table 2.1 Primers used for Real-Time Quantitative PCR Detection of Enteric Pathogens.

Microorganism	Primer	Sequence (5' to 3')	Target Gene	Function	Reference
Escherichia coli	Eco-F	GTCCAAAGCGGCGATTTG	*uidA*	Glucuronidase	Shannon *et al.*, 2007
	Eco-R	CAGGCCAGAAGTTCTTTTTCCA			
Enterococcus	g-Encoc-F	ATCAGAGGGGGATAACACTT	16S	16S rDNA	Matsuda *et al.*, 2009
spp.	g-Encoc-R	ACTCTCATCCTTGTTCTTCTC	rDNA		
***Salmonella* spp.**	Sal-F	CGTTTCCTGCGGTACTGTTAATT	*invA*	Invasion protein	Shannon *et al.*, 2007
	Sal-R	AGACGGCTGGTACTGATCGATAA			

2.7 Statistical Analyses and Data Manipulations

2.7.1 Structural Community Analysis

GelComparII: Gel Electrophoresis software (Applied Maths, Austin, Texas USA) was used to perform cluster analyses on DGGE-based banding patterns generated from each sample. The cluster analysis was performed using the un-weighted pair-group method with arithmetic mean (UPGMA) based on the Pearson correlation which considers the intensity of DGGE bands as well as the presence of a band at a given position (Brons and van Elsas, 2008). Banding patterns were also used to calculate community structural diversity under some circumstances (Chapter 6) using the Shannon Diversity index (H'; Shannon, 1948) (e.g. Gafan *et al.*, 2005).

2.7.2 Analysis of Community-Level Physiological Profiles

Absorbance measurements obtained from Biolog^{TM} EcoPlates at 96h after inoculation were used to determine several functional diversity indices proposed by Zak *et al.* (1994) (e.g. Weber *et al.*, 2008). Average well color development (AWCD) was determined by averaging the

net absorbance values of all carbon source containing wells after 96h. AWCD is used as an overall estimate of metabolic rate of the microbial community in question (Garland, 1997). The Shannon Diversity index (H'; Shannon, 1948) was used to determine the diversity of carbon sources used by each community (metabolic diversity) which was calculated using the equation $H' = -\sum pi \ln(pi)$ where *pi* is the ratio of the absorbance of a given carbon source divided by the sum of the absorbance values for all 31 carbon sources. A ratio of the absorbance for each carbon source divided by AWCD was also calculated for each Biolog^{TM} EcoPlate and used to perform a Principal Components Analysis (e.g. Choi and Dobbs, 1999) using XLSTAT Statistical Analysis Software (Addinsoft, Paris, France). Additionally, in chapters 5 and 6, absorbance values for each carbon source classified as either polymers, carbohydrates carboxylic/acetic acids or nitrogen containing compounds were added together and divided by the total absorbance within the entire Biolog^{TM} EcoPlate at 96h after incubation to determine the percent usage of different carbon source types for microbial communities (modified from Zak *et al.*, 1994). This assessment was used to further characterize changes occurring in microbial community carbon source utilization profiles during mesocosm treatments.

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Chapter 3

A Comparison of Microbial Communities Associated with *Phalaris arundinacea* along a Water Quality Gradient in the Grand River, Ontario

3.1 Introduction

Anthropogenic activities are increasingly impacting our freshwater ecosystems (Mekonnen and Hoekstra, 2015). Water quality in aquatic freshwater systems is a serious concern as eutrophication resulting from human activities has already been attributed to losses in biodiversity around the world (Tilman *et al.*, 2001; Hautier *et al.*, 2009; Hooper *et al.*, 2012). Wetlands represent a unique ecosystem that can help to ameliorate the effects of water pollution and eutrophication within freshwater ecosystems (Coveney *et al.* 2002). Water passing through natural or constructed wetland systems is subjected to filtration and a variety of biological and chemical processes which have been shown to remediate some of the effects of anthropogenic activity, such as inorganic nutrient pollution (Sheoran and Sheoran, 2006). Many of the beneficial ecosystem services provided by wetlands are the result of microbiological activity in association with wetland plants (e.g. Stottmeister *et al.*, 2003). The unique and diverse microhabitats created by the physiological processes occurring in plant roots create microhabitats suited to many different bacteria and microbial processes allowing for the rapid cycling of nutrients, thereby increasing the effectiveness of the remediation process naturally occurring in wetlands (Brix, 1997; Stottmeister *et al.*, 2003; Vymazal, 2007). As such, it is important to understand how microbial communities may be affected by changes in water quality within wetlands to better understand how increasing anthropogenic impacts may affect the ecosystem services they provide. Some of the methods we can use to assess changes in microbial

communities are by looking at changes to community composition (structure), which involves changes to the relative abundance of different groups of microorganisms, and by assessing changes to community function. Community function can be assessed by a variety of different methods, such as assessment of microbial enzyme activity and the degradation of various relevant substrates (e.g. Zak *et al.,* 1994; Kourtev *et al.,* 2002). Biolog^{TM} EcoPlates present a unique method of assessing community functional shifts by looking at the utilization of 31 different carbon sources by whole microbial communities (Garland and Mills, 1991; Zak *et al.,* 1994). They have been employed to assess functional community changes within a variety of different habitat types over a wide range of different environmental conditions (e.g. Insam, 1997; Weber *et al.,* 2008; Floch *et al.,* 2011). Very few studies, to the best of our knowledge, have investigated how water quality, particularly, inorganic nutrient pollution, affect microbial communities within wetland ecosystems (e.g. Ravit *et al.*, 2003; Mentzer *et al.,* 2006; Ahn *et al.,* 2007; Cao *et al.,* 2008). Of these studies, only a handful examine this question within natural wetland systems (e.g. Ravit *et al.*, 2003; Cao *et al.*, 2008). The findings from these studies have been mixed, with some studies indicating that nutrient pollution does impact microbial community structure and/or function (Ravit *et al.,* 2003; Mentzer *et al.*, 2006; Ahn *et al.*, 2007), while others have found no noticeable difference between impacted and unimpacted communities (e.g. Cao *et al.,* 2008). Clearly, more investigation is needed into this matter to better understand how increasing anthropogenic impacts are affecting the microbial communities in natural wetland systems.

In order to test the hypothesis that differences in water quality, particularly inorganic nutrient pollution, affect wetland microbial communities (both structurally and functionally), we examined microbial communities from the rhizosphere and rhizoplane of *Phalaris arundinacea*

located in riparian zones along the Grand River, Ontario (Canada). *P. arundinacea* is also known as reed canary grass and is considered to be native to North America (Apfelbaum and Sams 1987). *P. arduninacea* is abundant along the Grand River and throughout the Grand River Watershed and also has applications within constructed wetlands (e.g. Bernard and Lauve, 1995; Edwards *et al.*, 2006). We chose six different sites with classifications of high, moderate and poor water quality by the Grand River Conservation Authority based on a nutrient quality index (Loomer and Cooke, 2011). Microbial communities associated with water and *P. arduninacea* roots were sampled at each different site, multiple times throughout the year, to assess how differences in nutrient pollution affected community structure and function, using PCR-denaturing gradient gel electrophoresis (PCR-DGGE) and Biolog^{TM} EcoPlates. We also took a more in depth look specifically into the microbial community fraction associated with fecal contamination and potential human pathogenesis as microorganisms associated with fecal matter are directly related to water quality (Cabral, 2010). Some of the most significant human pathogens originating from fecal contamination found in freshwater systems include *Vibrio* spp., *Shigella* spp., *Salmonella* spp., *Enterococcus* spp. and *Eschericia coli* (Cabral, 2010). We chose to examine both total fecal coliform numbers, as well as a more specific look at *Salmonella* spp., *Enterococcus spp.* and *E. coli.* These microorganisms were specifically chosen due to their relevance in human health and because of existing information available about their presence and ecology within the Grand River Watershed (Thomas 2011; Morrison 2014).

3.2 Materials and Methods

3.2.1 Experimental Design and Field Sampling Protocol

Reed Canary Grass (*Phalaris arundinacea*) was selected as our study organism due to its abundance in the Grand River Water Shed. It was the only wetland plant that was located

directly adjacent to the river at all sampling sites, thus being significantly impacted by the water quality in the river. We selected six sampling locations along the main Grand River based on the GRCA nutrient quality index from the 2011 Water Quality Report for the Grand River Water Shed (Loomer and Cooke, 2011). Our sites were selected to reflect a gradient in water quality characteristics from good to poor, and were also chosen in conjunction with Provincial Water Quality Monitoring Network (PWQMN) sampling locations which provided us with additional historical sampling data for the sites. The two high water quality sites chosen were Shand Dam and West Montrose (Figure 3.1). Shand Dam (43.724658, -80.343832) was the northern most site located in Center Wellington, Ontario approximately 1 km downstream from the Bellwood Lake Reservoir Dam and was primarily surrounded by agricultural land. This site also had a weeping bed belonging to a residential building directly adjacent and slightly upstream of the sampling site. West Montrose (43.588219, -80.470979) was the second northern most site, located approximately 20 km downstream from Shand Dam. West Montrose was primarily impacted by agricultural land with a stream carrying agricultural effluent entering the main river about 50 m upstream and a residential trailer park located about 100 m downstream from the sampling location. The two intermediate water quality sites selected were Conestogo River and Bridge and Lancaster. The Conestogo River (43.530117, -80.543724) site is about 10 km south of West Montrose and impacted by both runoff from adjacent agricultural land and effluent from the St. Jacobs municipal waste water treatment plant (WWTP). The site at Bridge and Lancaster (43.481737, -80.481135) is approximately 10 km south of the site at Conestogo River, on the border between Kitchener and Waterloo, Ontario. It is within a heavily urbanized area and adjacent/slightly upstream of a municipal WWTP. The two poor water quality sites selected were Doon and Glen Morris. The Doon site (43.386393, -80.387462) is located in southeastern

Kitchener on the edge of Cambridge approximately 30 km downstream from Bridge and Lancaster. The land between these two locations is heavily urbanized. Effluent from the Doon WWTP enters the Grand River directly upstream from the sampling location. The sampling location at Glen Morris (43.277990, -80.343149) is located just outside of Cambridge, Ontario approximately 20 km downstream of the Doon site, and was our southernmost sampling location. The Glen Morris site is surrounded by agricultural land, but effluent from two municipal WWTP enters the Grand River between after the Doon sampling site before it reaches Glen Morris. At each site we chose four rectangular quadrats (50 x 120 cm) dominated by the study species. In the center of each quadrat we placed a piezometer (30 cm x 8 cm) which allowed for the sampling of porewater at each site. Each quadrat was further subdivided into 8 smaller quadrats (50 x 15 cm) to allow for destructive sampling at each sampling event. We chose 3 or the 4 quadrats to sample from and the corresponding subquadrats during event sampling event using a random number generator. Full plants were removed from sampling locations to ensure that we were sampling from the correct plant species. Entire root systems with attached soil were removed from the quadrats and placed in sterile Whirl-paks® (The Aristotle Corporation, Stamford, Connecticut USA) for transport to the lab. Samples of river water were collected from midstream approximately 30 cm below the water using pre-sterilized glass bottles. For water quality assessment, we collected water from the river as well as from the piezometers and stored it in Nalgene® bottles (Nalge Nunc International Corporation, Rochester, New York USA) to take back to the lab. We also made some water quality assessments at the site using a YSI Professional Plus Multiparameter Instrument (YSI 1700/1725, YSI a Xylem Brand, Yellow Springs, Ohio USA) and a Pro Plus Quatro Field Cable (YSI a Xylem Brand, Yellow Springs,

Ohio USA) for measuring water temperature, dissolved oxygen, conductivity, oxidation/reduction potential (ORP), pH and total suspended solids.

We attempted to collect samples at an interval of approximately once every six weeks from May 20, 2015 to November 11 2015, to capture variability during the seasons. Freezing of the ground and the river during the mid and later winter precluded sampling events at this time. During each sampling event we sampled all six sites over two days, due to the high volume of samples collected at each location and time constraints with processing samples. Each of the two days were never separated by more than 5 to 10 days and on each day we made sure to sample one site from each water quality classification.

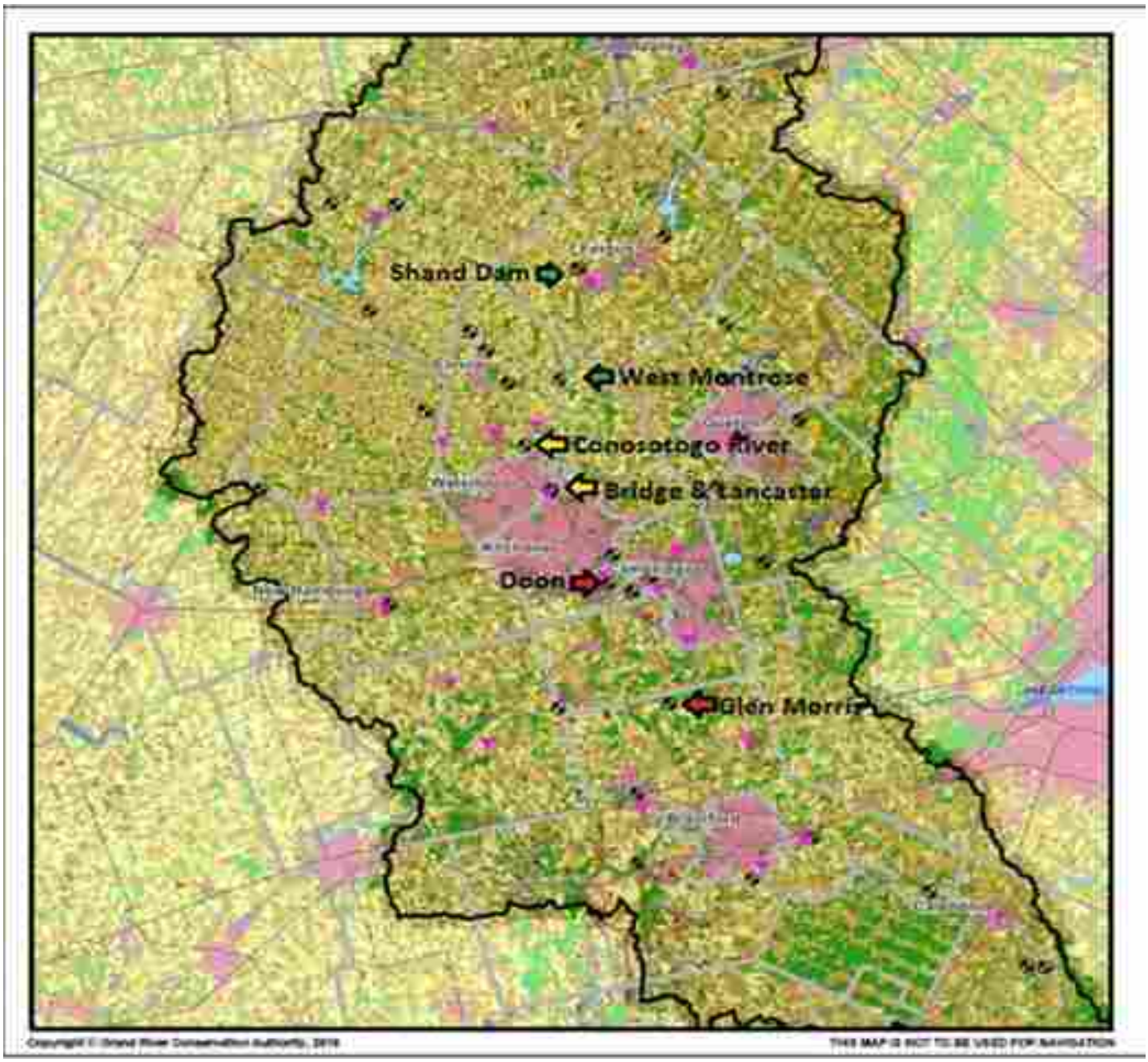

Figure 3.1 A map of the Grand River Watershed. Field sampling locations are indicated with arrows and correspond to provincial water quality monitoring sites. Each site has been given a water quality designation established by the Grand River Conservation Authority. High water quality sites (low impact) are indicated with green, intermediate water quality sites (medium impact) are indicated with yellow and poor water quality sites (high impact) are indicated with red.

3.3 Results

3.3.1 Structural Profiles of Microbial Communities Associated with *P. arundinacea* across a Water Quality Gradient

Structural similarity among all samples was only 3.1 % during the May 2015 sampling event (Figure 3.2). Structural profiles clustered into four primary groupings related to sample type. The highest degree of structural similarity (38%) was between the rhizosphere community structural profiles which exhibited three sub-clusters related to the Glen Morris, Doon (poor water quality) and West Montrose (high water quality) sites (77.7%). Another sub-cluster included community structural profiles from the Shand Dam site (74.8%). The third sub-cluster contained the rhizosphere structural profiles from both intermediate water quality sites, Bridge and Lancaster and Conestogo River (87%). The rhizoplane structural profiles clustered into two distinct groups which had only 17.3% similarity. The first group contained the rhizoplane structural profiles from Bridge and Lancaster and Conestogo River (48.7%), while the second clustered rhizoplane community profiles from the other four sites (Doon, Glen Morris, Shand Dam and West Montrose, 61%). The community structural profiles from river water at the six sites showed 35% similarity among the profiles from four of the sites, Bridge and Lancaster, Doon, West Montrose and Conestogo River. The river water community profiles from Shand Dam and Glen Morris were distantly related to the other four communities with only 3% similarity (38% similarity to each other), and showed more similarity to rhizosphere or rhizoplane community profiles.

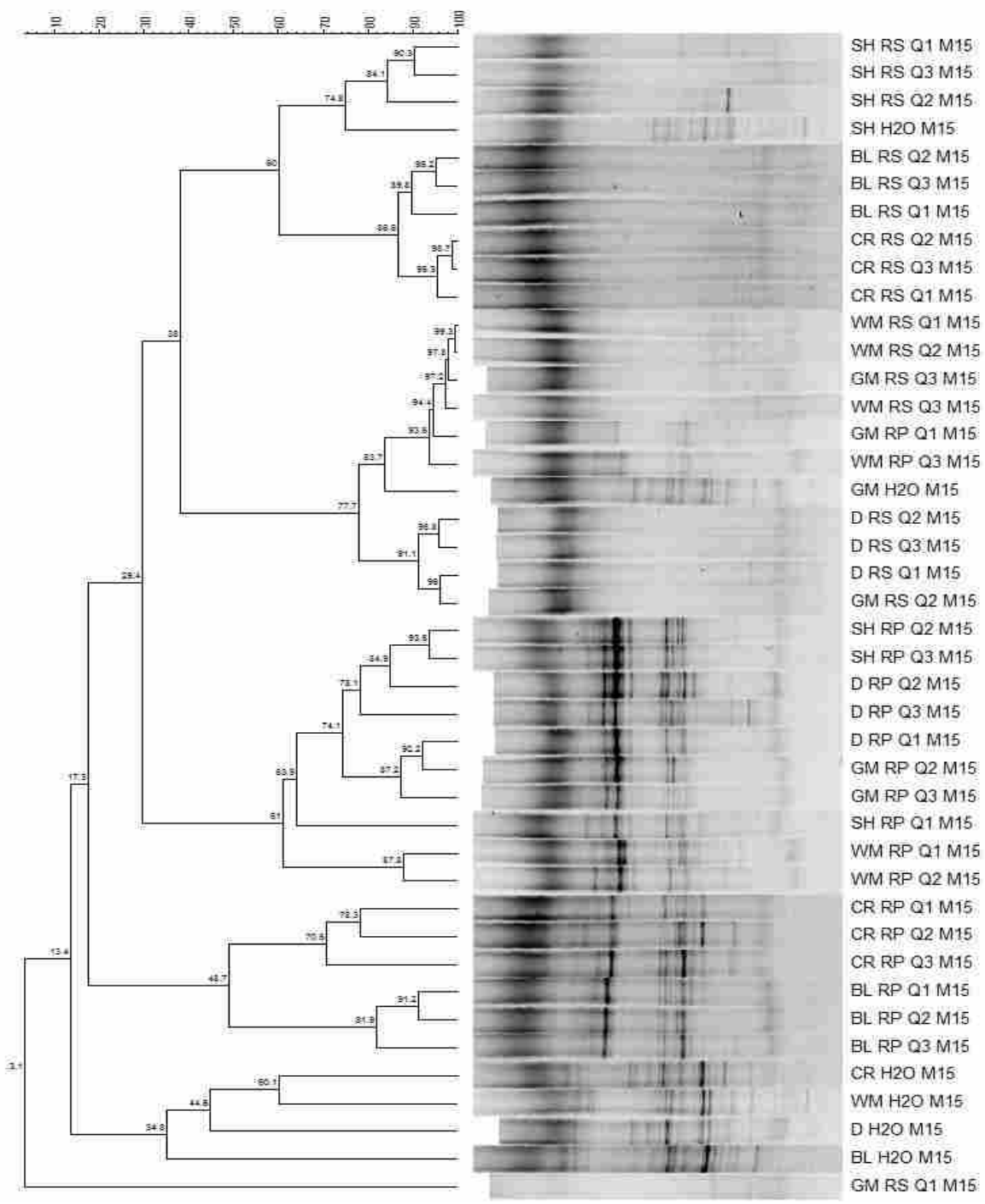

Figure 3.2 Hierarchical cluster analysis results of DGGE profiles obtained from microbial DNA extracted during sampling events in May 2015 (M 15) demonstrated graphically as a UPGMA dendrogram. High water quality sites included Shand Dam (SH) and West Montrose (WM). Intermediate water quality sites included Conestogo River (CR) and Bridge and Lancaster (BL). Low water quality sites included Doon (D) and Glen Morris (GM). Different sample types are indicated as H_2O (water), RS (rhizosphere) and rhizoplane (RP). Samples from different quadrats within a site are indicated with Q1-3. Numbers indicate percent similarity among clusters.

Structural community profiles from all sampling types at each of the six sampling locations showed much greater similarity among one another compared to the previous sampling event (48%) (Fig 3.3). As in the previous sampling event, community structural profiles clustered primarily by sampling type, with rhizosphere structural profiles exhibiting the highest degree of within group similarity (83%). Within this grouping, structural profiles grouped according to the three water quality designations. The Doon and Glen Morris (poor water quality) sites exhibited the highest similarity (94%). The intermediate quality sites (Bridge and Lancaster and Conestogo River) exhibited 91% similarity, although one of the Bridge and Lancaster profiles was an outlier and clustered with the poor water quality sites. The high water quality sites (Shand Dam and West Montrose) exhibited 87% similarity. The river water microbial communities exhibited the second highest amount of similarity among samples of the same type (75%), and the Bridge and Lancaster/Conestogo River communities were the most similar (97%). Rhizoplane communities exhibited 61% structural similarity (74% without outlier). Sub-groupings within the rhizoplane cluster did not correlate to water quality designations, although samples from the same sites (Q1-Q3) did tend to cluster together.

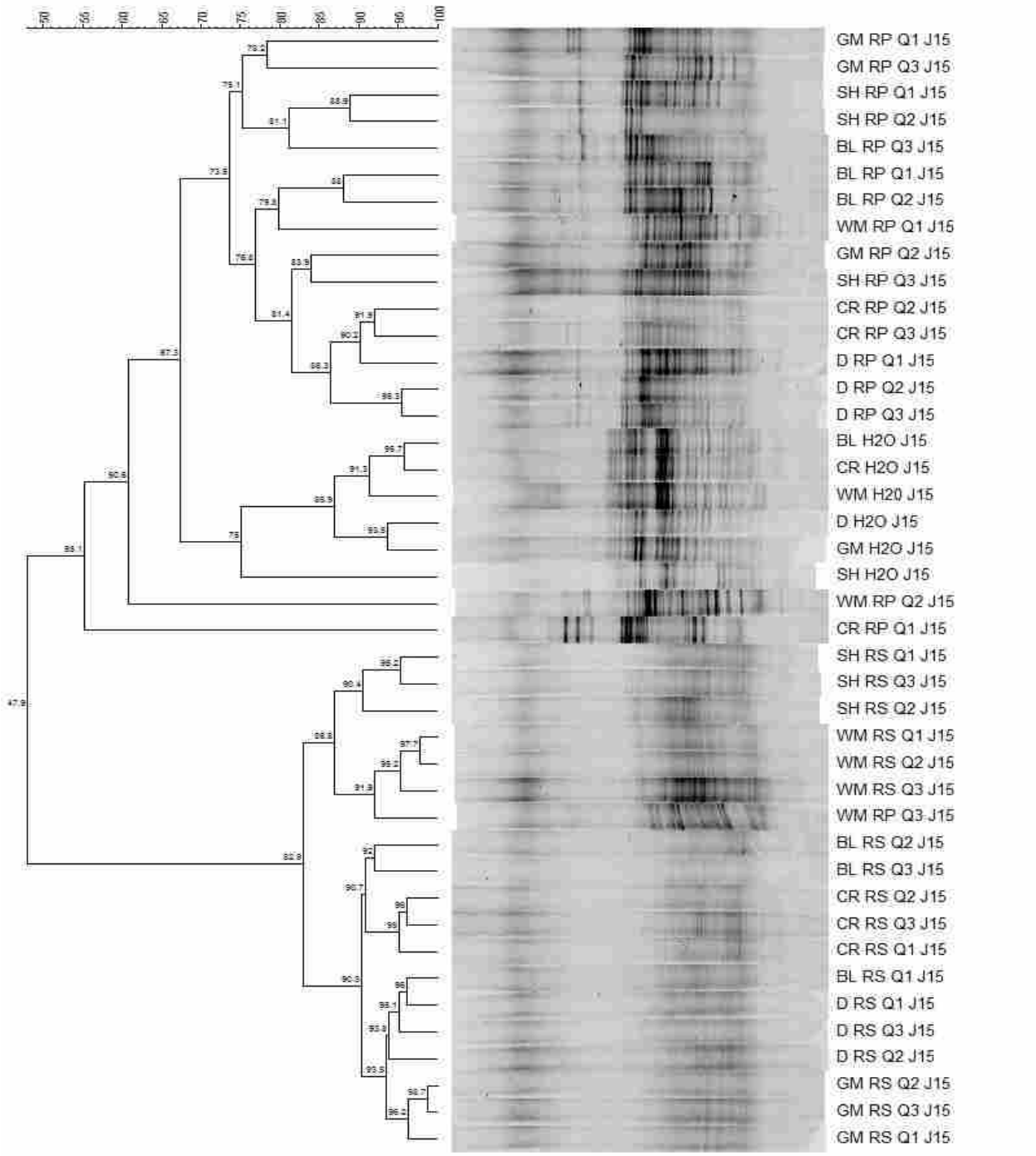

Figure 3.3. Hierarchical cluster analysis results of all of the DGGE profiles obtained from microbial DNA extracted during sampling events in July 2015 (J 15) demonstrated graphically as a UPGMA dendrogram. High water quality sites included Shand Dam (SH) and West Montrose (WM). Intermediate water quality sites included Conestogo River (CR) and Bridge and Lancaster (BL). Low water quality sites included Doon (D) and Glen Morris (GM). Different sample types are indicated as H_2O (water), RS (rhizosphere) and rhizoplane (RP). Samples from different quadrats within a site are indicated with Q1-3. Samples from different quadrats within a site are indicated with Q1-3. Numbers indicate percent similarity among clusters.

The August 2015 sampling event exhibited more variability among samples in terms of structural profiles than in July 2015 (1.9% similarity) (Fig 3.4). There was significant clustering corresponding to samples of the same community type, from different sampling locations, however, these associations were not as strong as those observed during previous sampling events. All six water community structural profiles clustered together with 35% similarity, although this cluster also contained some rhizoplane structural profiles from Bridge and Lancaster. The two high water quality sites (West Montrose and Shand Dam) were the most similar (76%), while the two poor water quality sites (Doon and Glen Morris) also formed a sub-grouping with 70% similarity. Rhizoplane structural profiles exhibited 25% structural similarity (33% without outliers). Samples from the same site tended to cluster together with the highest similarity being among rhizoplane communities from the Shand Dam site (86% similarity). Rhizosphere community profiles formed three sub-groupings, which were only related by 2% similarity. The most distantly related group contained the rhizosphere community profiles from Conestogo River (16% similarity). The second sub-grouping contained structural profiles from West Montrose and Bridge and Lancaster (40% similarity). The third subgroup was distantly related to the other two and contained rhizosphere structural profiles from Shand Dam, Doon and Glen Morris (14% similarity).

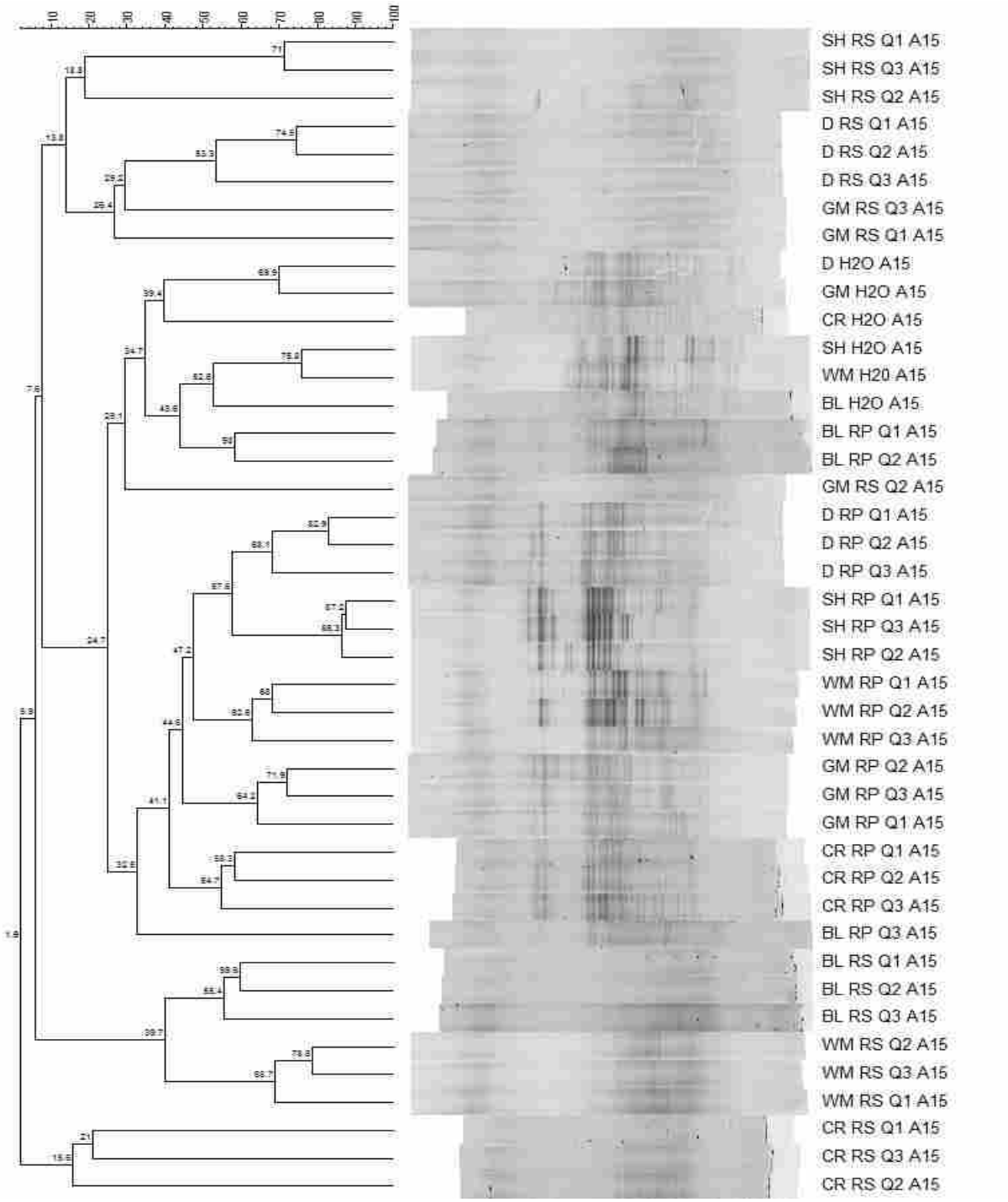

Figure 3.4 Hierarchical cluster analysis results of all of the DGGE profiles obtained from microbial DNA extracted during sampling events in August 2015 (A 15) demonstrated graphically as a UPGMA dendrogram. High water quality sites included Shand Dam (SH) and West Montrose (WM). Intermediate water quality sites included Conestogo River (CR) and Bridge and Lancaster (BL). Low water quality sites included Doon (D) and Glen Morris (GM). Different sample types are indicated as H_2O (water), RS (rhizosphere) and rhizoplane (RP). Samples from different quadrats within a site are indicated with Q1-3. Numbers indicate percent similarity among clusters.

Community structural profiles from all sample types at each of the six sites only shared 9% similarity during sampling in October 2015 (Fig 3.5). Rhizoplane community profiles exhibited the highest degree of within sample type similarity (41% excluding outliers). Within the rhizoplane cluster, samples from the same site tended to cluster together. There were two main sub-clusters, which contained rhizoplane community profiles from Bridge and Lancaster and Doon (48% similarity), or Glen Morris/Shand Dam/Conestogo River (46% similarity). There were several outliers, primarily from the West Montrose and Glen Morris sites, which were only distantly related to the other rhizoplane community structural profiles. Rhizosphere structural community profiles only exhibited 9% similarity among all of the six sites. There were four distinct groupings within the larger cluster relating to the different sites. These sub-groupings contained community profiles from Conestogo River (90% similarity), Bridge and Lancaster (83% similarity), West Montrose/Shand Dam (43% similarity) and Doon/Glen Morris (65% similarity). Unlike in the previous sampling events the structural profiles from river water communities did not cluster together strongly. One cluster contained structural profiles from Conestogo River, West Montrose and Doon (40% similarity), but the other three sites were structurally dissimilar and grouped with either rhizoplane or rhizosphere communities.

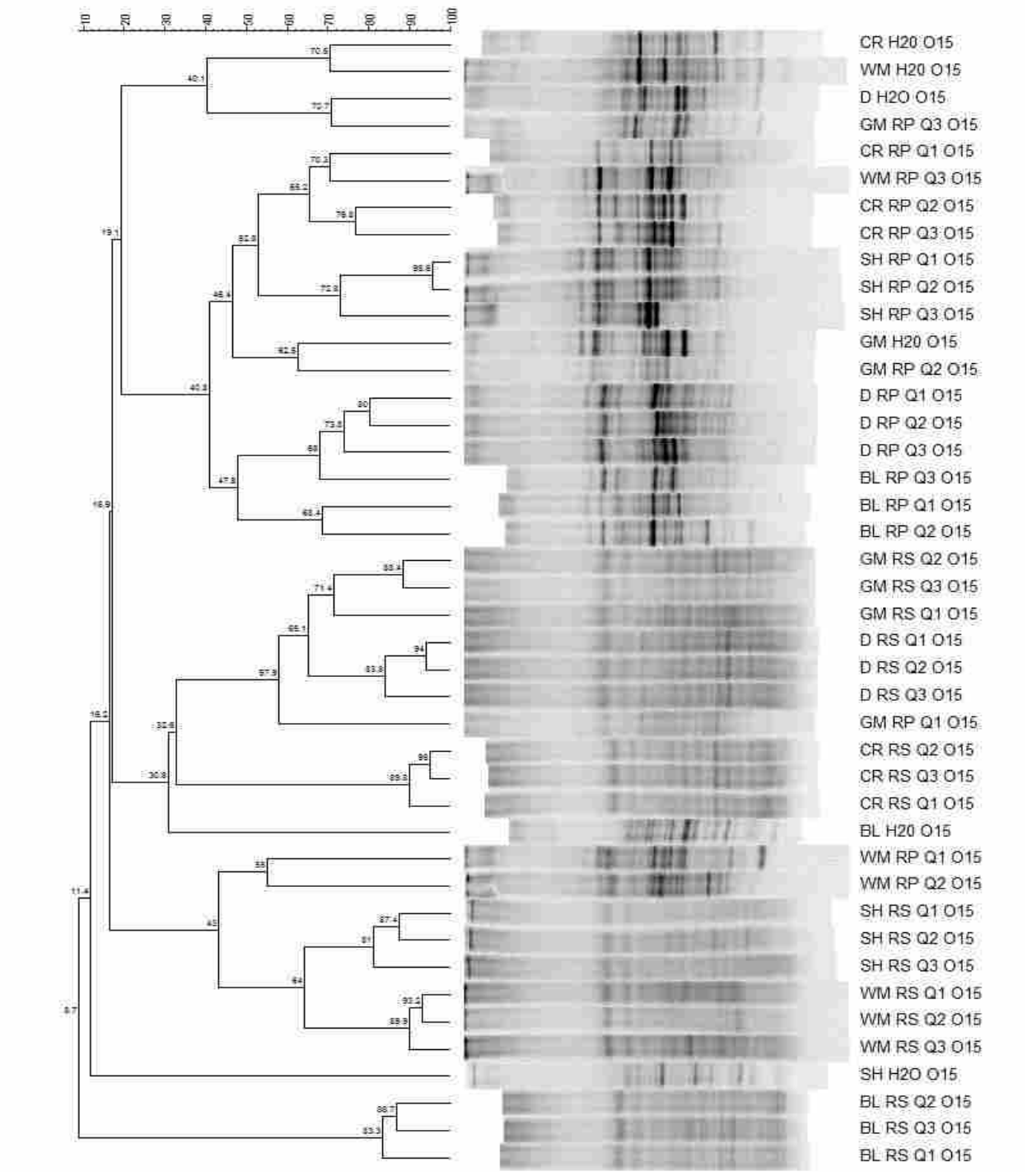

Figure 3.5 Hierarchical cluster analysis results of all of the DGGE profiles obtained from microbial DNA extracted during sampling events in October 2015 (O 15) demonstrated graphically as a UPGMA dendrogram. High water quality sites included Shand Dam (SH) and West Montrose (WM). Intermediate water quality sites included Conestogo River (CR) and Bridge and Lancaster (BL). Low water quality sites included Doon (D) and Glen Morris (GM). Numbers indicate percent similarity among clusters.

From the sampling event in November 2015, samples from each of the different sampling sites including each of the three different community types (water, rhizosphere and rhizoplane) shared 16% structural similarity (Fig 3.6). The structural profiles primarily clustered into the three different sample types with rhizosphere samples sharing 24% structural similarity, rhizoplane samples sharing 16% similarity (37% without outliers) and water samples sharing 50% similarity. Within the rhizosphere, structural community profiles exhibited a clustering pattern with two distinct groupings, the first shared 44% structural similarity and contained structural community profiles from both of the intermediate water quality sites (Conestogo River and Bridge and Lancaster) and Shand Dam (High water quality). The second cluster shared 32% structural similarity and had one sub-grouping containing structural profiles from the lower water quality sites (Glen Morris and Doon, 57% similarity) and another sub-grouping containing structural profiles from West Montrose (42% similarity). The rhizoplane structural community profiles were clustered in with the water structural profiles and formed two primary sub-groupings, the first contained structural profiles from Bridge and Lancaster, Conestogo River, Glen Morris, Doon and Shand Dam, the second contained structural profiles from Shand Dam, West Montrose and Doon. The second sub-grouping of rhizoplane community profiles contained all six of the water community profiles as well. Not all of the structural profiles from rhizoplane samples clustered together with other rhizoplane samples from the same sampling site, a trend that we had been observing at previous sampling events. Structural profiles from water microbial community fell into two groupings, the first contained the profiles from West Montrose, Conestogo River, Glen Morris and Bridge and Lancaster (78% similarity) while the second grouping contained the structural profiles from Shand Dam and Doon (68% similarity).

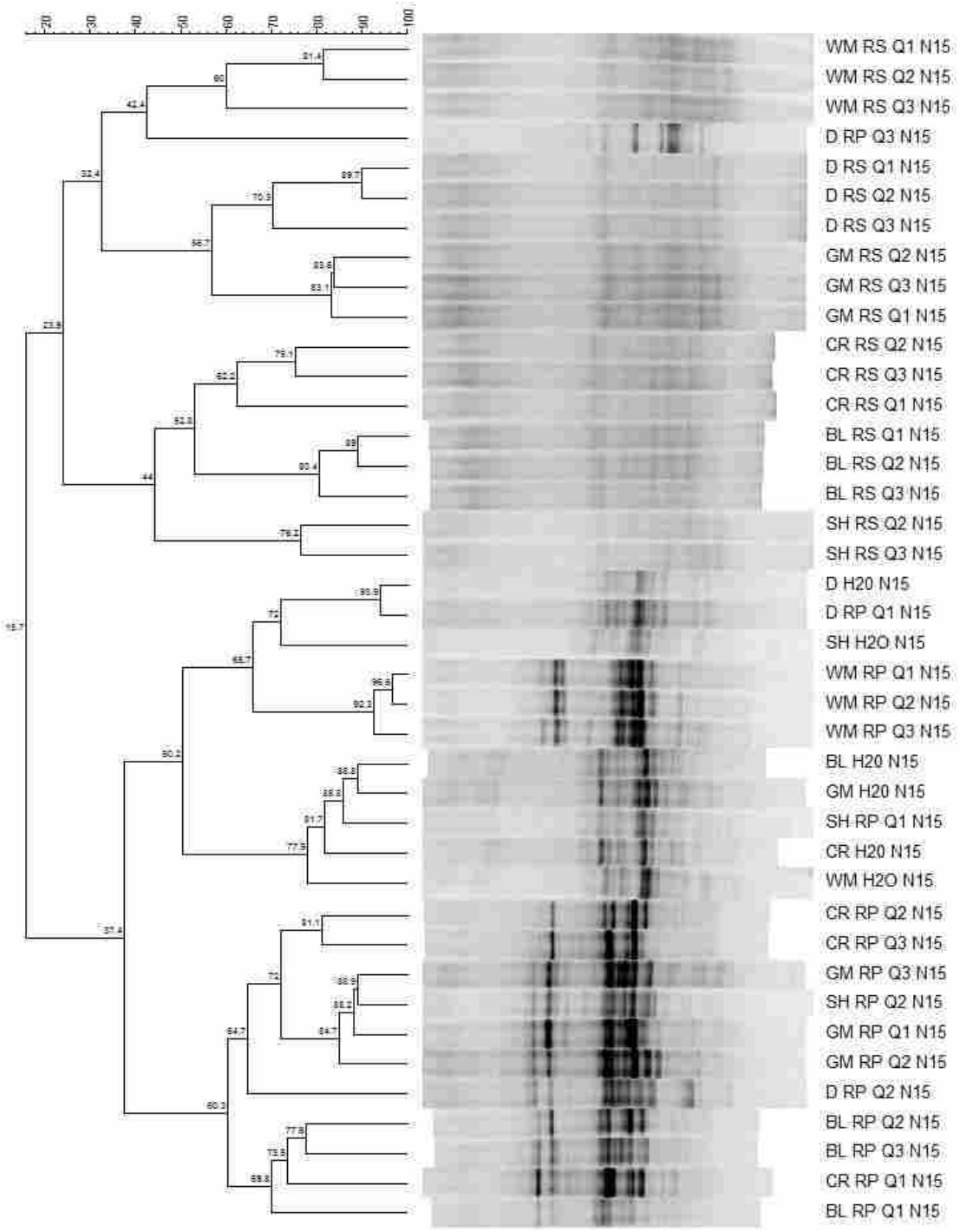

Figure 3.6 Hierarchical cluster analysis results of all of the DGGE profiles obtained from microbial DNA extracted during sampling events in November 2015 (N 15) demonstrated graphically as a UPGMA dendrogram. High water quality sites included Shand Dam (SH) and West Montrose (WM). Intermediate water quality sites included Conestogo River (CR) and Bridge and Lancaster (BL). Low water quality sites included Doon (D) and Glen Morris (GM). Different sample types are indicated as H_2O (water), RS (rhizosphere) and rhizoplane (RP). Samples from different quadrats within a site are indicated with Q1-3. Numbers indicate percent similarity among clusters.

Over the duration of the sampling season, structural similarity among rhizosphere microbial communities exhibited the greatest amount of variability between sampling events (Table 3.1). Similarity among rhizosphere microbial communities from different sampling locations was relatively high during the sampling events in May and July but very low in August and October. The rhizosphere exhibited the highest within-sample type structural similarity compared to the other sample types during May and July, but became much more variable throughout the rest of the year. Rhizoplane-associated microbial communities exhibited the least amount of variation in structural similarity and within sample type clustering between sampling events. Furthermore, there was a large increase in within sample type structural similarity for all community types during the sampling event in July.

Table 3.1 Summary of Within Sample Type Percent Similarity for Microbial Community Structural Profiles Associated with *P. arundinacea* in the Grand River, ON as determined by PCR-DGGE-Based Hierarchical Cluster Analysis

	May	**July**	**August**	**October**	**November**
Rhizoplane	17.3%*(2)	73.5%*(2)	32.5%*(2)	40.8%*(4)	37.4%
Rhizosphere	38%*(1)	82.9%	1.9%	8.7%	23.9%
Water	34.8%(2)	75%	34.7%	8.7%	37.4%

*Percent similarity value excludes outliers and the numbers of outliers excluded are indicated in the brackets

3.3.2 Functional Profiles of Microbial Communities Associated with *P. arundinacea* across a Water Quality Gradient

3.3.2.1 Rhizoplane Communities

Principal component analysis of carbon source utilization profiles obtained from rhizoplane microbial communities associated with *P. arundinacea* at our six sampling sites along the Grand River did not reveal any consistent trends correlating with sampling site water quality classifications (Fig. 3.7). Correlations between carbon source utilization profiles from communities associated with different sampling sites varied depending on sampling date. For example, during the sampling event in May 2015, rhizosphere communities from West Montrose and Conestogo River were very similar (Fig. 3.7 A), however in each of the other sampling events, carbon source utilization profiles established by communities obtained at these two sites were very distinct (Fig. 3.7 B,C,D and E). Carbon source utilization profiles from rhizoplane communities at Shand Dam, Glen Morris and Bridge and Lancaster were similar during most sampling events (Fig. 3.7 B,C and E) despite being spatially very distinct, representing the extremes of the sampling sites, with Shand Dam representing most northern site and Glen Morris the most southern. The sampling event in October 2015 (Fig. 3.7 D) revealed the most distinct carbon source utilization profiles for rhizoplane communities at the six different sampling sites. During this time point, carbon source utilization profiles at Doon and Glen Morris were closely related as were Shand Dam and Bridge and Lancaster. Doon, Glen Morris and West Montrose were separated from Conestogo, Shand Dam and Bridge and Lancaster along the PC1 axis. Conestogo River and West Montrose were distinct from the other four sites along the PC2 axis. The West Montrose rhizoplane community was distinct from the communities at other sites due to strong usage L-serine and 2-hydroxybenzoic acid (Table A1). The Conestogo River community was distinct due to the usage of Tween 40 and pyruvic acid methy ester.

Communities from Doon/Glen Morris were distinguished by usage of itaconic acid, N-acetyl-D-glucosamine and L-serine while those from Shand Dam and Bridge and Lancaster were associated with usage of Tween 80, D,L-α-glycerol phosphate and L-threonine. All other sampling time points yielded very high overlap among the different sites.

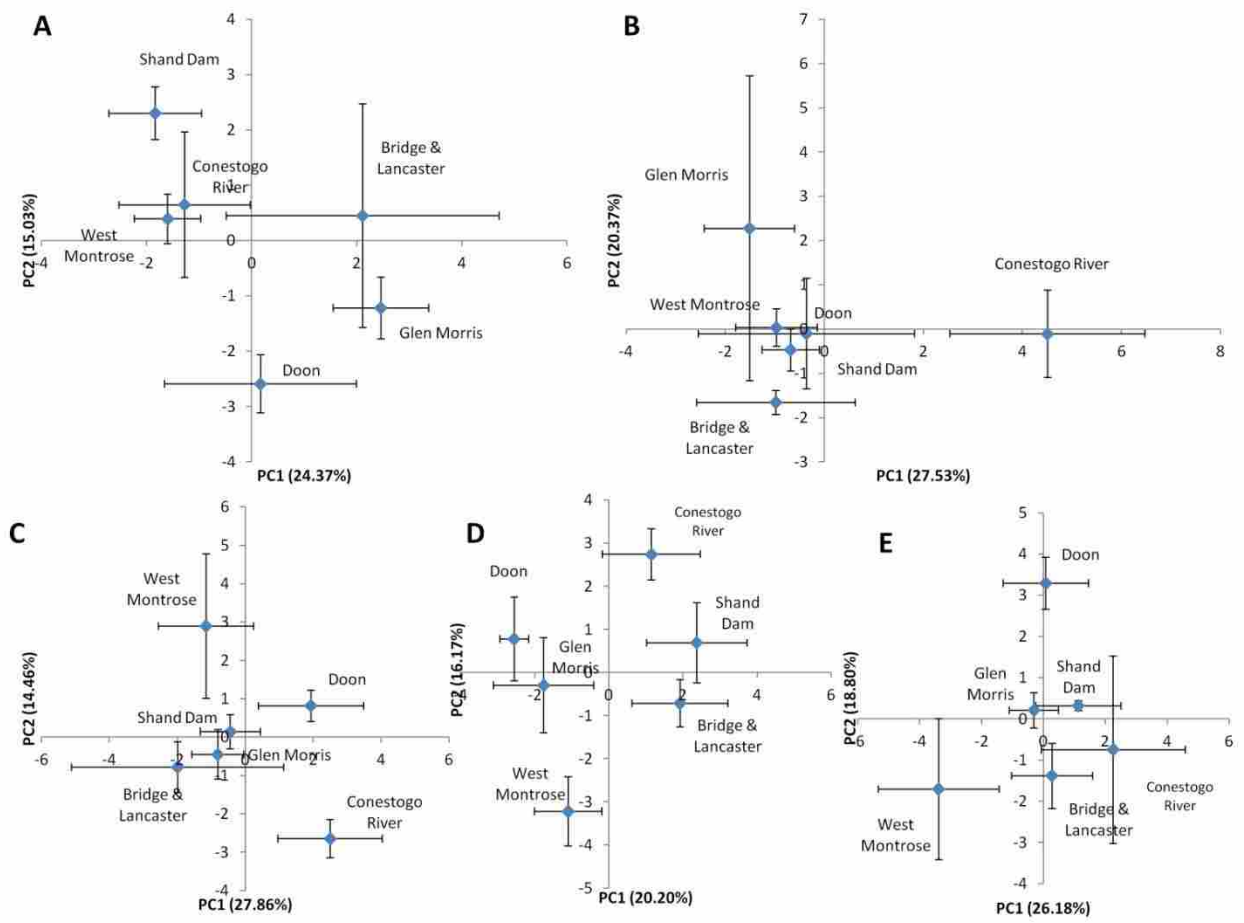

Figure 3.7 PC1 vs. PC2 plot of Biolog™ carbon source utilization by microorganisms present in rhizoplane samples from *Phalaris arundinacea* obtained in May (A), July (B), August (C), October (D) and November (E) of 2015. High water quality sites included Shand Dam and West Montrose. Intermediate water quality sites included Conestogo River and Bridge and Lancaster. Low water quality sites included Doon and Glen Morris. Each data point represents an average PC1 and PC2 score from three experimental replicates and three technical replicates. Error bars represent standard error from experimental replicates only.

3.3.2.2 Rhizosphere Communities

Principal component analysis of carbon source utilization profiles obtained from rhizosphere microbial communities associated with *P. arundinacea* at our six sampling sites along the Grand River did not exhibit trends related to site water quality designations (Fig. 3.8). Carbon source utilization profiles from rhizosphere communities obtained at different locations along the Grand River tended to be similar, with a high degree of overlap among sites during the sampling events in August and November 2015 (Fig. 3.8 C and E). However, distinct carbon source utilization profiles were associated with most sampling locations during sampling events in May, July and October of 2015 (Fig. 3.8 A, B and D). Despite having distinct carbon source utilization profiles associated with most sampling sites, during these times, different carbon sources are associated with distinguishing each site during different sampling events (Table A2). Furthermore, the relationship among the different communities represented on the PC1 vs. PC2 plot also changes depending on the sampling date. For example, during sampling in May 2015 (Fig. 3.8 A) Bridge and Lancaster and Glen Morris (most negative PC1 scores) are separated from Conestogo River and Doon (neutral PC1 scores), as well as Shand Dam and West Montrose (positive PC1 scores) along the PC1 axis. However, in July 2015, this relationship changes and Bridge and Lancaster and Doon (negative PC1 scores) are separated from West Montrose (neutral PC1 scores), as well as Conestogo River, Glen Morris and Shand Dam (positive PC1 scores along the PC1 axis. This indicates that the carbon source utilization profiles associated with the rhizosphere communities at these sites are dynamic (not static) and influenced by more factors than just those that relation to water quality and geographic location.

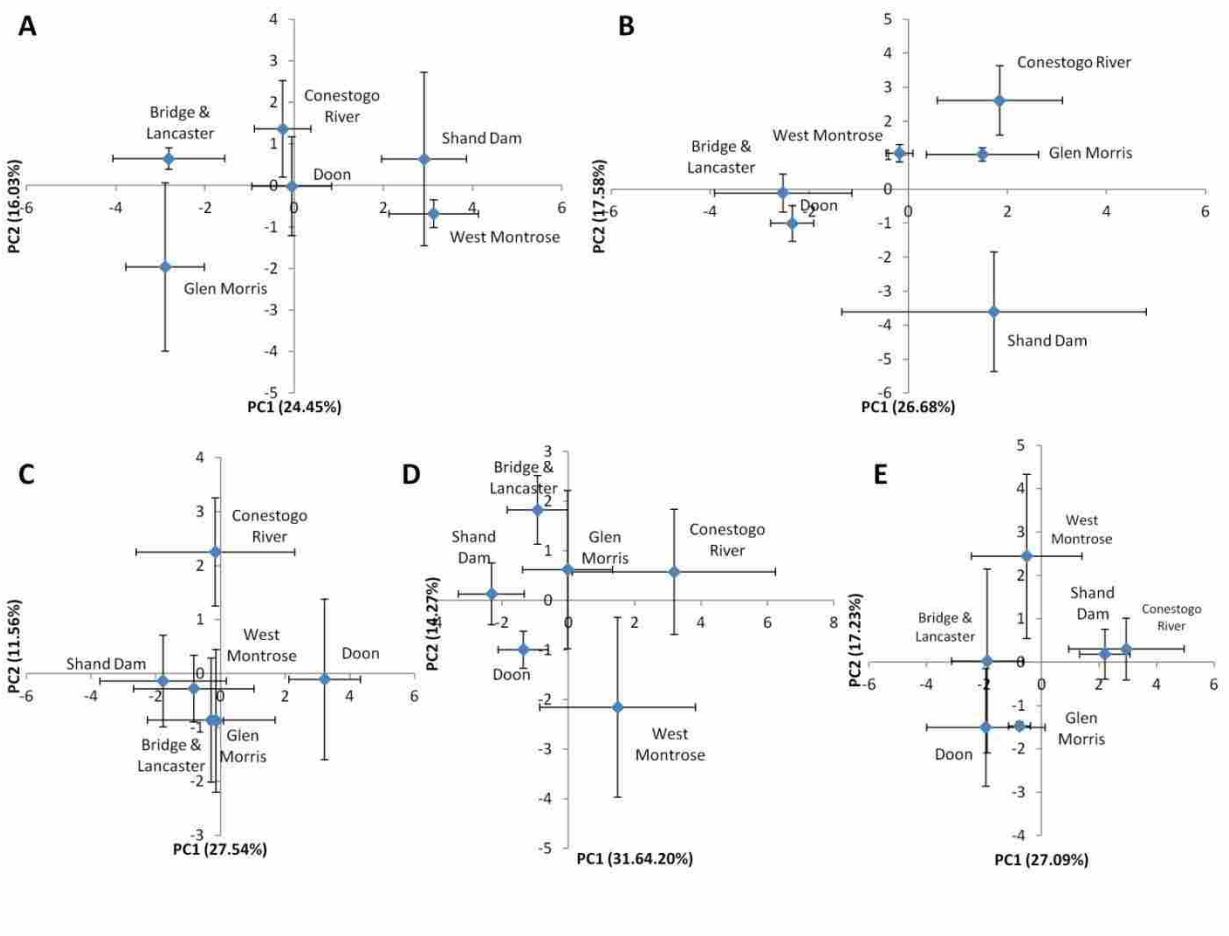

Figure 3.8 PC1 vs. PC2 plot of Biolog^{TM} carbon source utilization by microorganisms present in rhizosphere samples from *Phalaris arundinacea* obtained in May (A), July (B), August (C), October (D) and November (E) of 2015. High water quality sites included Shand Dam and West Montrose. Intermediate water quality sites included Conestogo River and Bridge and Lancaster. Low water quality sites included Doon and Glen Morris. Each data point represents an average PC1 and PC2 score from three experimental replicates and three technical replicates. Error bars represent standard error from experimental replicates only.

3.3.2.3 River Water Communities

Principal component analysis of carbon source utilization profiles established by river water microbial communities collected from six sampling sites along the Grand River, between May and November of 2015, did not show trends related to the water quality designations assigned to the different sites (Fig. 3.9). The relationships among the carbon source utilization profiles established by river water communities at each site changed among sampling events. Sites which exhibited very similar PC1 and PC2 scores during one sampling event did not necessarily exhibit equally similar carbon source utilization profiles (and PC1 and 2 scores) during another sampling event. Furthermore, carbon sources used to distinguish water communities from one another also changed from sampling event to sampling event (Table A3).

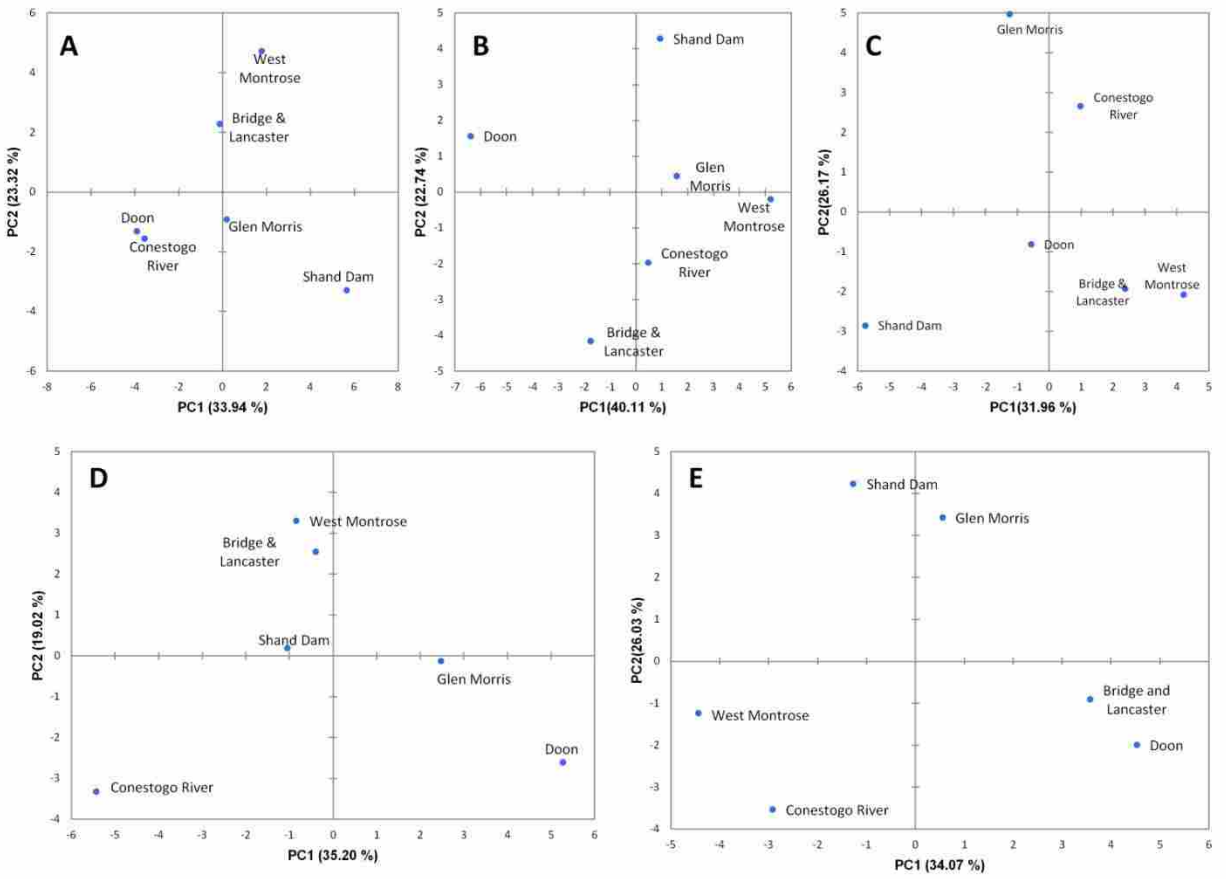

Figure 3.9 PC1 vs. PC2 plot of Biolog^{TM} carbon source utilization by microorganisms present in water column samples obtained in May (A), July (B), August (C), October (D) and November (E) of 2015. High water quality sites included Shand Dam and West Montrose. Intermediate water quality sites included Conestogo River and Bridge and Lancaster. Low water quality sites included Doon and Glen Morris. Each data point represents an average PC1 and PC2 score from three technical replicates.

3.3.2.4 Combined Community Types (Rhizoplane, Rhizosphere and Water)

When carbon source utilization profiles from all sample types were included within the same principal component analysis a consistent trend emerged during all sampling events (Fig. 3.10). Separation of carbon source utilization profiles by sample type (rhizosphere, rhizoplane and water) was consistent across sampling dates. Water communities were consistently associated with positive PC1 scores, while rhizosphere and rhizoplane samples were associated with negative PC1 scores. Carbon source utilization profiles associated with rhizoplane or rhizosphere communities were separated along the PC2 axis. Several carbon sources distinguish microbial communities of the three different sample types consistently across all sampling events (Table A4). River water microbial communities were consistently associated with pyruvic acid methyl ester, Tween 40, glycogen, β-methyl-D-glucoside and D,L-α-glycerol phosphate**.** These carbon sources fall into the Biolog^{TM} EcoPlate classification of polymers and carbohydrates primarily. Alternatively, rhizosphere communities were commonly associated with D-galacturonic acid, D-glucosaminic acid, 2-hydroxybenzoic acid, 4-hydroxybenzoic acid, L-argnine, L-serine, phenyl ethylamine and putrescine. These carbon sources primarily fell into the categories of phenolic compounds, carboxylic acids, amines and amino acids. Rhizoplane microbial communities were associated with a smaller subset of the same carbon sources that were correlated with rhizosphere communities including, 2-hydroxybenzoic acid, L-serine, phenyl ethylamine and putrescine (phenolic compounds, amino acids and amines).

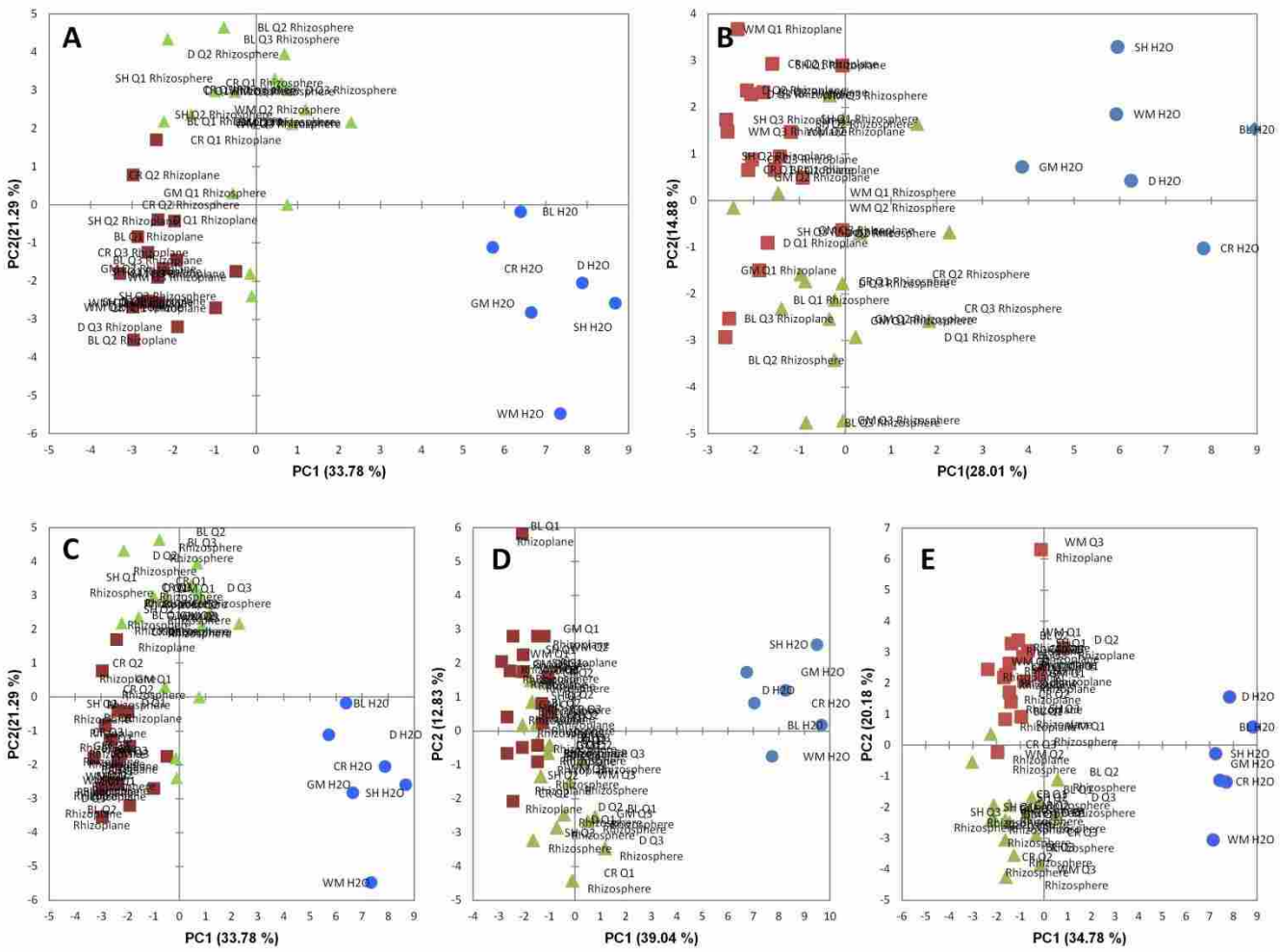

Figure 3.10 PC1 vs. PC2 plot of Biolog^{TM} carbon source utilization by microorganisms present in rhizoplane (red square), rhizosphere (green triangle) and water column samples (blue circle), obtained in May (A), July (B), August (C), October (D) and November (E) of 2015. High water quality sites included Shand Dam (SH) and West Montrose (WM). Intermediate water quality sites included Conestogo River (CR) and Bridge and Lancaster (BL). Low water quality sites included Doon (D) and Glen Morris (GM). Each data point represents an average of PC1 and PC2 scores from 3 technical replicates. Samples collected from the same site and a different quadrat are indicated with Q1-3.

3.3.2.5 Metabolic Diversity

The metabolic diversity of the microbial communities at each site was calculated using the Shannon Diversity index (H`) applied to Biolog^{TM} EcoPlate data from the three different community types at each different water quality site. Differences were observed between the metabolic diversity of the microbial communities associated with river water, *P. arundinacea* rhizosphere or rhizoplane, with diversity in the rhizoplane being the highest followed by the rhizosphere and then the water communities (Fig. 3.11). There were no consistent differences in metabolic diversity among the six different sites for any of the community types (Fig 3.11 A, B, C). Some seasonal differences in metabolic diversity were observed with seasonal variations being the most pronounced in the rhizosphere and rhizoplane. In the rhizosphere, community metabolic diversity tended to be highest in August and November and lowest in October. Alternatively, in the rhizoplane, metabolic diversity tended to be highest in July and November, and lowest in October.

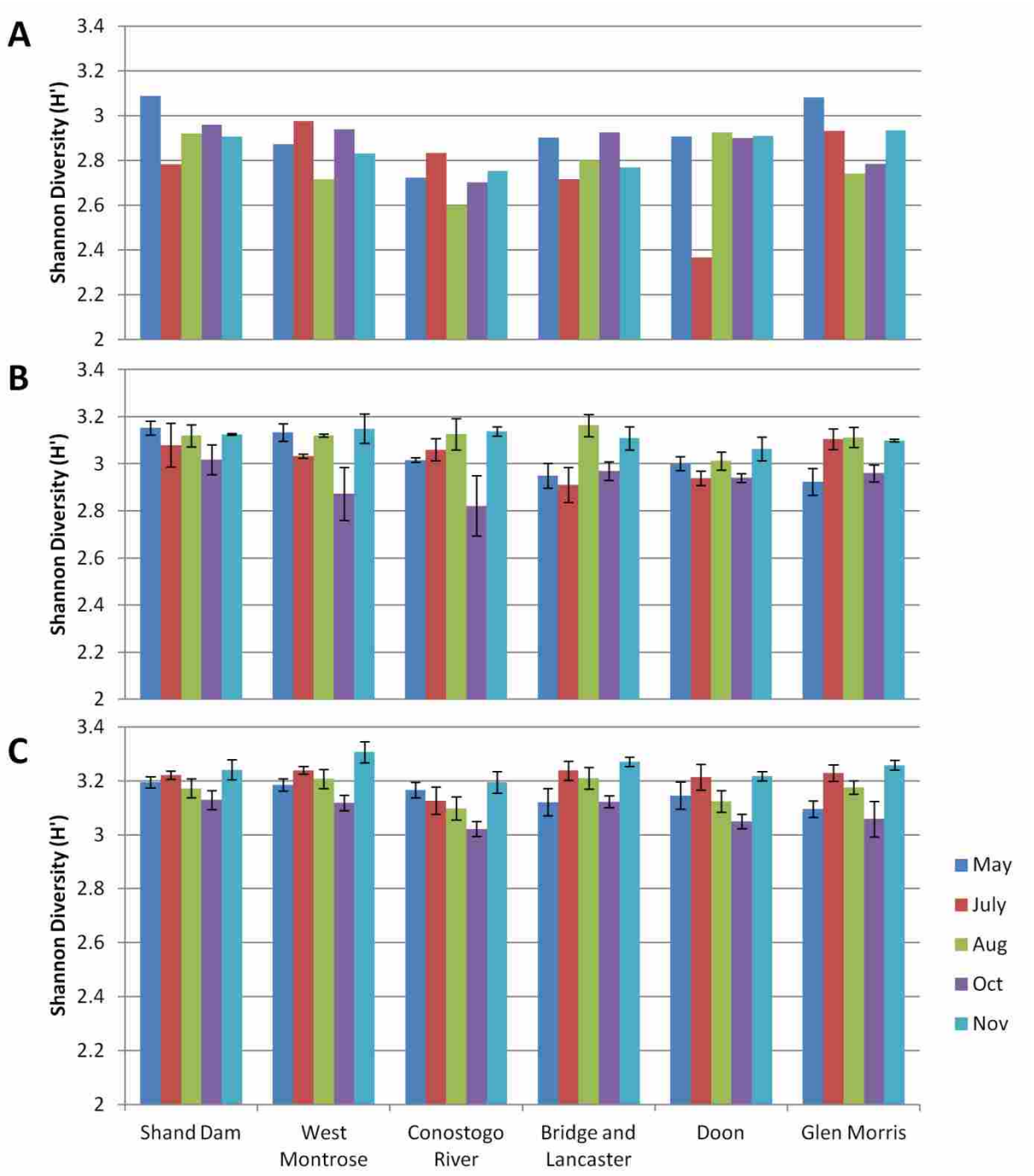

Figure 3.11 Metabolic diversity of microbial communities in river water (A), the rhizosphere (B) and the rhizoplane (C) as determined by Biolog^{TM} plate carbon source usage following 96h of incubation. Error bars are representative of standard error for 3 experimental replicates. Each bar is the average of 3 technical replicates (A) and 3 experimental replicates (B and C only) for each treatment.

3.3.3 Water Chemisty

When a PCA was performed to assess trends in water chemistry related to water quality parameters among the six water quality sites over the entire sampling period (Fig 3.12), the closest correlation was between the two high water quality sites, Shand Dam and West Montrose, which were both associated with negative PC1 and negative PC2 scores. Intermediate water quality sites, Conestogo River and Bridge and Lancaster, also were closely related in terms of water quality measurements, and both had positive PC2 scores. However, Conestogo River water chemistry had a PC1 score close to zero (but negative), while Bridge and Lancaster had a positive PC1. The two designated poor water quality sites did not share similar water chemistry, with regards to water quality parameters, and Glen Morris was actually more similar to the intermediate water quality sites than to Doon, the other poor water quality site. Glen Morris was associated with negative PC1 and positive PC2 scores, while Doon was associated with positive PC1 and negative PC2 scores. Water quality parameters that were correlated with positive PC1 scores included pH, dissolved oxygen and high inorganic nutrient concentrations for nitrite, ammonium and phosphorus (Table 3.2). Positive PC2 scores were associated with pH, oxidation reduction potential, and nitrate concentration. Negative PC2 values were associated with total dissolved solids and ammonium concentration.

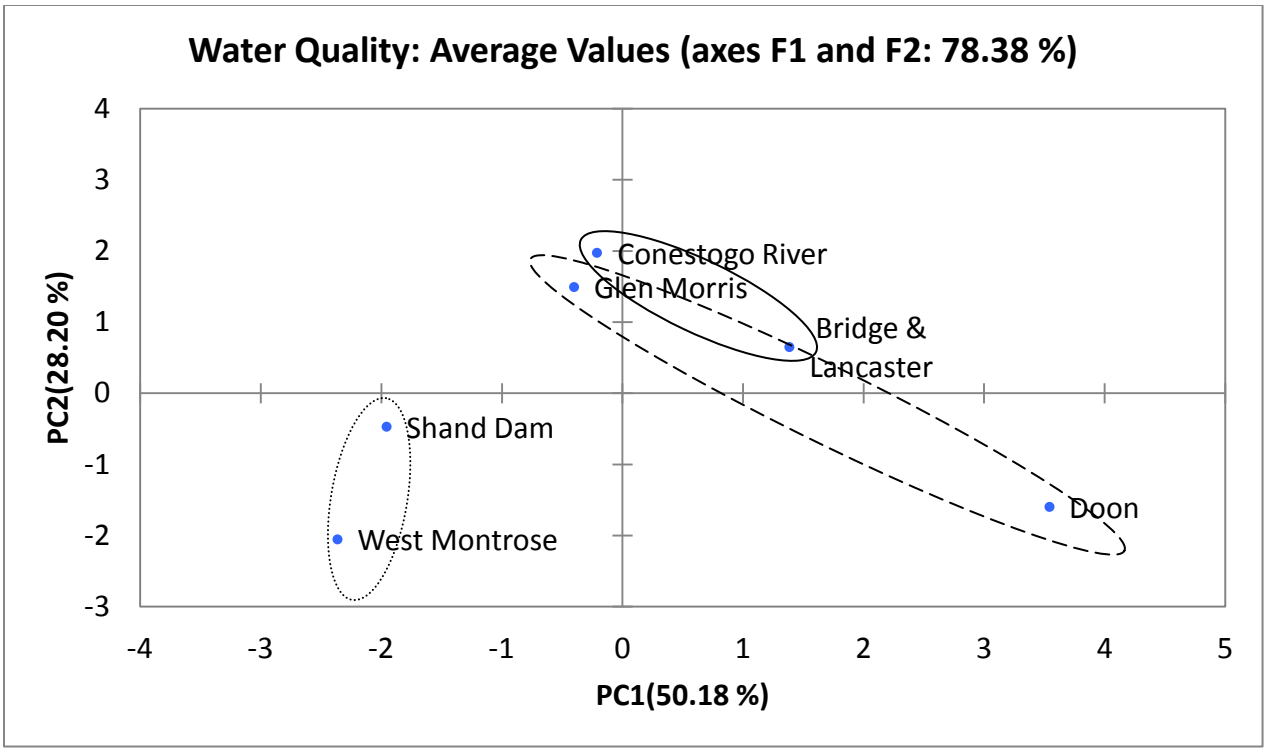

Figure 3.12 PC1 vs. PC2 plot of the physicochemical properties of pore-water averaged over the experimental sampling period (May to November) for all of the Grand River sampling sites. High water quality sites (as defined by the Grand River Conservation Authority) included Shand Dam and West Montrose (circled with short dashed lines). Intermediate water quality sites included Conestogo River and Bridge and Lancaster (circled with a soil line). Low water quality sites included Doon and Glen Morris (circled with large dashed lines).

Table 3.2 Factor Loadings and Eigenvalues for the First Two Principal Components of the PCA of Physicochemical Properties of Pore-Water Averaged Over the Experimental Sampling Period (May to November) for all of the Grand River Sampling Sites.

	F1	F2
pH	**0.717**	**0.540**
Dissolved Oxygen	**0.917**	-0.129
Total Dissolved Solids	0.317	-0.541
Oxidation Reduction Potential	0.371	**0.787**
Nitrite	**0.905**	-0.318
Nitrate	0.441	**0.772**
Ammonium	**0.747**	-0.582
Phosphorus	**0.921**	-0.008
Eigen Values	4.014	2.256

*Factors contributing significantly to positive principal component scores are bolded while factors contributing to negative principal component scores are underlined

Measurements of inorganic nutrient concentrations from pore water at each of the six water quality sites exhibited seasonal trends and trends associated with GRCA water quality designations. Nitrate concentrations tended to be lowest at the two high water quality sites (Shand Dam and West Montrose) and highest at Conestogo River (intermediate water quality) and Glen Morris (poor water quality) (Fig 3.13A). Nitrate concentrations were intermediate at Bridge and Lancaster (intermediate water quality) and Doon (poor water quality). For the majority of sampling sites, nitrate concentrations were highest in October or August and lowest in July. Nitrite concentrations exhibited distinct trends compared to nitrate. Season differences in nitrite concentrations were less dramatic at most sites (Fig 3.13B). As well, nitrite concentrations were comparable across the six water quality sites at most time points, although Bridge and Lancaster and Doon showed trends towards higher values at most sampling events. Ammonia concentrations were much higher at the Doon site compared to the other five sampling sites during all sampling events other than in August (Fig 3.13C). Ammonia concentrations were comparable between the other sites and seasonal trends were not consistent among sites. Total reactive phosphorus measurements tended to be lowest at Shand Dam and West Montrose during most sampling events, and highest at Doon (Fig 3.13D). Seasonal trends were not consistent between sites. Overall, inorganic nutrient loads tended to be highest at the Doon site, while Shand Dam and West Montrose tended to have the lowest values. Interestingly, despite being classified as a poor water quality site, Glen Morris tended to have low or intermediate inorganic nutrient levels for most compounds evaluated, aside from nitrate.

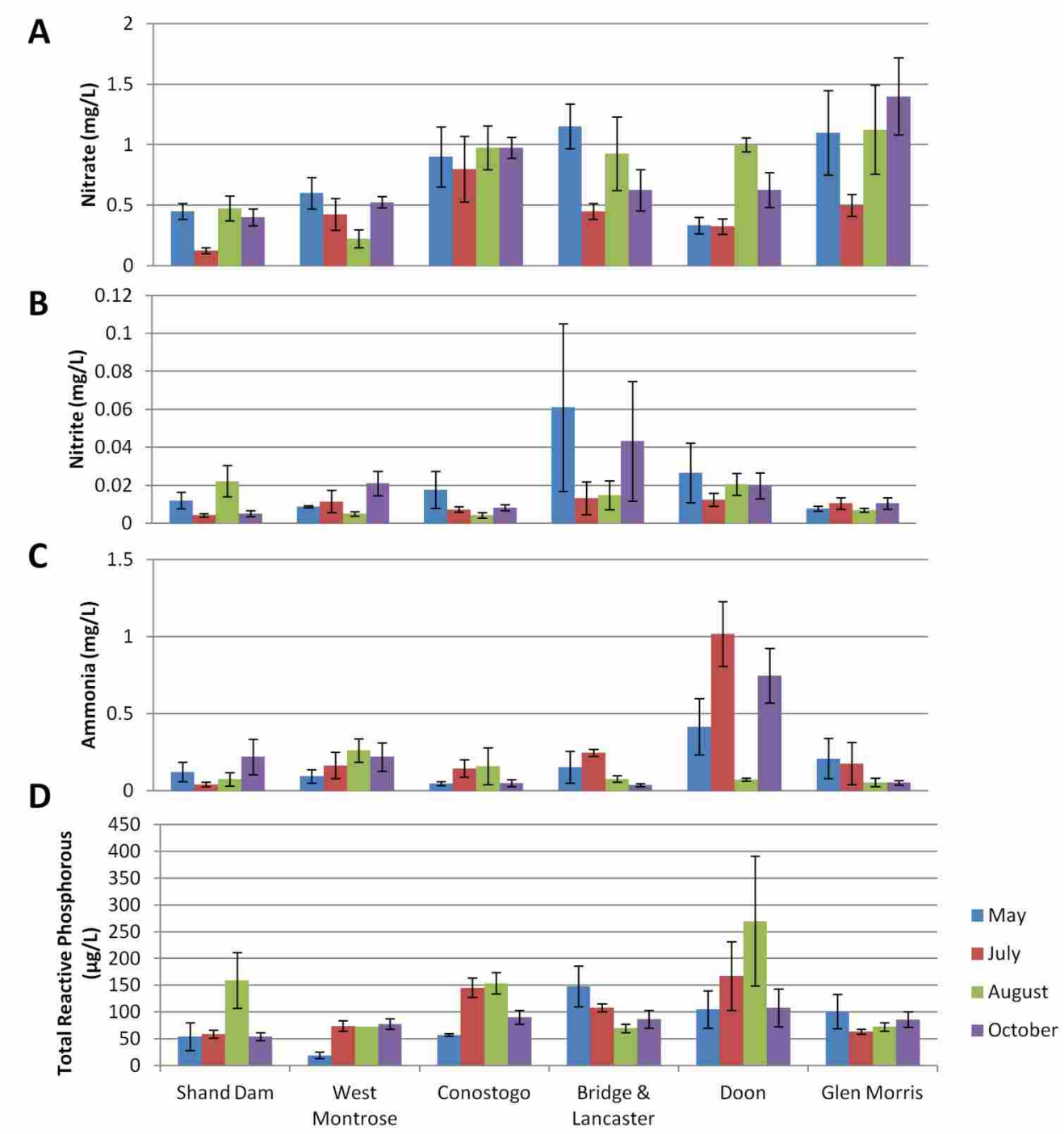

Figure 3.13 Average pore-water concentrations of inorganic nutrients including nitrate (A), nitrite (B), ammonia (C) and total reactive phosphorus (D). Bars are the average of four measurements taken from four different quadrats at each site. Error bars represent standard error from the four measurements.

3.3.4 Microbiological Indicators of Water Quality

3.3.4.1 Culture-Based Methods

Heterotrophic plate counts (HPC) conducted on R2A media revealed that per mL or gram of relevant material; culturable bacterial concentrations were significantly higher in the rhizosphere and rhizoplane than in the water. Heterotrophic plate counts from water were significantly lower at Shand Dam compared to the other sampling sites (Fig 3.14A). Consistent reliable seasonal trends across sampling sites in heterotrophic plate counts were not observed for any of the sample types, although there was some evidence for higher HPCs in the rhizosphere during the November sampling event (Fig 3.14 15B). Site specific differences related to water quality were not observed among the different sampling events (Fig 3.14).

A similar number of fecal coliforms were cultured from all sample types at each of the six different sites (Fig 3.15). There was a lot of variability in the quantity of fecal coliforms detected in river water between sampling events, however, no seasonal trends were consistently identified at all of the sites (Fig 3.15A). Fecal coliform detection tended to be highest in July at most sites for rhizosphere and rhizoplane samples, and lowest in either October or November (Fig 3.15 B and C).

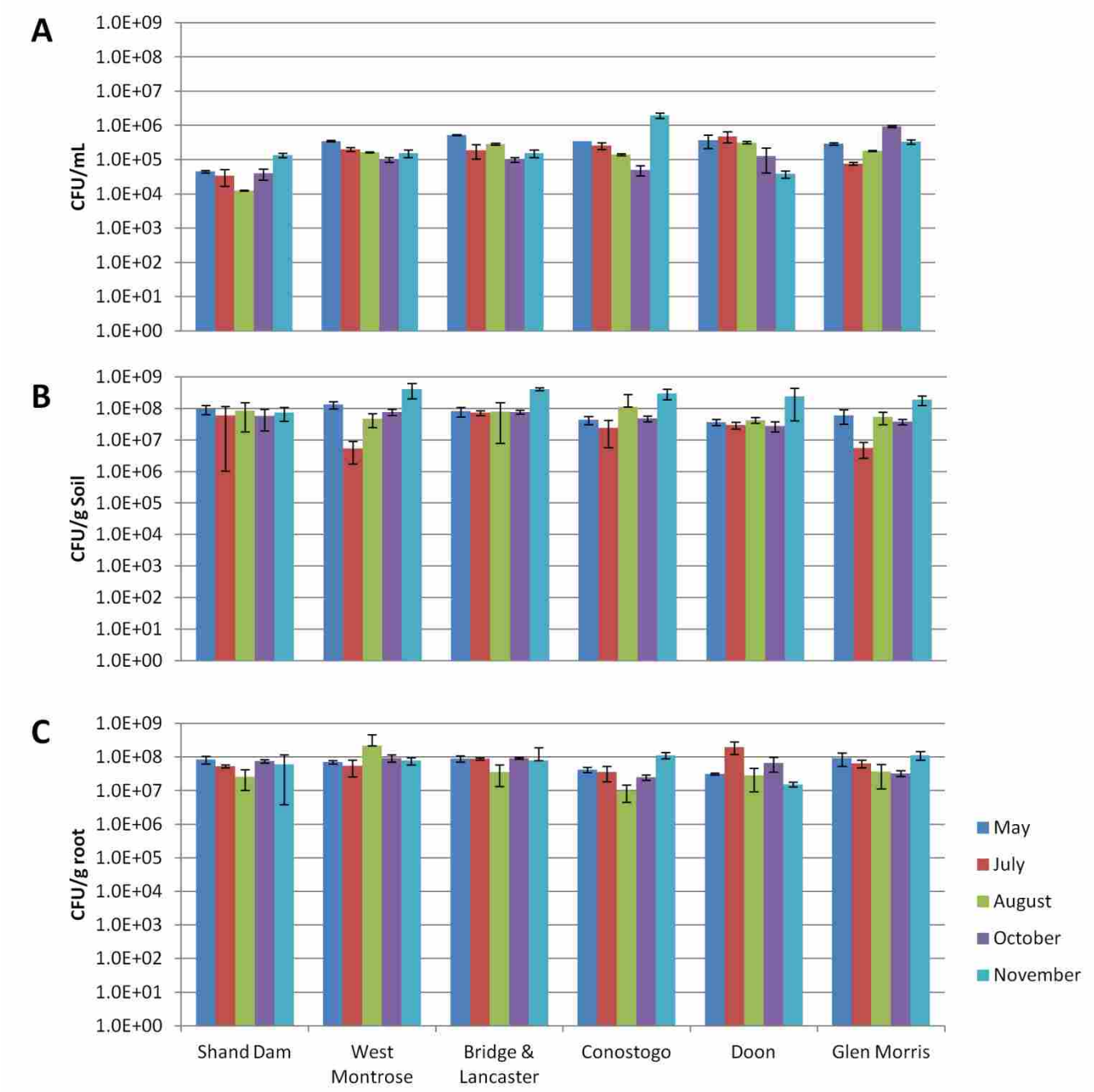

Figure 3.14 Colony Forming units per mL or gram of material obtained from river water (A), *P. arundinacea* rhizosphere soil (B) or roots (C) grown on R2A media at room temperature for 120 hours. Bars represent the average of three technical replicates (A only) and three experimental replicates from three quadrats within a site (B and C only). Error bars represent standard error from technical replicates (A only) or experimental replicates (B and C).

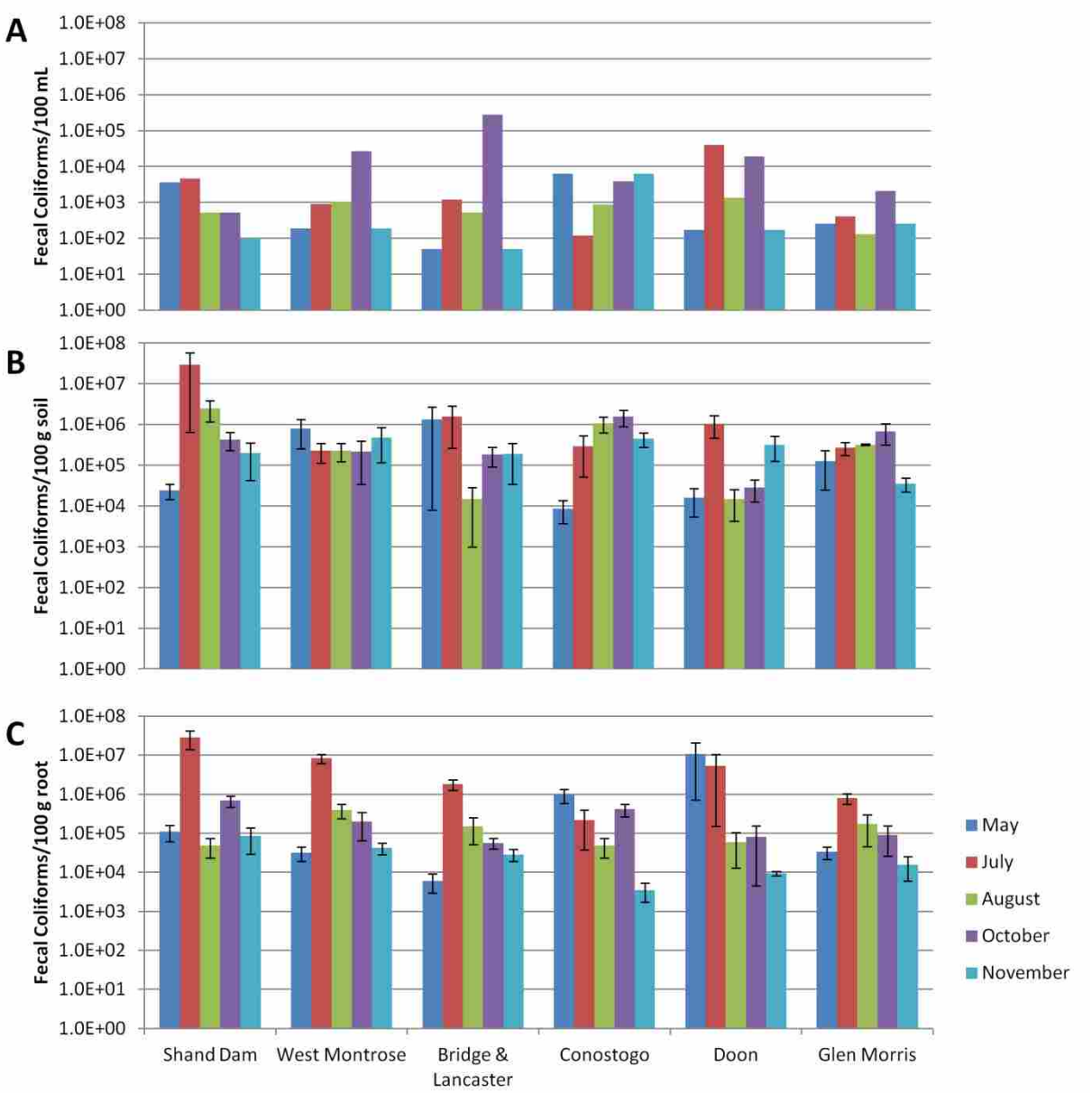

Figure 3.15 Fecal Coliforms per 100 mL or gram of material obtained from river water (A), *P. arundinacea* rhizosphere soil (B) or roots (C) grown on m-FC media. Bars represent the average of three experimental replicates from three quadrats within a site (B and C only). Error bars represent standard error from experimental replicates (B and C).

3.3.4.2 Molecular-Based Methods

Molecular detection of fecal indicator organisms in river water at each of the six different water quality sites identified several trends related to seasonality. Although the quantity of *Salmonella* genetic material did not differ significantly among sites, each site exhibited the same seasonal trends in organism abundance, with lowest levels being observed in May and August, and increases occurring in July, October and November. *Salmonella* detection was highest in November across all sites (Fig 3.16A). Similarly, *E. coli* abundance was not significantly different across the sites, but each site exhibited the same seasonal trends in detection with highest abundance occurring in August and October. Similar quantities of *E. coli* were detected in May, July and November at most sites, which were approximately 5-6 orders of magnitude lower than peak abundance (Fig 3.16D). The detection of *Enterococcus* in the river water communities was less consistent than with the other two organisms. *Enterococcus* was most commonly detected at the Shand Dam and Doon sites (all sampling events), and least likely to be detected at Bridge and Lancaster, being observed at only 3 of the 5 sampling events (Fig 3.16G). *Enterococcus* was not present or present in levels below detection limits in 75% of the sampling sites in October, and tended to be present in higher quantities during July and November.

Molecular detection of *Salmonella* genetic material in the rhizosphere of *P. arundinacea* indicated trends towards highest abundance at the Bridge and Lancaster site as compared to the other sampling locations, which exhibited comparable quantities (Fig 3.16B). Similar to observations made from the water communities, *Salmonella* tended to be detected in higher abundances in November. Lowest detection levels were observed in May and October. *E. coli* abundances were similar across all sampling sites, with seasonal trends in abundance observed across all locations (Fig 3.16E). *E. coli* was consistently detected in higher amounts during

sampling in August and October, while November exhibited the lowest abundance in the rhizosphere of *P. arundinacea. Enterococcus* was present in the rhizosphere more frequently than it was detected in the water (Fig 3.16H). Detection was most consistent at the Conestogo River site, and least frequent at Doon, which is perplexing as *Enterococcus* was consistently found in the water at the Doon site during all sampling events (Fig 3.16H). Abundance was lowest during August and October and comparable at the May, July and November sampling events.

Salmonella was consistently detected in the rhizoplane microbial community of *P. arundinacea* (Fig 3.16C). Similar quantities of *Salmonella* were detected at each of the six sampling locations. Seasonal variation in abundance was similar among the sampling sites and was similar to observations in the rhizosphere, with higher quantities of *Salmonella* genetic material in November and lower quantities in May and October. Trends in *E. coli* abundance were similar across the six sampling sites and seasonal trends were reflective of those from water and rhizosphere communities (Fig 3.16F). *E. coli* abundance was highest in August and October and lowest in November. *Enterococcus* was detected more consistently in the rhizoplane than in either of the other 2 community types (Fig 3.16I). There were no significant differences among the six sampling locations in terms of *Enterococcus* and seasonal variations were inconsistent.

Across all three community types, *Salmonella* was detected in the lowest quantities, compared to either *E. coli,* which was the most abundance, or *Enterococcus. Enterococcus* was typically present in higher quantities than *Salmonella* when detected, but was far less consistently present in the samples. Comparable quantities of all three indicator organisms were detected in the rhizosphere and rhizoplane, while water samples exhibited moderately reduced loads.

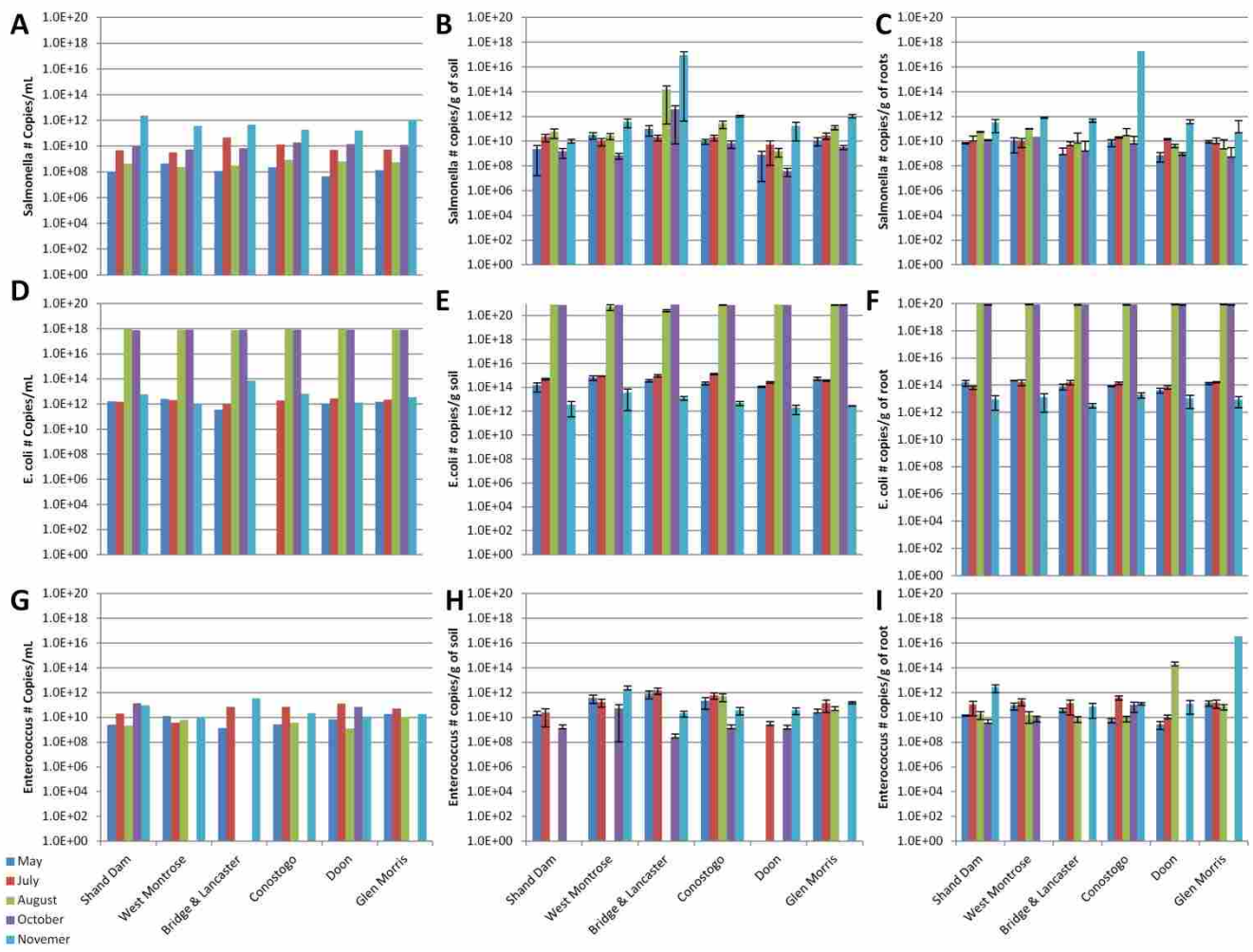

Figure 3.16 Average relative abundance (target gene copies/mL) of bacterial pathogens (*Salmonella* (A-C), *E. coli* (D-F) and *Enterococcus* (G-I)) obtained from samples associated with Grand River water (A, D, G), and *P. arundinacea* rhizosphere (B, E, H) and rhizoplane (C, F, I) material obtained at sampling sites during the 2015 sampling period. Bars are the average of three samples obtained from each site (except for water samples where n=1). Error bars represent standard error of three samples.

3.4 Discussion

We hypothesized that water quality would influence the structure and function of microbial communities associated with wetland plants (specifically *P. arundinacea*) growing along a water quality gradient. If our hypothesis was correct we would have expected to see microbial communities from comparable water quality sites exhibiting similar structural community profiles and similar carbon source utilization patterns as measured by Biolog™ EcoPlates. We did not observe this effect to the same extent that we had expected. Instead we found that the primary driver of community structure and function was sample type. Structural and functional community profiles were consistently grouped according to whether the community was from the river water, rhizosphere or rhizoplane. Water quality, and/or site specific effects were observed within the structural profiles of the rhizosphere during some sampling events, but these patterns were mostly absent from the carbon source utilization profiles. Furthermore, pathogen presence in these communities was not influenced by water quality, with communities from all six sites exhibiting similar quantities and seasonal trends. Thus we can conclude that community habitat type and rhizo-compartment, is a stronger driver of community structure and function than water quality in the case of *P. arundinacea.* Furthermore, water quality may be a lesser factor influencing community structure under some circumstances.

Structural analysis of microbial communities from the water, rhizosphere and rhizoplane of *P. arundinacea* at each of the six different water quality sites along the Grand River, indicated that community habitat type was the most important influence driving community structure. These findings agree with the limited other studies that have compared microbial community structures across rhizo-compartments under various environmental

conditions (Edwards *et al.*, 2014; Santos-Medellín *et al.*, 2017; Wang *et al.*, 2017; Xiao *et al.*, 2017; Wu *et al.*, 2018). Microbial community structure also appeared to be influenced by site location and water quality, to a lesser extent, among some communities. Structural similarity among samples obtained from sites of similar water qualities was most predominant in the rhizosphere, and least in the water and rhizoplane communities. In the rhizosphere, samples taken from the same site typically clustered together exhibiting similar community structural profiles. These clusters sometimes correlated with water quality classifications, especially during the sampling events in July, October and November. Rhizoplane communities exhibited a fair degree of variability and did not always cluster together by site, and never by water quality classification. Similarly, water community structures did not exhibit similarity by water quality designation, despite the fact that sites sharing similar water quality designations were directly up or downstream from each-other and closest in proximity.

It is possible that we observed a water quality dependent effect on the microbial community structure in the rhizosphere but not in the rhizoplane of *P. arundinacea*, because the community in the rhizoplane was being primarily shaped by the plant. The rhizoplane microbial community is directly attached to the plant root via biofilm and should be more strongly influenced by plant processes than rhizosphere communities in the adjacent soil as plant specific effects occur in a gradient starting at the plant root surface and emanating outwards (Vymazal *et al.*, 2007; Hartmann *et al.*, 2009). Thus, if the physiology of the plant is not being affected by the site specific characteristics (including water quality), the rhizoplane microbial community structure may be unaffected by the water conditions at the site. Unfortunately, it is impossible to say if the difference in microbial community response by rhizo-compartment is common among other study types and for other environmental influences, due to the fact that most studies focus

solely on the rhizosphere community. Many studies have found site specific influences in the rhizosphere community of different plant species. These differences have been attributed to soil type (Marschner *et al.,* 2001, Berg and Smalla, 2009; Edwards *et al.,* 2015), soil cultivation practices (Edwards *et al.,* 2015; Wu *et al.,* 2018) and degree of disturbance and anthropogenic impact (Ravit *et al.,* 2003).

The similarity between microbial community structures from *P. arundinacea* rhizosphere samples at different water quality sites was dependent on the sampling date. The correlation between site water quality designation and rhizosphere community structure was the strongest in July and October. We believe that this is because of the specific water chemistry at those sites during these sampling times. During the sampling events in July and October, our water chemistry measurements exhibited a strong linear gradient in several water quality parameters from high to low water quality sites, that was not as pronounced (or absent) during other sampling events. In July, water temperature, pH, dissolved oxygen, total dissolved solids and conductivity showed an increasing trend from Glen Morris to Shand Dam (Glen Morris/Doon>Bridge and Lancaster/Conestogo River>West Montrose/Shand Dam) (Table A5 and A6). Similarly, this water chemistry gradient was also strong during the sampling event in October for dissolved oxygen, total dissolved solids, conductivity and nitrate/nitrite concentrations. The water quality characteristics used to designate these sites as either high, moderate or low water quality were based on average water quality measurements over a period of years, thus, variability in measurements within a site on a monthly basis would not be uncommon (Loomer and Cooke, 2011). Since we observed a correlation between the strength of the water chemistry differences between the sites and the degree of water quality influence on rhizosphere community structure, water quality does in fact influence community structure

within the rhizosphere. However, due to fluctuating conditions over time, which are typical of any natural system, these influences may or may not be apparent at any given time depending on the magnitude of differences in chemical parameters among the study sites. Interestingly, although site water quality classifications were based on the level of inorganic nutrient impact, the concentrations of nitrate, nitrite, ammonia and phosphate did not often exhibit the same gradient as the other chemical parameters that we measured. For example, ammonia concentrations were significantly elevated at Doon (poor water quality), but comparable among the other sites. Nitrate levels tended to be highest at the intermediate water quality sites and Glen Morris. Nitrite concentrations were comparable among most of the sites, but elevated at Bridge and Lancaster and Doon, and phosphate concentrations tended to follow the established water quality gradient from Shand Dam to Doon, but concentrations at Glen Morris were more comparable to the good water quality sites. As such, it is likely that the other water chemistry parameters we measured such as pH, dissolved oxygen and conductivity played a larger role in structuring the rhizosphere community profiles we observed than the nutrient quality at the sites. pH has been established as a significant driver of microbial community structure within various habitat types including river sediments (Xiong *et al.,* 2012; Liu *et al.,* 2015), soil (Lauber *et al.,* 2009; Rousk *et al.,* 2010) and freshwater lakes (Lindström *et al.,* 2005). Dissolved oxygen has been identified as a factor influencing microbial community structure in sequence batch reactors (Guo *et al.,* 2009). Additionally, dissolved oxygen has been established as a driving factor in the structure of denitrifying microbial communities (Desnues *et al.* 2007; Knapp *et al.* 2009; Graham *et al.* 2010). Furthermore, in a study by Hollister *et al.,* (2010) the concentration of dissolved oxygen in the lake soils and sediments was found to be significantly correlated with microbial community structure. The role of electrical conductivity in influencing microbial community

structure has not been established in the literature; however, there is some evidence that electrical conductivity may influence Archaea community characteristics within soil and sediment environments (Hollister *et al.,* 2010).

Community functional characteristics were established based on sole carbon source utilization patterns for the three different community types at each of the six water quality sites. There was a strong association between microbial community habitat type and carbon source utilization profiles. As with community structural profiles, habitat type seems to be the dominant driver of community function, independent of water quality characteristics or seasonal variations. We found distinct differences in the types of carbon sources preferred by each of the different community types. Specifically, water microbial communities exhibited a preference for polymers and carbohydrates, while rhizosphere communities predominantly preferred phenolic compounds, amines, amino acids and carboxylic acids and rhizoplane communities exhibited a preference for phenolic compounds, amines and amino acids (a less diverse subset of the preferred substrates from the rhizosphere community). Carboxylic acids and amino acids were also identified as the preferred carbon substrates for microbial communities associated with subsurface soil environments while groundwater and surface soil microbial communities exhibited a preference for carbohydrates in a study carried out by Lehman *et al.* (1995). Carboxylic acids and carbohydrates were also identified as important carbon sources during sole carbon source utilization analysis of microbial communities from grassland soils (Zak *et al.,* 1994). Guanghua *et al.* (2008) found that soil microbial communities preferred amino acids and carboxylic acids, while the addition of chemical fertilizers to the community caused a shift to preference for carboxylic acids, carbohydrates and polymers. Alternatively, Rutgers *et al.* (2016) found a correlation between sole carbon substrate utilization preferences of microbial

communities of different soil types and land use patterns. Amino acid usage was positively correlated with the sand content of the soil, and arable land was associated with the usage of carboxylic acids. Rhizosphere microbial communities from a constructed wetland containing combinations of up to sixteen different species of wetland plants were associated with strong usage of polymers, amines, carbohydrates and amino acids when compared to microbial communities in unplanted controls (Zhang *et al.,* 2010). Furthermore, in a study by Osem *et al.* (2007) which looked at rhizoplane (*Typha domingensis* and *Cyperus alopcuroides*) microbial community carbon source utilization profiles within a wastewater treatment reactor, they found that rhizoplane communities exhibited a preference for utilization of 4-hydroxybenzoic acid, D-malic acid, L-asparagine and phenylethyl amine compared to microbial biofilm communities formed on particulate support medium. Interestingly, our rhizoplane communities of *P. arduninacea* also exhibited a consistent preference towards phenolic compounds, amino acids and amines (2-hydroxybenzoic acid, L-serine and phenylethyl amine), so the association with plant roots may be driving some of these community preferences in carbon source utilization profiles. Plant root exudates, which serve as a significant carbon source for microorganisms living in association with plant roots, typically consist of simple sugars in combination with organic acids, amino acids and phenolics (Walker *et al.,* 2003).; as such, it makes sense that we would see increased usage of these types of carbon sources by plant associated microbial communities. Thus soil characteristics, inorganic nutrient loading, and association with plants appear to influence microbial carbon source utilization profiles, and amino acids and carboxylic acids seem to be commonly used substrates among most microbial communities in association with either plant roots or soil. In reference to aquatic based microbial communities, in a study by Choi and Dobbs (1999) carbon source utilization patterns using Biolog^{TM} EcoPlates were

examined for water-based microbial communities and it was shown that differential use of polymers, carbohydrates and carboxylic acids distinguished among freshwater, groundwater and saltwater communities. Similarly, Lyons and Dobbs (2012) examined carbon source utilization profiles for heterotrophic bacterial communities associated with river water and found that these communities were primarily associated with usage of carbohydrates and polymers, specifically Tween 40 and pyruvic acid methyl ester. Interestingly, when these communities were compared to those of organic aggregate-associated heterotrophic microbial communities they found that the primary difference among these two groups was usage of nitrogen containing carbon sources, which were utilized by the aggregate associated communities to a significantly higher extent than the water associated communities. The water associated bacterial communities from our study share these common carbon source utilization profile characteristics, with a preference for usage of carbohydrates and polymers as carbon sources over nitrogen containing compounds, suggesting that these community characteristics may be common among various water associated microbial communities. The findings from Lyons and Dobbs (2012) suggest that these characteristics may be in part, related to the lifestyle of these microorganisms, compared to those in the soil, sediment and root communities which form complex attached biofilms associated with organic matter.

Despite some evidence for site specific and water quality associated effects on microbial community structure, especially during specific sampling events, such effects were not present in the functional community profiles during the same time period. Although the relationship between microbial structure, structural diversity and community function are currently poorly understood, microbial community functional characteristics are generally thought to reflect community structure (Torsvik and Øvreås, 2002). Some examples of structural

community characteristics being reflected in community function include a study by Yu *et al.,* (2015) which examined the effects of long term fertilizer application on the structural and functional diversity of microbial communities in the rhizosphere of mulberry (*Morus* spp.). They found that soil microbial communities associated with fertilizer treatment consisting of organic and inorganic nutrient additions differed significantly from non-fertilized microbial communities in bacterial abundance and structural diversity measurements. Furthermore, the microbial community associated with the fertilizer treatment exhibited unique carbon source utilization profile characteristics including a greater utilization of carbohydrates and carboxylic acids than the soil community without fertilizer addition. Metabolic diversity calculated from carbon source utilization data obtained from Biolog^{TM} analysis indicated that the soil community receiving the fertilizer treatment also had higher metabolic diversity. However, this is not always the case, as seen in a study by Marschner *et al.,* (2003), which examined the effects of long term organic and inorganic nutrient enrichment on microbial soil communities, and found that community structural characteristics were distinct between the various types of nutrient additions as reflected by DGGE analysis. However, these structural changes were not accompanied by discernible functional community changes as measured by altered microbial enzyme activities (arylsulfatase, protease and phosphatase). In another example, O'Donnell *et al.,* (2001) explored the relationship between soil microbial community structural and functional characteristics in response to the addition of fertilizers and found that differences in structural profiles from soil communities receiving various inorganic nutrient amendments were not reflected in the Biolog^{TM} EcoPlate carbon source utilization profiles observed from the same communities. So as you can see, the literature is full of conflicting examples of how well observed trends in structural community characteristics are mirrored in functional community measurements. Part of this

disagreement can likely be attributed to how well genetic studies can actually predict ecosystem functions. The presence and abundance of particular organisms or operational taxonomic units (OTUs) within a microbial community may not be a reliable indicator of community functional traits (Fuhrman, 2009). Many genes are only expressed by a particular organism under a specific set of circumstances which may or may not be present in the environment of interest (Zehr and Ward, 2002). As well, a closely related group of organisms can have extremely different genomes (Cohan, 2002). Furthermore, different methodologies can yield extremely different results which influence how we interpret community level characteristics. For example, in one of the studies cited above, Marschner *et al.* (2003), the influence of fertilizer addition on soil community structural changes were assessed using both DGGE and phospholipid fatty acid (PLFA) profile analysis. Both techniques are commonly used to assess changes in microbial community structures; while DGGE examines community fingerprints by sorting microbial genetic material by the guanine-cytosine content of a PCR amplified fragment of DNA, PLFA profiles look at the relative abundance of different fatty acid methyl-esters via chromatography, as different groups of microorganisms have different fatty acid signatures (Tunlid and White, 1992). Marschner *et al.* (2003) found significant differences among soil microbial community structural profiles receiving different fertilizer treatments when community structure was assessed using DGGE, but not with PLFA analysis. Additionally, the Biolog^{TM} method of measuring functional characteristics of microbial communities has been criticized for having the same limitations that all culturing techniques do, in that it only reflects the activity of fast growing eutrophic microorganisms while the organisms with slow growth and more complex nutritional and environmental growth requirements will not be reflected (Konopka *et al.*, 1998). As such, only a fraction of the total microbial community will be represent in the results, making

it easy to overlook potential changes in functional community characteristics carried out by the uncultured portion of the microbial community that would be represented in the structural profile but missed by Biolog^{TM} analysis.

A common indicator of water quality is the presence of potentially harmful human pathogenic microorganisms, or indicator microorganisms, which signify fecal contamination (Maybeck *et al.,* 1989; Jamieson *et al.,* 2004; Harwood *et al.,* 2005). Surprisingly, despite differences in the degree (and type) of anthropogenic impact at each of the different study sites, we did not identify any differences among the sites in terms of the quantity of fecal coliform bacteria detected through m-FC culturing methods or the molecular detection of indicator microorganisms (*Salmonella, E. coli, Enterococcus*). There were no consistent differences among the sites across all seasons, and seasonal trends in detection or abundance of relevant organisms followed similar trends at all sites. This is perplexing as theoretically the poor water quality sites, impacted by effluent from multiple waste water treatment plants, should exhibit elevated quantities of these microorganisms. However, it is important to also consider alternate sources of fecal contamination, such as agricultural sources and wildlife (e.g. birds) (e.g. Edge and Hill, 2005). Surprisingly, multiple incidences reported in the literature suggest that the correlation between quantities of fecal indicator bacteria in environmental samples and actual fecal contamination may not be as strong as we had previously assumed. In a study by Litton *et al.* (2010) markers of fecal contamination were evaluated at multiple distances (up to 550 m) from the discharge site of a WWTP (domestic and industrial waste water) into a river in southern California which receives no other runoff or discharge during dry weather. The study found that concentrations of fecal indicator bacteria, using culture dependent and independent methods, actually increased in water column and river sediment samples as distance from the discharge

site increased. Litton *et al.* (2010) concluded that the source of the microorganisms was actually from *in situ* growth in streambed sediments and not from wastewater effluent. Similarly, in an experiment by Drummond *et al.* (2014) where synthetic effluent containing *E. coli* was injected into a freshwater stream simulating point source contamination, they found that the majority of the *E. coli* injected into the stream was retained over long term sampling (several months) within the top 3 cm of the streambed sediment and among submerged macrophytes stands. They also noted that there was a reservoir for *E. coli* within the streambed where *E. coli* was retained and re-suspended into the water column continually, causing *E. coli* to be detected in quantities higher than those present at the site of effluent injection. There have been several additional studies that have noted the persistence and re-establishment of microorganisms associated with fecal contamination in soil (Van Donsel *et al.,* 1967) and sediment (Howell *et al.,* 1996) ecosystems. As such, the detection of fecal coliforms and indicator microorganisms in soil and water ecosystems may not always be associated with recent fecal contamination. There is an increased need to understand the nature of fecal indicator bacteria from natural sources (Jamieson *et al.,* 2004), as fecal bacteria have been detected in quantities as high as $2x10^3$ CFU/g of soil and $5x10^2$ CFU/mL of runoff water collected from sites not receiving any known source of fecal contamination (Entry *et al.,* 2000a, Entry *et al.,* 2000b).

Several interesting trends were noted throughout the sampling period, across the different sites, with regards to seasonal abundance of the different pathogenic indicator bacteria. Similar seasonal variations in organism abundance were noted among the different sampling sites along the Grand River. In particular, the molecular detection of *Salmonella* indicated significantly higher quantities in the water column, rhizosphere and rhizoplane during November sampling events. The increased prevalence of an organism typically associated with optimal growth

temperatures between 35 and 36 °C, and a thermal niche of 28-40 °C, during the colder season's sounds abnormal, however, several studies have duplicated this seasonal trend when isolating *Salmonella* from natural environments (Bronikowski *et al.,* 2001). Thomas (2011) isolated *Salmonella* from surface water environments using culturing techniques and found that detection rates were highest in November, December and July. The serotypes which were most commonly detected during the colder seasons included Typhimurium, Infantis and Agona during the fall (September to November) and Montevideo and Hadar during the winter (December to February). In a study by Rhodes and Kator (1988) that examined the survival of *Salmonella* under environmental conditions related to different seasons found that the survival of *Salmonella* was negatively influenced by predation and antagonistic interactions with the resident microorganisms at warmer temperatures. In contrast, *E. coli* abundance was greatest at all sites and in all sample types between August and October. Ishii *et al.* (2007) found a similar pattern in the seasonal abundance of *E. coli,* with the quantity of *E.coli* being detected in samples of sand and sediment from Lake Superior increasing during the summer and early fall seasons. Compared to *Salmonella, E. coli* has a poor tolerance for lower temperatures despite having a similar optimal growth temperature (35-36°C) and thermal niche (29-41 °C) (Bronikowski *et al.,* 2001). Rhodes and Kator (1988) found that the recovery of *E. coli* from river water was significantly reduced at temperatures below 10°C, with less than 6% of the inoculated *E. coli* being recovered after 1 week in these conditions, compared to 83-100% for *Salmonella.* This would explain why we observed the lowest quantities of *E. coli* in our samples during the months that correlated with the coolest water temperatures (May and November). Alternatively, *Enterococcus* did not exhibit the same type of consistent seasonal trends among sampling sites and sample types as the other two microorganisms. Unlike *Salmonella* and *E. coli, Enterococcus*

was not detected during all sampling events or at all sites during an individual sampling event. Detection was most consistent and typically in higher quantities during sampling events in July and November, and within the rhizoplane. Various studies of *Enterococcus* under freshwater conditions have indicated that warm temperatures may lead to quicker die-off of these microorganisms (Sinton *et al.*, 2002; Noble *et al.*, 2004; Jenkins *et al.*, 2011). Wajugi and Harwood (2012) found that the persistence of *Enterococcus* was negatively impacted by competition and predation from the natural microbiota present in freshwater and sediment environments at room temperature. *Enterococcus* has been shown to grow at temperatures ranging from 5 to 45 °C, thus decreased survival at higher temperatures may be an effect of increased competition and predation (Zanoni *et al.*, 1993; Byappanahalli *et al.* 2012). We found that Enterococci were most common in the rhizoplane, which could suggest that they compete better in this environment compared to either the soil (rhizosphere) or the water column. *Enterococcus* has been isolated from the surface of plant structures belonging to over 47 different taxa (Mundt, 1963). Furthermore, *Enterococcus* was identified as composing a significant portion of the rhizoplane microbiome of another species of grass (Para grass, *Urochloa mutica*) (Mukhtar *et al.*, 2016). Thus, the rhizoplane may act as an environmental reservoir for *Enterococcus* spp., but further study including a greater diversity of plant species would be needed to confirm this.

It is also worthy of note that all fecal coliforms, on both m-FC media and *Salmonella* and *E. coli* with molecular detection methods, were present in greater numbers within the rhizosphere and rhizoplane compared to the water column. Culturable fecal coliforms isolated on m-FC media were more abundant within the rhizosphere and the rhizoplane by approximately two orders of magnitude. Comparatively, quantitative detection of *E. coli and Salmonella,* showed an

increase in abundance within the rhizosphere and rhizoplane by an average of three orders of magnitude and four orders of magnitude respectively. As previously mentioned, it has been established in the literature that fecal coliforms are capable of persistence and growth within soil and sediment environments (e.g. Litton *et al.,* 2010; Drummond *et al.,* 2014). However, association with plants presents another set of environmental conditions that may provide a favorable environment for these microorganisms. *Salmonella* have been found occurring naturally (i.e. not through accidental contamination or experimental introduction) within the rhizosphere of several different plant species including strawberry (Berg *et al.,* 2002), oilseed rape (Berg *et al.,* 2002), wheat (Germida and Siciliano, 2001) and common bean (Maougal *et al.,* 2014). Furthermore, *E. coli* isolated from various natural soil environments have actually been shown to alter rhizosphere microbial community characteristics in maize and show plant-growth promoting properties (Nautiyal *et al.,* 2010). Thus, the view of these organisms as primarily limited to reproduction within animal host environments is changing, and natural environments represent more than just vehicles for dissemination of these microorganisms.

3.5 Conclusions

Structural and functional community profiles of microorganisms associated with water, rhizosphere and rhizoplane environments from *P. arundinacea* were strongly influenced by habitat type and not significantly influenced by water quality at our six sites along the Grand River. Site dependent effects associated with GRCA site water quality classifications influenced microbial structural community profiles to a limited extent but only in the rhizosphere during certain sampling time points. This may be the result of other gradients in water chemistry across the sampling sites and not due to water quality related factors. We did not identify an obvious and consistent difference among our water quality sites in terms of indicators of fecal

contamination. We found a consistently higher number of fecal coliforms within the rhizosphere and rhizoplane of *P. arundinacea* compared to water column measurements. We believe that this may be due to naturalization of these microorganisms within the plant root-soil environment.

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Chapter 4

Site-Specific Differences in Microbial Community Structure and Function within the Rhizoplane and Rhizosphere of Wetland Plants is Plant Species-Dependent

4.1 Introduction

Increasing urbanization and pollution related to anthropogenic activities pose a serious threat to the health of our freshwater ecosystems (Tilman *et al.,* 2001; Hautier *et al.,* 2009; Hooper *et al.,* 2012; Mekonnen and Hoekstra, 2015). Wetlands play an integral role in preventing the degradation of freshwater ecosystem health (Coveney *et al.,* 2002). A combination of biological, chemical and physical processes occurring within wetlands facilitates their remedial functions (Sheoran and Sheoran, 2006). Wetland plants play a vital role in the ability of natural and constructed wetland systems to improve water quality. Experimentation with constructed wetland systems has underlined the importance of plants in improving wetland efficiency, despite the fact that the majority of contaminant removal is attributed to microbial activity and physical/chemical processes occurring within the wetland substrate (Tanner *et al.,* 1995; Brix, 1997; Stottmeister *et al.,* 2003; Vymazal, 2007; Read *et al.,* 2008; Brisson and Chazarenc, 2009). In addition to providing physical stability of wetland substrates, insulation during winter frosts and reduction in water current velocities, plant root systems increase the efficiency of wetlands by providing unique microhabitats for soil microflora (Brix, 1997; Stottmeister *et al.,* 2003; Brisson and Chazarenc, 2009). These microhabitats associated with the biological processes occurring within plant root systems provide niche environments suited to many different groups of microorganisms facilitating the rapid cycling of nutrients (Brix, 1997; Stottmeister *et al.,* 2003; Vymazal, 2007). The area of soil surrounding plant root systems that is

actively influenced by the physiological processes occurring within the plant is called the rhizosphere. Plant roots also provide surfaces for attachment of microbial biofilms, forming a community called the rhizoplane. Rhizosphere and rhizoplane communities are shaped by plant-mediated changes in environmental conditions such as gradients in oxygen, pH, carbon/nitrogen and water availability (e.g. Philippot *et al.,* 2013).

The biggest risk to human health associated with water quality is the presence of disease causing microorganisms (Chapman, 1996). Some of the most significant human pathogens originating from fecal contamination in freshwater systems include *Vibrio* spp., *Shigella* spp., *Salmonella* spp., *Enterococcus* spp. and *Escherichia coli* (Cabral, 2010). In addition to providing a means to improve the chemical composition of water (e.g. removal of inorganic nitrogen, phosphorus and heavy metals) wetlands have also been implicated in reducing the pathogen load of contaminated water. Studies that have observed the efficiency of fecal indicator microorganism reduction through wetland treatment ranges from 90 to 99% reduction which is comparable to traditional treatment methods used to remediate pathogen contaminated wastewater (Miescier and Cabelli, 1982; Wolverton, 1989; Watson *et al.,* 1990).

A great deal of interest has arisen with respect to the selection of different plant species for use in constructed wetlands in order to optimize efficiency (Brisson and Chazarenc, 2009). Structural and functional differences in rhizosphere and rhizoplane microbial communities among different plant species has been established in the literature (Garland, 1996; Grayston *et al.,* 1998; Marschner *et al.,* 2001; Wieland *et al.,* 2001 Marschner *et al.,* 2004). Thus it is not surprising that differences in contaminant removal processes have been observed when comparing constructed wetlands planted with different species (Coleman *et al.,* 2001; Stein *et al.,* 2006; Inamori *et al.,* 2007; Read *et al.,* 2008; Brisson and Chazarenc, 2009; Bissegger *et al.,*

2014). For example two to four-fold differences among plant species were observed with respect to the ability of the associated wetland to reduce total suspended solids, total nitrogen, phosphorus and copper from storm water (Read *et al.*, 2008). Plant species differences in reductions of nitrous and nitric oxide, ammonia, manganese, lead and iron concentrations from wastewater effluents were 20-fold or more (Read *et al.*, 2008).

Understanding how wetland plants and root-associated microbial communities interact with water quality associated factors will lead to a greater understanding of the biological dynamics occurring within wetlands, how to optimize natural wetland restoration and how to increase constructed wetland efficiency. With these goals in mind, we tested the following hypotheses:

1) Natural wetland systems highly impacted by anthropogenic activities will differ from less affected systems in both microbial community composition and functional characteristics.
2) The microbial community composition associated with wetland plants will differ among species. Different plant species will harbour unique microbial communities with contrasting community compositions, functionality and responses to perturbations in water quality.

In order to test these hypotheses, we chose two sites with contrasting water quality characteristics and three wetland plant species occurring at both sites. We used PCR-denaturing gel gradient electrophoresis (DGGE) to look at structural differences between the rhizosphere and rhizoplane microbial communities associated with each plant species across the two sites. We established carbon source utilization profiles using Biolog^{TM} EcoPlates for each community type (rhizosphere, rhizoplane and water) associated with the different plant species at the two

sites to establish functional differences among microbial communities. A combination of culture and molecular based-methods were also employed to assess plant species and site-specific effects on fecal indictor bacteria and potentially pathogenic microorganisms associated with fecal contamination.

4.2 Materials and Methods

4.2.1 Sampling Design

Two sites were chosen along the Grand River to represent contrasting water quality. These included West Montrose (43.588219, -80.470979) and a site on the Rare Charitable Research Reserve (43.384513, -80.385331). From historical data provided by the Provincial Water Quality Monitoring Network (PWQMN - www.ontario.ca/data/provincial-stream-water-quality-monitoring-network), as well as water samples obtained during the field sampling season in spring and summer of 2015, we classified West Montrose as a high water quality site and Rare as a poor water quality site. These classifications were based primarily on level of anthropogenic impact and measured concentrations of inorganic nutrients (nitrogen, phosphorus). West Montrose was primarily impacted by agricultural land with a stream carrying agricultural runoff entering the main river about 50 m upstream and a residential trailer park located about 100 m downstream from the sampling location. The sampling site at Rare was located approximately 40 km downstream from the West Montrose site, passing through highly urbanized areas in Waterloo and Kitchener, ON. Between West Montrose and the Rare sampling site, the Grand River receives input from five municipal waste water treatment plants (WWTPs), with the discharge from the Kitchener WWTP entering the Grand River approximately 4 km upstream from the sampling location.

Three different wetland plant species occurring at both sampling locations were selected for this study, which included *Potamogeton natans, Veronica spicata* and *Iris versicolor*. *P. natans* is a perennial aquatic plant species belonging to the family Potamogetonaceae and is commonly found in calm freshwater habitats (plants.usda.gov). These plants produces both floating and submerged leaves attached to roots anchored in the sediment by long petioles. *V. spicata* belongs to the family Scrophulariaceae and is a perennial flowering herbaceous plant which is introduced to this region and not native (plants.usda.gov). *I. versicolor* is a perennial herbaceous wetland plant belonging to the family Iridaceae and native to this area (plants.usda.gov).

Samples of Grand River water, rhizosphere soil and plant roots were collected from both sites over two separate sampling events on October 23, 2015 and November 11, 2015. Samples were obtained from three separate quadrats at each site, using the same selection method as previously described in Chapter 3 Materials and Methods. This provided a total of three different samples from each plant species at each site, with one or two of each collected per sampling event. All samples were processed for microbiological culture-based (heterotrophic plate counts and m-FC), molecular-based (PCR-DGGE, qPCR) and functional (Biolog^{TM} EcoPlate) community assessments as previously described in Chapter 2. River water was also analyzed for water chemistry parameters using a YSI Professional Plus Multiparameter Instrument (YSI 1700/1725, YSI a Xylem Brand, Yellow Springs, Ohio USA) and a Pro Plus Quatro Field Cable (YSI a Xylem Brand, Yellow Springs, Ohio USA) for measuring water temperature, dissolved oxygen, conductivity, oxidation/reduction potential (ORP) and pH. As well, water samples were taken back to the lab to perform further assessment of inorganic nutrient concentrations using

HACH® reagents (nitrite, nitrate, ammonia and total reactive phosphorus). Complete methodology is described in Chapter 2.

4.3 Results

4.3.1 Structural Profiles of Microbial Communities Associated with *I. versicolor, P. natans* and *V. spicata* at a High and Low Water Quality Site

PCR-DGGE derived community structural profiles of microbial communities from water, rhizoplane and rhizosphere samples at West Montrose and Rare from *Potamogeton natans, Veronica spicata* and *Iris versicolor* shared 13% similarity among all sample types (Fig 4.1). Structural similarity between the water communities from West Montrose and Rare was 22%. Structural profiles did not cluster together based on plant species or sample type. Cluster analysis revealed several smaller sub-groupings of structural profiles which contained generally a mixture of microbial communities from different sample types and different plant species, but from the same sampling location (e.g. either West Montrose or Rare). Microbial communities from the Rare site formed three different sub-groupings, the first with the highest degree of structural similarity (88%) contained rhizosphere and rhizoplane communities from *I. versicolor* and *P. natans.* The second cluster shared 59% similarity and contained three community profiles from rhizosphere and rhizoplane communities associated with *V. spicata.* The third cluster only shared 36% structural similarity and contained rhizosphere and rhizoplane samples from all three plant species. The structural community profiles from West Montrose clustered into six smaller groups. The first had 78% similarity and contained microbial community structural profiles associated with *I. versicolor* rhizosphere and *P. natans* rhizoplane material. The second, third and fourth groupings with 71, 67 and 52% structural similarity, respectively, contained microbial community structural profiles associated with samples from *P. natans* rhizosphere and

rhizoplane, *P. natans* rhizoplane and *V. spicata* rhizoplane, respectively. The final two groupings had 48 and 61% similarity and contained *I. versicolor* rhizoplane and *V. spicata* rhizosphere microbial community profiles, respectively.

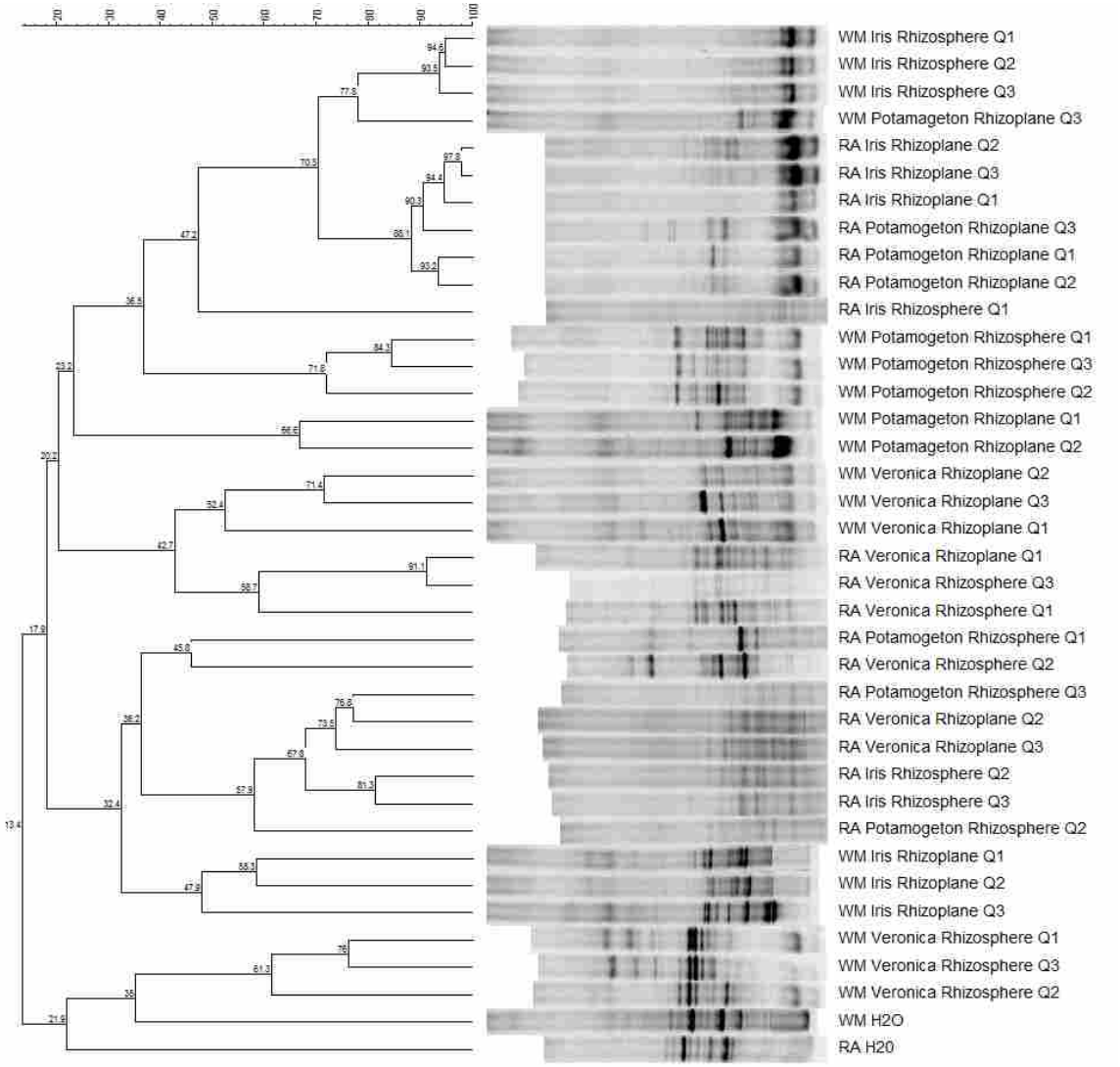

Figure 4.1 Hierarchical cluster analysis results of all DGGE profiles obtained from microbial DNA extracted from *Iris versicolor, Potamogeton natans* and *Veronica spicata* at two sites with contrasting water quality demonstrated graphically as a UPGMA dendrogram. West Montrose (WM) was the higher water quality site while Rare (RA) was the poor water quality site. Different sample types are indicated as H_2O (water), RS (rhizosphere) and rhizoplane (RP). Samples from different quadrats within a site are indicated with Q1-3. Numbers indicate percent similarity.

Cluster analysis of PCR-DGGE derived structural community profiles obtained from water microbial communities and rhizosphere and rhizoplane microbial communities associated with *I. versicolor* exhibited the greatest degree of structural similarity by sample location and sample type (Fig 4.2 A). All of the samples obtained from Rare shared 33% structural similarity and occurred within the same sub-grouping. West Montrose community profiles fell into two different clusters based on sample type (e.g. rhizosphere or rhizoplane community), which shared only 8% structural similarity. Rare rhizosphere microbial community profiles shared 33% similarity, while West Montrose rhizosphere community profiles shared 94% similarity. Rhizoplane communities from Rare exhibited 94% structural similarity, while rhizoplane communities from West Montrose shared 48% similarity.

Cluster analysis of PCR-DGGE derived structural community profiles obtained from water microbial communities and rhizosphere and rhizoplane microbial communities associated with *P. natans* from West Montrose and Rare formed clusters based on sample type and sample location (Fig 4.2 B). Rhizoplane samples from Rare exhibited the highest degree of structural similarity (90%). West Montrose rhizoplane microbial communities shared only 52% structural similarity. Rhizosphere microbial communities from West Montrose exhibited 72% similarity, while rhizosphere communities from Rare only exhibited 11% similarity. Rhizoplane microbial communities from Rare and rhizosphere microbial communities from West Montrose clustered together with 52% similarity, while rhizoplane communities from West Montrose and rhizosphere communities from Rare formed another cluster with 29% similarity.

Cluster analysis of PCR-DGGE derived structural community profiles obtained from water microbial communities and rhizosphere and rhizoplane microbial communities associated with *V. spicata* from West Montrose and Rare revealed clustering patterns based on sample type

as well as sample location (Fig 4.2 C). West Montrose rhizosphere samples shared 61% similarity and clustered with the West Montrose and Rare water microbial communities (35% and 22% similarity respectively). A second smaller cluster (24% similarity) contained three branches with structural community profiles from Rare rhizoplane and rhizosphere communities (33% similarity), West Montrose rhizoplane communities (52% similarity) and Rare rhizosphere and rhizoplane communities (59% similarity).

Within the West Montrose site, all rhizosphere and rhizoplane samples from *I. versicolor, P. natans* and *V. spicata* exhibited 8, 19 and 16% similarity respectively. Within the Rare site, all rhizosphere and rhizoplane samples from *I. versicolor, P. natans* and *V. spicata* exhibited 33, 11 and 23% similarity respectively. Similarity among samples obtained from the same site tended to be higher at Rare. Alternatively, the similarity among all microbial communities associated with *V. spicata* (16 %) was higher than for the other two plant species (*P. natans* 11%, *I. versicolor* 8%).

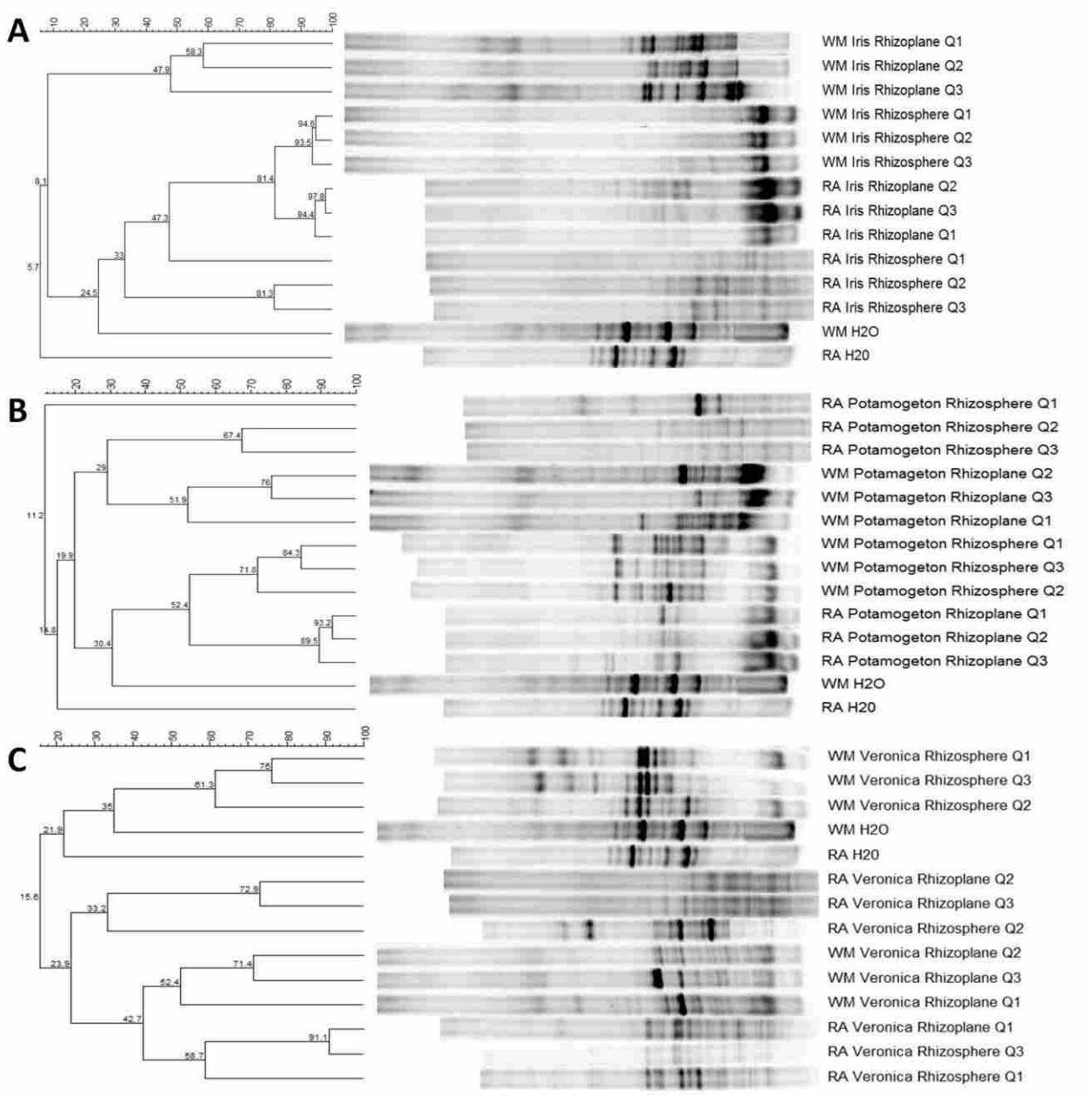

Figure 4.2 Hierarchical cluster analysis results for DGGE profiles obtained from microbial DNA extracted from (A) *Iris versicolor*, *(B) Potamogeton natans* and C) *Veronica spicata* at two sites with contrasting water quality demonstrated graphically as a UPGMA dendrogram. West Montrose (WM) was the higher water quality site while Rare (RA) was the poor water quality site. Different sample types are indicated as H_2O (water), RS (rhizosphere) and rhizoplane (RP). Samples from different quadrats within a site are indicated with Q1-3. Numbers indicate percent similarity.

4.3.2 Functional Community Profiles Associated with *I. versicolor, P. natans* and *V. spicata* at Two Sites with Contrasting Water Quality

4.3.2.1 Principal Component Analysis of Carbon Source Utilization by Rhizoplane, Rhizosphere and Water-Associated Microbial Communities from West Montrose and Rare

A PCA was performed on carbon source utilization profiles of microbial communities from water, rhizoplane and rhizosphere samples at West Montrose and Rare from *Potamogeton natans, Veronica spicata* and *Iris versicolor* (Fig 4.3). Carbon source utilization profiles for water samples from both sites were similar, each with negative PC1 and negative PC2 scores. Rhizoplane and rhizosphere samples from both sites and all plant species were separated from the water samples along the PC1 axis, primarily exhibiting positive PC1 scores. There was no visible distinction among the different plant species or between carbon source utilization profiles from the rhizosphere when compared to the rhizoplane. However, carbon source utilization profiles from West Montrose exhibited primarily positive PC2 scores, while microbial communities from Rare primarily exhibited negative PC2 scores. The top five carbon sources associated with positive PC1 scores included D-cellobiose, 4-hydroxybenzoic acid, L-asparagine, L-serine and putrescine (Table A7). The only carbon source exhibiting a negative factor loading for PC1 was Tween 40. Carbon sources contributing strongly to positive PC2 scores included Tween 40, Tween 80, D-mannitol, glucose-1-phosphate and L-threonine. Negative PC2 scores were associated with the carbon sources i-erythritol, 2-hydroxybenzoic acid, 4-hydroxybenzoic acid, itaconic acid and phenylethyl-amine.

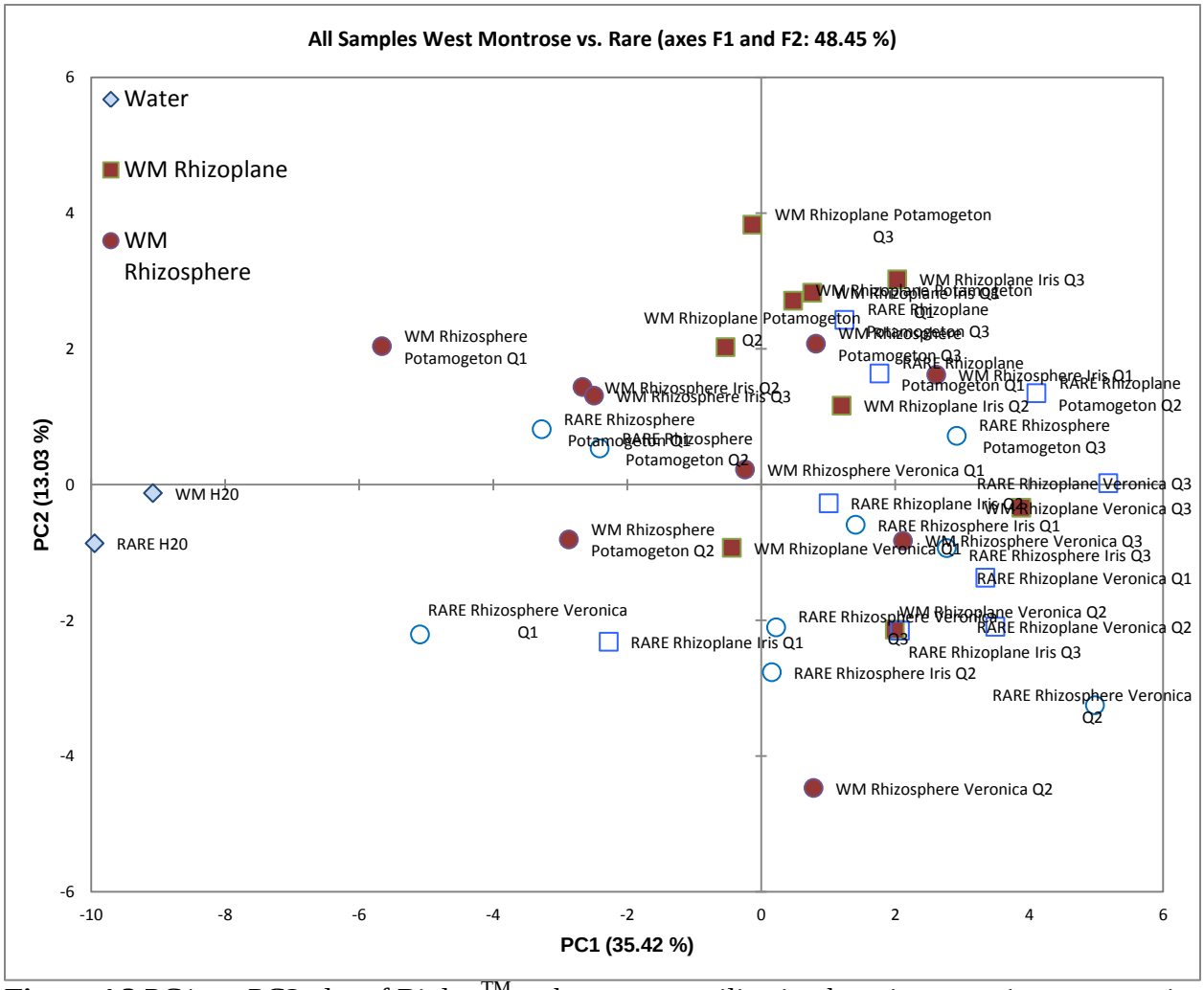

Figure 4.3 PC1 vs. PC2 plot of Biolog™ carbon source utilization by microorganisms present in all sample types obtained from *Iris versicolor*, *Potamogeton natans* and *Veronica spicata*. Samples were obtained from either West Montrose (WM) or Rare Charitable Research Reserve (RARE) during October and November, 2015. Samples from different quadrats within a site are indicated with Q1-3.

The PCA of microbial carbon source utilization profiles obtained from water, rhizosphere and rhizoplane microbial communities associated with *I. versicolor* exhibited the highest degree of differentiation between the two water quality sites (Fig 4.4 A). All sample types obtained from West Montrose exhibited positive PC2 scores while all sample types obtained from Rare exhibited negative PC2 scores. As in previous analyses, both water samples exhibited strong negative PC1 scores. Rhizoplane and rhizosphere microbial communities were separated from the water microbial communities along the PC1 axis, with these community types exhibiting PC1 scores, which were either negative or close to zero. *I. versicolor* microbial communities exhibited the least amount of differentiation between rhizosphere and rhizoplane communities of the plant species investigated, with no clear distinction between the carbon utilization profiles from the two different sample types (rhizo-compartments).

The PCA of microbial carbon source utilization profiles obtained from water, rhizosphere and rhizoplane microbial communities associated with *P. natans* at West Montrose and Rare exhibit distinct profiles both by sample type and by sample location (Fig 4.4 B). Carbon source utilization profiles from water communities at Rare and West Montrose were very similar, exhibiting negative PC1 and PC2 scores. Rhizosphere and rhizoplane community profiles from the two different water quality sites were primarily separated along the PC2 axis, with communities from West Montrose exhibiting primarily positive PC2 scores and communities from Rare exhibiting primarily negative PC2 scores. Rhizosphere microbial communities exhibited more differentiation between the two sampling locations than the rhizoplane microbial communities. Rhizoplane microbial communities from both sites were primarily associated with positive PC1 scores, while rhizosphere microbial communities had negative PC1 scores exclusively.

The PCA of microbial carbon source utilization profiles obtained from water, rhizosphere and rhizoplane microbial communities associated with *V. spicata* did distinguish between microbial communities obtained from the two different water quality locations but did exhibit differentiation by sample type (Fig 4.4 C). Water communities from West Montrose and Rare exhibited similar carbon source utilization profiles which were associated with negative PC1 and positive PC2 scores. Rhizosphere and rhizoplane microbial communities were primarily separated from water communities along the PC2 axis. Microbial communities established by West Montrose rhizoplane samples exhibited variable PC1 scores and negative PC2 scores. Rhizosphere communities from West Montrose were associated with positive PC1 and positive PC2 scores. Rare microbial communities isolated from rhizoplane samples had highly variable carbon source utilization profiles with either positive or negative PC1 and PC2 scores. Alternatively, Rare-associated microbial communities from the rhizosphere exhibited positive PC1 scores and either positive or negative PC2 scores. In general, rhizosphere communities tended to exhibit positive PC2 scores, while rhizoplane microbial communities exhibited primarily negative PC2 scores.

The water microbial communities were distinguished from the other community types primarily through their usage of the carbon source Tween 40 (Table A7). Furthermore, across the different plant species, rhizosphere and rhizoplane communities from West Montrose were consistently differentiated by usage of the carbon sources glucose-1-phsophate and Tween 40, while itaconic acid was consistently associated with microbial communities from the Rare sampling location.

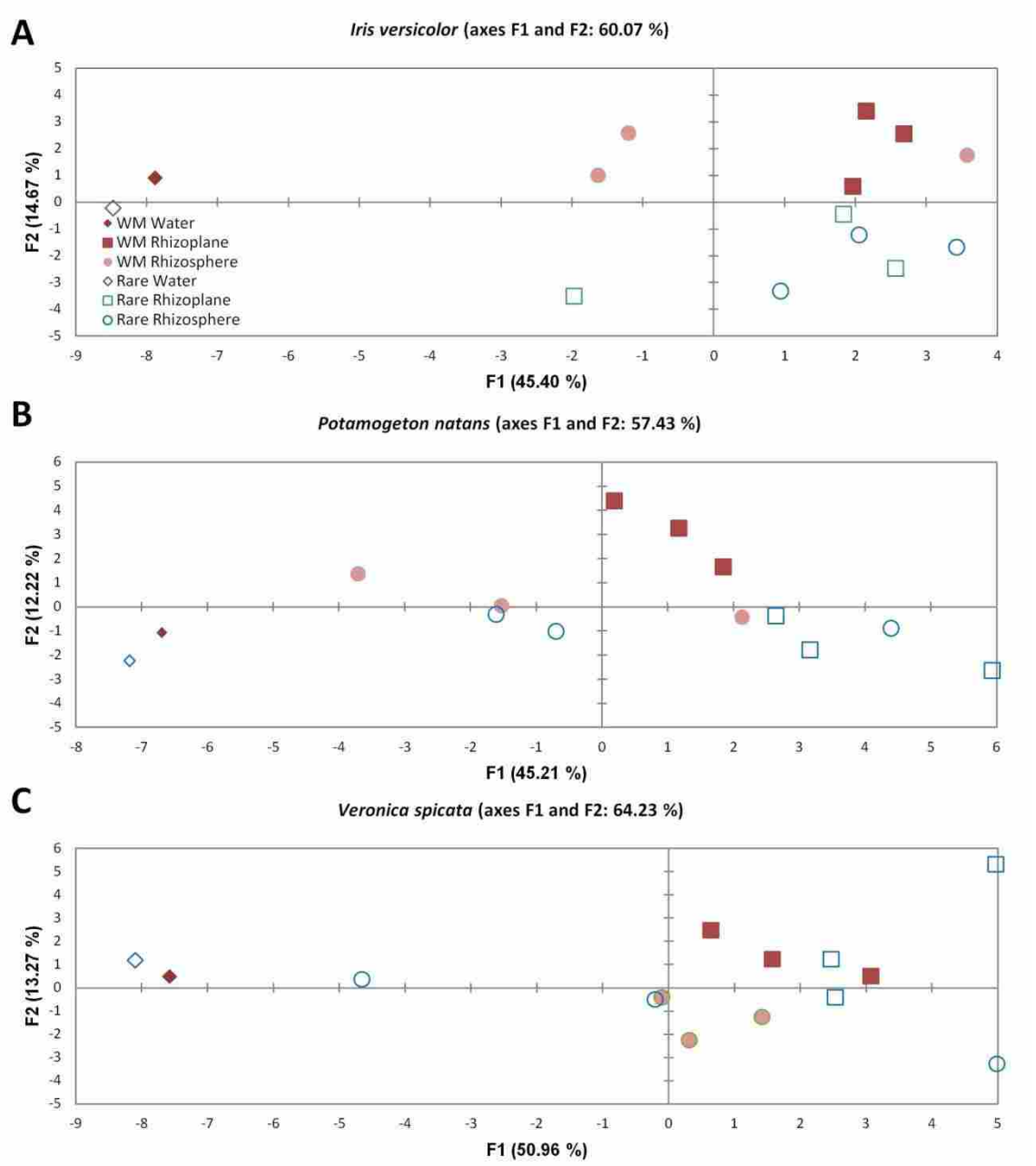

Figure 4.4 PC1 vs. PC2 plots of Biolog™ carbon source utilization by microbial communities from river water, rhizosphere or rhizoplane samples associated with A) *Iris versicolor*, B) *Potamogeton natans* and C) *Veronica spicata* at West Montrose (WM) and Rare Charitable Research Reserve (Rare). Each data point represents an individual quadrat containing that plant species within the sampling site and is an average of three sets of PC scores from triplicate technical replicates.

4.3.2.2 Average Well Color Development and Metabolic Diversity

Average well color development (AWCD), the average absorbance measured from a microbial community across all 31 carbon sources, showed much higher values for rhizosphere and rhizoplane communities as compared to water communities across all plant species examined (Fig 4.5 A). AWCD was similar between community types (rhizosphere and rhizoplane) and between the two sampling locations for *I. versicolor*. At both sites, AWCD was higher in the rhizoplane of *P. natans* compared to the rhizosphere. AWCD was higher in the rhizoplane at Rare compared to the rhizosphere for *V, spicata.* Within the same community type (rhizosphere or rhizoplane), AWCD values were similar between Rare and West Montrose for the most part.

Metabolic diversity was consistently higher among rhizosphere and rhizoplane communities compared to water microbial communities (Fig 4.5 B). Metabolic diversity measurements for communities associated with *I. versicolor* were similar at both sampling locations and within the rhizosphere and rhizoplane. Conversely, *P. natans* exhibited higher metabolic diversity at the Rare sampling location compared to West Montrose, within both the rhizosphere and rhizoplane microbial community. Metabolic diversity for *V. spicata* associated microbial communities was similar between sampling locations within the same sample type. However, at the Rare site only, metabolic diversity was higher in the rhizoplane compared to the rhizosphere.

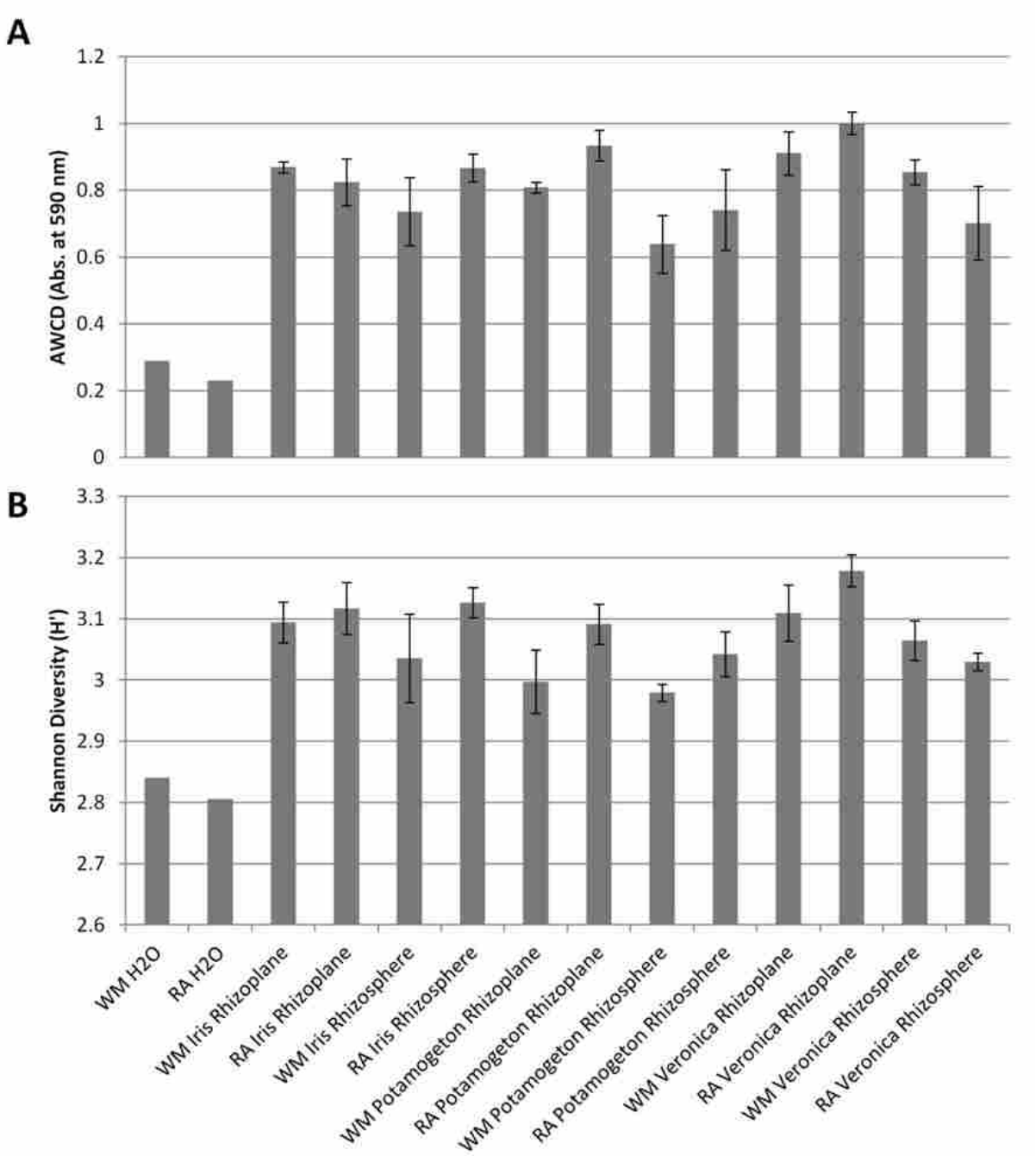

Figure 4.5 (A) Average well color development (AWCD), defined as the average absorbance of all 31 Biolog^{TM} EcoPlate carbon sources at 590 nm after 96 hours of incubation at room temperature and (B) metabolic diversity of microbial communities associated with the water, rhizosphere and rhizoplane of *I. versicolor*, *P. natans* and *V. spicata* calculated from Biolog ^{TM} carbon source utilization using Shannon Diversity (H'). Each bar represents the average of three technical replicates for water samples, and three experimental replicates and three technical replicates for rhizoplane and rhizosphere samples. Error bars are representative of standard error only.

4.3.3 Water Chemistry at West Montrose and Rare During Sampling Events

Water chemistry was assessed at both the Rare and West Montrose water sampling locations during sampling in October and November 2015 (Table 4.1). Rare, the poor water quality site, exhibited higher concentrations of all inorganic forms of nitrogen (nitrite, ammonia, nitrate), however total reactive phosphorus was measured in similar concentrations at both sites. Measurements of pH and dissolved oxygen were not significantly different between the two sites. However, conductivity was much higher at Rare than at West Montrose.

Table 4.1 Water Chemistry Data Obtained from River Water at Rare Charitable Research Reserve and West Montrose during Sampling in October and November of 2015.

	Rare	**West Montrose**
pH	8.34±0.025	8.42 ± 0.075
Nitrite (mg/L)	0.071 ± 0.0025	0.007 ± 0
Nitrate (mg/L)	3.045 ± 0.89	1.355 ± 0.766
Ammonia (mg/L)	0.178 ± 0.112	0.031 ±0.006
Total Reactive Phosphorus (mg/L)	0.006 ± 0.001	0.005 ± 0.001
Dissolved Oxygen (mg/L)	12.78 ± 2.26	14.77 ± 2.26
Conductivity (Cus/cm)	937 ± 46	490 ± 60

* Values are averages from the two sampling events ± standard error.

4.3.4 Microbiological Indicators of Water Quality

4.3.4.1 Culture-Based Methods

Culture-based microbiological measurements associated with water quality, including heterotrophic plate counts and membrane fecal coliform measurements, exhibited some species-specific and site-specific differences. Total heterotrophic plate counts (HPCs) from river water were higher at the Rare site than at West Montrose (Fig 4.6 A). *I. versicolor* was the only plant species to exhibit differences in HPCs between the two sites, where total bacterial counts on R2A media were higher in the Rare site for both the rhizosphere and the rhizoplane, as compared to counts at West Montrose. For *P. natans* and *V. spicata,* HPCs were similar between the rhizosphere and the rhizoplane and between Rare and West Montrose. Fecal coliform counts were higher in river water from West Montrose, compared to Rare (Fig 3.6 B). However, within most plant species and most sample types (rhizosphere and rhizoplane), fecal coliforms tended to be higher at Rare. For *I. versicolor,* fecal coliform abundance in the rhizoplane was similar between the two sites, however within the rhizosphere, higher numbers of fecal coliforms were detected at Rare. *P. natans* exhibited greater numbers of fecal coliforms at Rare within rhizoplane samples, but measurements within the rhizosphere were similar between the two sampling locations. *V. spicata* exhibited a greater number of fecal coliforms at the Rare site, within both rhizosphere and rhizoplane samples.

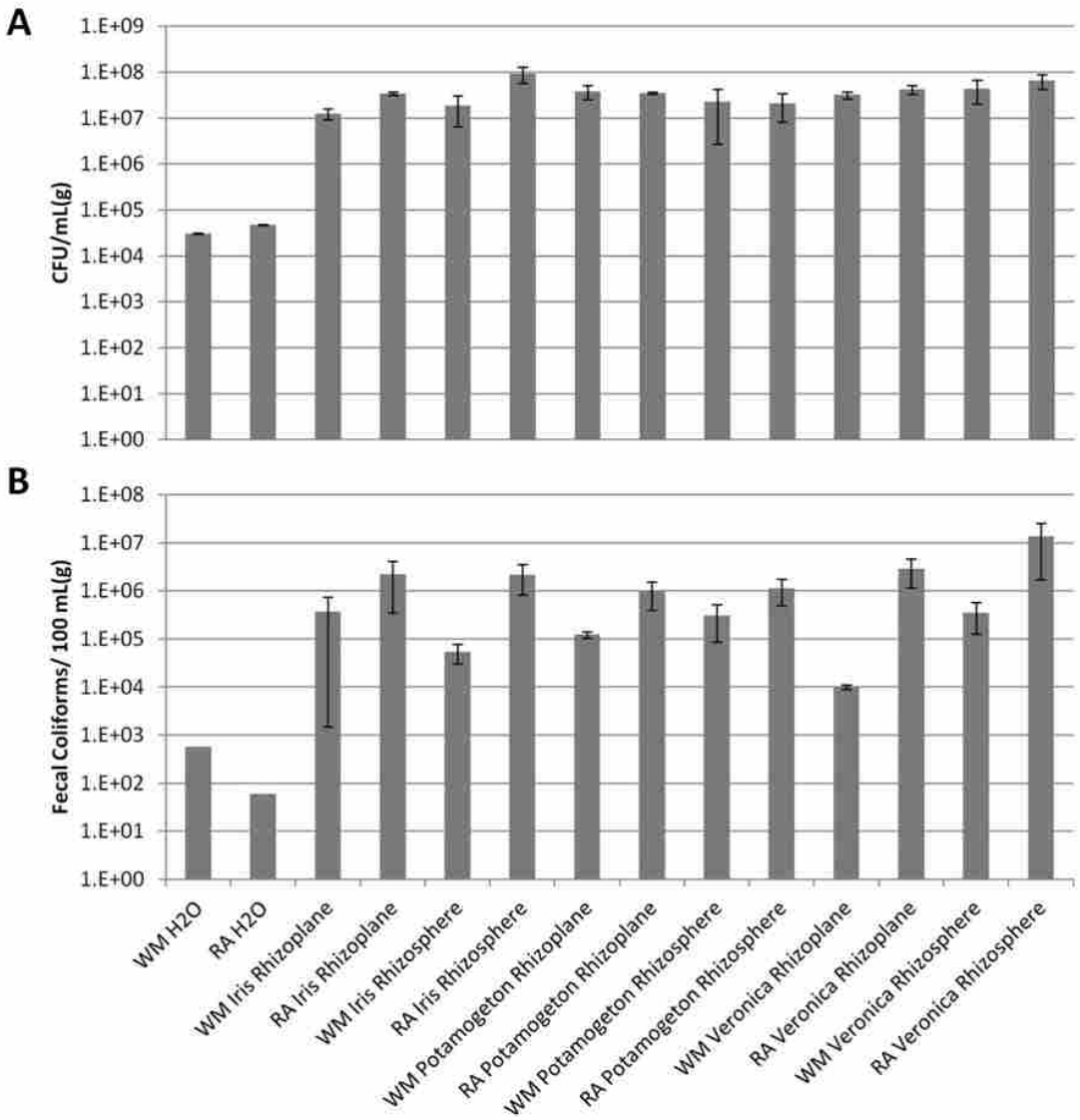

Figure 4.6 Heterotrophic plate counts (A) and membrane fecal coliform counts (B) in Grand River water and plant rhizoplane or rhizosphere material from West Montrose or Rare. (A) Colony forming units are per mL of river water or per gram of rhizosphere or rhizoplane material associated with *I. versicolor, P. natans* and *V. spicata* at West Montrose (WM) or Rare (RA) grown on R2A media at room temperature for 120 hours. Bars represent averages from two technical replicates (water only) and three experimental replicates (rhizosphere and rhizoplane). (B) Fecal coliform counts determined using the m-FC method after incubation at 44.5°C for 48 hours for 100 mL of river water or 100 g of rhizosphere or rhizoplane material from *I. versicolor, P. natans* and *V. spicata* at West Montrose and Rare. Bars represent averages from two technical replicates (water only) and three experimental replicates (rhizosphere and rhizoplane). Error bars represent standard error from experimental replicates only.

4.3.4.2 Molecular-Based Methods

Quantification of DNA from pathogenic microorganisms associated with fecal contamination revealed several distinct trends related to site, sample type and plant species (Fig 4.7). *Salmonella* quantities were greater in West Montrose water compared to Rare (Fig 4.7 A). Quantities of *Salmonella* detected in rhizosphere and rhizoplane samples from different plant species at the two sites were similar in most cases. The only exception was *I. versicolor* which had greater *Salmonella* quantities present in the rhizosphere at West Montrose compared to the rhizosphere community at Rare.

The results of qPCR based quantification of *E. coli* DNA showed that the abundance of this microorganism was similar at the two different sites (Fig 4.7 B). Similar quantities of *E. coli* DNA were isolated from the rhizosphere and rhizoplane at Rare and West Montrose for all plant species sampled with the exception of *V. spicata*, where *E. coli* was detected in greater quantities at the Rare site within the rhizosphere compared to the rhizosphere at West Montrose.

Enterococcus genetic material was detected in greater quantities in the river water at the West Montrose sampling location compared to Rare (Fig 4.7 C). Within sample type comparisons between the two sampling locations did not reveal differences for any plant species except *V. spicata,* where greater *Enterococcus* numbers were present in Rare rhizoplane samples compared to West Montrose rhizoplane samples. Furthermore, *Enterococcus* was completely absent from the rhizosphere microbial community associated with *P. natans* at both sites.

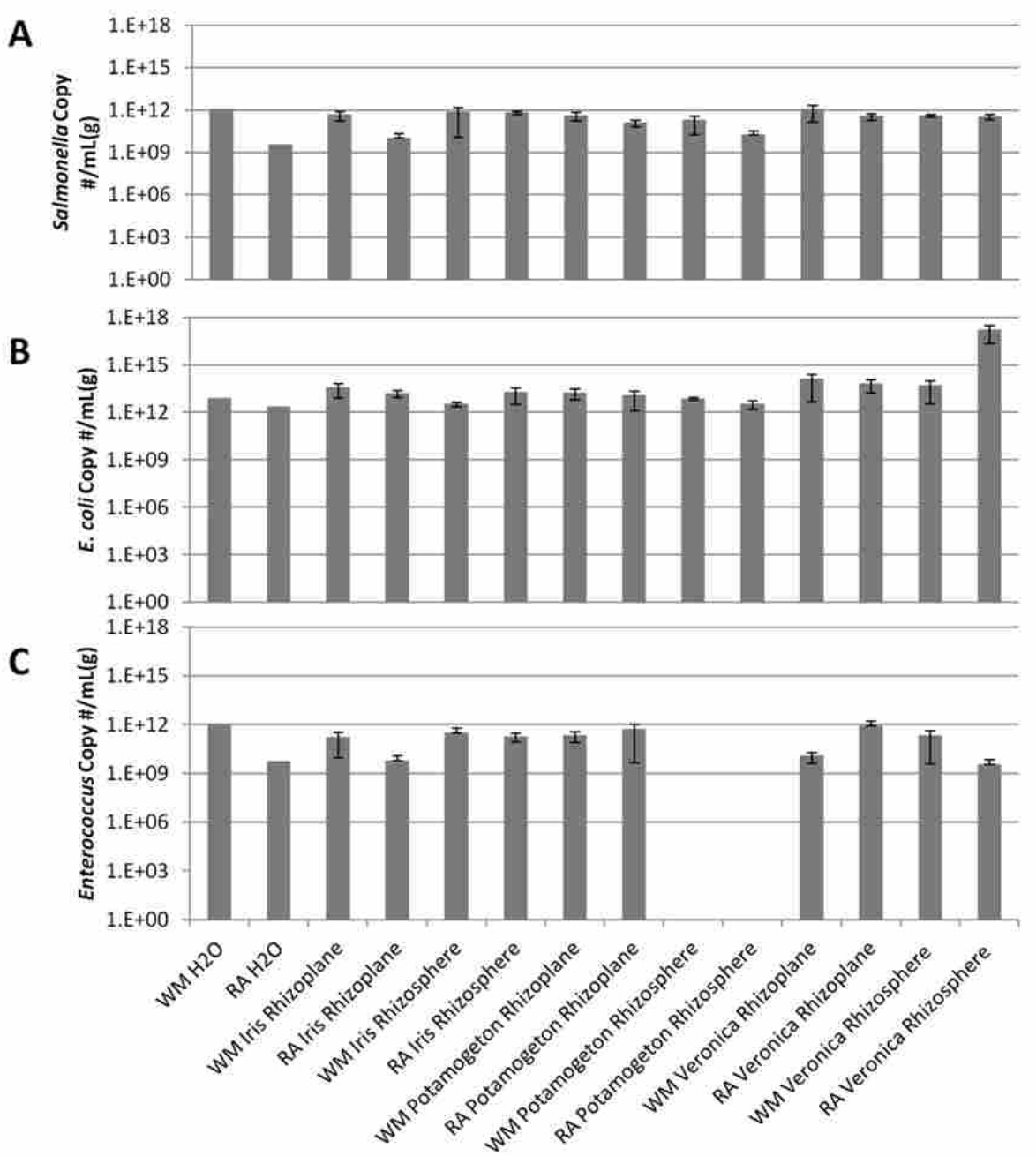

Figure 4.7 Average relative abundance (target gene copies/mL) of bacterial pathogens (*Salmonella* spp. (A), *E. coli* (B) and *Enterococcus* spp. (C)) obtained from water samples, rhizoplane and rhizosphere material from *I. versicolor*, *P. natans* and *V. spicata* at West Montrose (WM) and Rare (RA). Bars represent averages from three experimental replicates and two technical replicates. Error bars represent standard error of experimental replicates only.

4.4 Discussion

Structural profiles associated with river water, rhizoplane and rhizosphere samples tended to form clusters within the same sampling location, plant species and sample type (e.g. samples from Q1-3). Beyond that, larger clusters tended to form between different plant species and community types, within the same sampling location. This indicates that sampling location was a significant driver of community structure during our sampling period. The exception to this trend was the microbial communities associated with *V. spicata.* Rhizoplane communities from Rare associated with *V. spicata* clustered with rhizosphere communities from West Montrose. Rare rhizoplane communities associated with *V. spicata* clustered with other Rare sample types from different plant species, however rhizosphere communities associated with *V. spicata* from the West Montrose sampling location exhibited structural similarity with water-associated communities (Fig 4.1). The structure of microbial communities associated with *I. versicolor* exhibited the greatest dissimilarity between sampling locations, with *V. spicata* exhibiting the least.

The literature has established that microbial structural profiles associated with the rhizosphere of different plant species tend to be unique (e.g. Grayston *et al.*, 1998; Marschner *et al.*, 2001, Marschner *et al.*, 2004). This is also true of microbial rhizoplane communities, although considerably fewer studies have examined this community type (e.g. Wieland *et al.*, 2001; Nunan *et al.*, 2005; Ofek-Lalzar *et al.*, 2014). Of the studies that have been performed comparing community structures between the two different rhizo-compartments, findings have indicated that communities are selected by the plant, from the bulk soil, in a stepwise manner, first in the rhizosphere and then in the rhizoplane in such a way that each community type selects for enrichment of certain groups or species while depleting others (Bulgarelli *et al.*, 2012; Ofek-

Lalzar *et al.*, 2014; Edwards *et al.*, 2015; Zhang *et al.*, 2017). Thus, as the rhizosphere and rhizoplane microbial community are a function of both the selection process by the plant, and the available microorganisms for selection in the bulk soil at each site, it makes sense that we would see community structural profiles clustering by plant species, community type and location. This, of course, assumes that the bulk soil microbial community is different at both locations, but I think we can assume this to be true as this is a wetland environment, the water-associated microbial communities at each site were dissimilar, and we saw differences among communities of the same type at the two sites (Fig 4.1 and 4.2). We were not able to obtain structural profiles for bulk soil communities at either location as there was no soil unimpacted by plant roots available. Furthermore, other studies have found that microbial community structure in bulk soil is variable by location within the same environment type (e.g. Horner-Devine *et al.*, 2004).

Varying degrees of similarity exist between communities of the same rhizo-compartment between sampling locations, depending on the associated plant species. *V. spicata* exhibited the greatest amount of structural similarity between rhizosphere (16%) and rhizoplane (24%) microbial communities at West Montrose versus Rare. *P. natans* exhibited the second greatest amount of similarity (11% in the rhizosphere, 19% in the rhizoplane) while microbial communities associated with *I. versicolor* were the most dissimilar by site (8% in the rhizosphere, 8% in the rhizoplane). This suggests that plant species differ in their ability to recruit root-associated microorganisms, and may be affected differently by variability in environmental conditions, such as differences in water quality and water chemistry. It should also be noted that the rhizoplane microbial community seems to exhibit less variability than the rhizosphere between sites, which could be a function of the degree of plant-specific influence. Hypothetically physical proximity to the plant root promotes a degree of consistency across

different environmental conditions external to the immediate plant root environment. This may lend itself to a more consistent microbial community within the rhizoplane. To the best of our knowledge, no other studies have examined the effects of water quality and plant species on microbial community structure in wetland microbial communities. However, several studies have observed how differences in soil type can affect rhizosphere microbial communities in association with different plant species. For example, Marscher *et al.*, (2001) examined rhizosphere microbial community structures associated with chickpea (*Cicer arietinum*), canola/rapeseed (*Brassica napus*) and Sudan grass (*Sorghum bicolour*) grown in sandy, loam and clay soils. They found that Sudan grass exhibited the most variability in rhizosphere microbial community structure by soil type. Chickpea rhizosphere microbial communities grown in sandy soil were distinct from loam and clay-based communities, while rapeseed rhizosphere microbial communities grown in clay were distinct from sand and loam-based communities. The three different soil types exhibited differences in the concentrations of inorganic nutrients and organic matter. Similarly, Marschner *et al.*, (2004) examined differences in rhizosphere microbial communities of barley (*Hordeum vulgare*) and cucumber (*Cucumis sativus*) under conditions of nitrogen and phosphorus deficiency, and showed that cucumber rhizosphere communities exhibited greater variability in rhizosphere community structures between the nutrient availability treatment types. Furthermore, when they examined rhizosphere microbial communities associated with chickpea, Sudan grass and canola (*Brassica* sp.) under conditions of deficient soil phosphorus and phosphorus enrichment (organic and inorganic) they found that rhizosphere communities associated with chickpea and canola varied by fertilization treatment while those associated with Sudan grass were relatively unaffected. These studies indicate that soil microbial communities may be affected by differences in nutrient availability, which is

similar to differences in wetland-associated microbial communities experiencing different water quality conditions (specifically inorganic nutrient loads). Furthermore, these studies also mirrored our results in that microbial community changes in response to altered environmental conditions is partly determined by the plant species the community is associated with. Further research will be required to understand which plant species-specific characteristics contribute to root-associated microbial community stability across changing environmental conditions.

Functional community characteristics assessed using carbon source utilization profiles of rhizosphere and rhizoplane communities were separate from water communities for all three plant species. Carbon utilization profiles only differed between the rhizoplane and the rhizosphere microbial communities for *V. spicata* and *P. natans* (Fig 4.4). Site specific differences in carbon source utilization profiles between microbial communities were only present for *I. versicolor* and *P. natans.*

Garland (1996) was the first study to illustrate that rhizosphere microbial communities associated with different plant species exhibited distinct carbon source utilization profiles. When Biolog^{TM} carbon source utilization profiles from rhizosphere microbial communities associated with white potato, soybean, wheat and sweet potato were analyzed using detrended correspondence analysis (DCA) distinct profiles were observed among the different microbial communities associated with each plant species. Grayston *et al.,* (1998) also demonstrated differences in carbon source utilization profiles among rhizosphere microbial communities associated with different plant species. Biolog^{TM} carbon source utilization profiles for rhizosphere microbial communities in association with ryegrass (*Lolium perenne*), bentgrass (*Agrostis capillaries*), wheat (*Triticum aestivum*) and clover (*Trifolium repens*) were compared to those of non-rhizosphere microbial communities from un-amended soil and soil supplemented

with additional carbon as sucrose using canonical variate analysis (CVA). Differences among these microbial communities were established for the rate of carbon utilization, as well as for the types of carbon sources preferred by each community. In contrast, our findings did not identify clear plant species-specific carbon source utilization profiles among the rhizosphere or rhizoplane microbial communities from *I. versicolor*, *P. natans* and *V. spicata* when analyzed together with all community types from the two different sampling locations (Fig 4.3). However, when analyzed individually by rhizo-compartment within the same sampling location, the differences in carbon source utilization profiles emerged for all plant species and rhizo-compartment associated microbial communities (data not shown). This indicates that the differences in carbon source utilization profiles between sites and rhizo-compartments was a greater source of variation than the differences in carbon source utilization by the microbial communities associated with the different plant species. The previously mentioned studies used different methods of statistical analysis (CVA and DCA vs. PCA), different Biolog^{TM} plates (GN vs. EcoPlate) with different carbon sources, and conducted their studies under controlled laboratory conditions instead of under field conditions, all of which is likely to contribute to the differences between our findings.

Very few studies published in the literature have compared carbon source utilization profiles of microbial communities between the rhizosphere and the rhizoplane. We observed that some plant species possess root-associated microbial communities with unique carbon source utilization profiles for each rhizo-compartment (*V. spicata* and *P. natans*), while others may not display distinct functional profiles between the rhizosphere and the rhizoplane (*I. versicolor*). Grayston (2000) found that carbon source utilization profiles produced by rhizoplane microbial communities associated with three species of trees (Larch, Spruce and Sycamore) were distinct

from those of the associated rhizosphere microbial communities for all three species. Similarly, when Baudoin *et al.* (2001) compared carbon source utilization profiles between bulk soil, rhizosphere and rhizoplane communities using Biolog^{TM} plates, they found that the biggest difference in substrate utilization was between the bulk soil community and the rhizoplane community. The rhizosphere microbial community used carbon sources that were intermediate between the two community types. Substrate utilization by rhizosphere and rhizoplane microbial communities were similar in many respects, and primarily differed in the relative usage of amino acids and amines-amides (Table A7). Rhizodeposits, which commonly contain nitrogenous carbon sources including amino acids, amines and amides, represent the most readily available source of organic nitrogen in root impacted soil, which would be seen by rhizoplane microbial communities before reaching the rhizosphere, thus exposing the rhizoplane microbial community to these compounds more frequently and in greater concentrations compared to the rhizosphere (e.g. Richardson *et al.*, 2009). Thus, it appears that the distinction between metabolic profiles of microbial communities in the rhizoplane compared to the rhizosphere may be the result of exposure to varying concentrations of rhizodeposits as a function of distance from the root surface. To the best of our knowledge, these are the only two additional studies in the literature (Grayston, 2000; Baudoin *et al.*, 2001) that have looked at the relationship between the rhizosphere and the rhizoplane with respect to carbon source utilization profiles. However, concluding from the evidence currently available, distinct metabolic profiles among rhizo-compartments may be commonplace. Thus, *I. versicolor* which lacks the distinction among root-associated microbial community types is an exception to what has been typically observed. In *I. versicolor* the structural community profiles established by PCR-DGGE revealed differences between the rhizoplane and rhizosphere microbial communities (Fig 4.2). However, this was not

reflected in the functional profiles of these different community types. This disagreement could be the result of the unique root exudation and rhizodeposit profile associated with *I. versicolor*. Alternatively, the lack of distinction between these two community types could also be the result of a seasonal change in plant rhizodeposition, as the literature has shown that the quantity and composition of plant root exudates can vary seasonally (Edwards *et al.,* 2018). Another explanation is that functional differences in carbon source utilization profiles between the two rhizo-compartments existed, but could not be detected using Biolog™ plates. This could be the case depending on the nature of the structural differences between the communities, as Biolog™ plates only detect the activity of a portion of the total microbial community present in the initial inoculum (Smalla *et al.,* 2001).

Our findings indicated that metabolic profiles of microbial communities associated with the rhizosphere and the rhizoplane of *I. versicolor, P. natans* and *V. spicata* responded to the distinct environmental pressures associated with two sampling locations with contrasting water quality differently by plant species. *I. versicolor* exhibited the greatest site specific-effect on carbon source utilization profiles, while *V. spicata* exhibited no clear differences in metabolic capabilities between communities at the two sites. To the best of our knowledge, this is the first time carbon source utilization profiles have been used to compare functional community characteristics between wetland-associated microbial communities under contrasting water quality conditions. We have established that plants play a role in structuring their root-associated microbial communities, and as such these communities are species- specific (e.g. Grayston *et al.,* 1998; Marschner *et al.,* 2001, Marschner e*t al.,* 2004). Due to the unique nature of these microbial communities, it makes sense that their stability (structural and functional) across changing environmental conditions may also vary by plant species. Stability of the

functional community profile associated with *V. spicata* rhizosphere and rhizoplane microbial communities across variable environmental conditions may make it a good candidate for use in constructed wetlands, however, more work would need to be done to support this. Evidence from the literature suggests that root-associated microbial communities can contribute to the successful establishment of invasive plant species beyond their native range (e.g. Reinhart *et al.*, 2003; Callaway *et al.*, 2004; Reinhart and Callaway 2004). *V. spicata* is an introduced plant species throughout central and eastern Canada (Brouillet *et al.*, 2010). The ability to form a functionally stable root-associated microbial community under dynamic physico-chemical conditions may be a contributing factor in its success at colonizing and becoming established in new environments.

By integrating structural and functional microbial community data, we were able to establish agreement between the two different measures of microbial community change. The structural community data, obtained through PCR-DGGE-based microbial community fingerprinting, revealed that *V. spicata* showed the greatest amount of structural similarity between the West Montrose and Rare sites (Fig 4.2). This agreed well with the functional community data, which did not indicate clear differences in carbon source utilization profiles between microbial communities at the different sites (Fig 4.4). The root-associated microbial communities of *P. natans* had intermediate levels of structural similarity between the two sites, among the plant species tested. Similarly, these microbial communities exhibited intermediate levels of differentiation in functional community data between the two sites. Furthermore, the rhizosphere microbial community associated with *P. natans* exhibited more structural differentiation between sites, as compared to the rhizoplane. This agrees with the functional community data, with the clearest functional differences being noted between the rhizosphere

microbial communities at Rare and West Montrose. *I. versicolor* exhibited the least structural similarity between rhizosphere and rhizoplane communities at the two sites, and also exhibited distinct functional community profiles associated with West Montrose and Rare microbial communities. The ability of functional community data obtained using carbon source utilization profiles to reflect differences in community structure seems to be dependent on the nature of the genetic community level changes being detected. Biolog™ analyses have all of the biases inherent in culture-based methods, ultimately reflecting the activity of a small subset of the microbial community. Smalla *et al.* (1998) confirmed that the microbial populations contributing to carbon source utilization profiles measured using Biolog™ plates were structurally distinct from those present in the initial inoculum and were skewed towards non-fastidious microorganisms from the subclass γ-Proteobacteria. Thus, if shifts in community structure are related to this group of microorganisms we can expect to also see changes in Biolog™ carbon source utilization profiles. Findings by Buyer *et al.* (2002) support this; they observed structural and functional community changes occurring within the rhizosphere of corn and soybean (compared to the bulk soil community) and found that functional community data reflected structural community changes, and that the heterotrophic microorganisms capable of rapid growth under aerobic conditions being detected with Biolog™ substrate utilization assays were the same types of microorganisms being enriched in the rhizosphere. As such, the agreement between our structural and functional community data is likely a reflection of changes occurring in these types of microbial populations from the rhizosphere and rhizoplane communities of our study plants. Furthermore, differences in nutrient conditions between the two water quality sites may have contributed further to the ability of Biolog™ analysis to detect changes in community functional profiles. Smalla *et al.,* (2001) found that microbial communities from different

environments (activated sludge fed with glucose and peptone versus potato rhizosphere) experienced different community level effects upon inoculation into the Biolog^{TM} plates. All dominant members of the microbial community in the activated sludge were represented in at least one of the Biolog^{TM} plate wells, while many of the dominant populations from the potato rhizosphere were absent from the wells. The authors suggest that this is due to differences between the two environments in terms of substrate variety and concentration.

Culturable fecal coliforms measured by growth on m-FC media, revealed a trend between the two water quality sites. All plant species exhibited an increase in the number of fecal coliforms cultured from the rhizosphere, rhizoplane or both community types, at the Rare study site (Fig 4.6). Due to the noted proximity of the Rare sampling location to a WWTP discharge this should be the obvious explanation for the observed trend. However, we cultured a greater number of fecal coliforms from the river water at West Montrose, as compared to the river water at Rare (Fig 4.6). This was also true for molecular measurements of *Salmonella* and *Enterococcus* DNA obtained via qPCR, which were also higher at West Montrose (Fig 4.7). Thus, at least during these sampling events, there does not seem to be a greater number of fecal coliforms being introduced into the Rare site (at least not from the water in the river). Therefore, an alternative explanation as to why fecal coliform numbers are more abundant in some root – associated microbial communities at the low water quality site must exist. One possibility is that they persisted from a previous introduction at the Rare site, from the river water or otherwise. Another possibility is that the environmental conditions present at Rare are more conducive to fecal coliform establishment in the rhizosphere and/or rhizoplane. We have only limited water chemistry data from the two sites, but the biggest differences we observed were related to higher inorganic nitrogen concentrations, lower dissolved oxygen and higher conductivity at Rare as

compared to West Montrose. Indirect effects via site-specific conditions on plant growth and root exudation profiles may be a factor in the greater prevalence of fecal coliforms at the Rare site.

One of the most interesting findings resulting from this study was the complete absence of the genus *Enterococcus* from the rhizosphere of *P. natans* (Fig 4.7). This was true for all three quadrats sampled for this species at both study sites. Several different mechanisms have been suggested to explain why certain microorganisms (specifically fecal indicator microorganismss) may be reduced within the rhizosphere and these include: 1) increased oxygenation in the rhizosphere (Vymazal, 2005), 2) competition with resident microflora (Cooley *et al.*, 2003; Cooley *et al.*, 2006), and 3) secretion of antimicrobial compounds in the rhizosphere of plant or microbial origin (Gopal and Goel, 1993; Neori *et al.*, 2000; Axelrood *et al.*, 2006; Fett, 2006). The first mechanism of elimination, as well as secretion of plant derived antimicrobial substances, can be dismissed as probable explanations. The reason being that despite being absent from the rhizosphere, *Enterococcus* was detected in the rhizoplane of *P. natans* in significant quantities. Thus, it is likely that the failure of *Enterococcus* to persist in the *P. natans* rhizosphere was due to competition with the rhizosphere microbial community and/or the presence of antimicrobial compounds of microbial origin being produced in the rhizosphere of *P. natans*. Competition with resident microorganisms was shown to reduce loads of fecal indicator microorganisms (specifically *Salmonella enterica* and *E. coli* O157:H7) in the rhizosphere of *Arabidopsis thaliana* and lettuce (*Wausteria paucula*) (Cooley *et al.*, 2003; Cooley *et al.*, 2006). Additionally, plant-associated strains of *Pseudomonas* have been shown to produce antimicrobial compounds inhibiting the growth of multiple serovars of *Salmonella enterica* (Fett, 2006). Furthermore, a strain of *Bacillus subtilis* (SK.DU.4) isolated from rhizosphere soil was shown to

produce antimicrobial peptides with activity specific to Gram positive bacteria including *Staphylococcus aureus , Streptococcus mutans, Micrococcus luteus and Listeria monocytogenes* (Baindara *et al.*, 2013).

4.5 Conclusions

We hypothesized that microbial communities associated with the roots of wetland plants would be affected by water quality and that these effects would be different based on the associated plant species. Site-specific differences at the two contrasting water quality sites affected the structure and function of rhizosphere and rhizoplane microbial communities differently depending on which wetland plant they were associated with. Microbial communities associated with *V. spicata* were more structurally and functionally similar between the two sampling locations, while those associated with *I. versicolor* exhibited the greatest site-specific differences. Site-specific differences in community structure and function also varied depending on the community type. Rhizoplane microbial communities tended to be more structurally/functionally similar between sites than rhizosphere communities. Differences between the two contrasting water quality sites with respect to fecal coliform presence was also observed, with fecal coliform abundance being greater in most plant-associated microbial communities at the poor water quality site (Rare). Furthermore, plant species-specific effects on fecal indictor microorganisms were observed. Specifically, *Enterococcus* was completely excluded from the rhizosphere of *P. natans.* It should be noted that although we selected quadrats with the same soil type and plant community composition at both sites, to the best of our ability, this was still a field study. As such, the influence of other factors aside from those related to water quality occurring at each site may have also impacted microbial community structure and function and thus, cannot be ruled out as factors affecting our results.

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Chapter 5

Structural and Functional Changes to Plant Root-Associated Wetland Bacterial Communities Exposed to Different Water Qualities in Lab-Scale Constructed Mesocosms

5.1 Introduction

The human population is placing an increasing demand on available supplies of potable water, which are being negatively impacted by pollutants originating from industrial, agricultural and anthropogenic activities. In addition to potentially harmful contaminants of anthropogenic origin, such as personal care pharmaceutical products, pesticides, heavy metals, petroleum and volatile phenolics, the addition of high levels of inorganic nutrients to aquatic ecosystems originating from human activities poses a serious threat to the health of aquatic ecosystems (Vymazal, 2007; Wang *et al.,* 2007). Inorganic forms of nitrogen and phosphorus are among the most common inorganic nutrients entering aquatic ecosystems in excess and can have detrimental effects on ecosystem dynamics and the health of aquatic plants and animals (Lamers *et al.,* 2002; Wang *et al.,* 2007; Faulwetter *et al.,* 2009). Anthropogenic and agricultural effluents are also associated with the presence of disease-causing microorganisms originating from human and animal fecal matter. Some of the most significant human pathogens originating from fecal contamination found in freshwater systems include *Salmonella* spp., *Enterococcus* spp. and *Escherichia coli* (Cabral, 2010). Natural wetlands have long been recognized for their ability to remove contaminants from polluted waters before they enter receiving water systems (Johnston, 1991). The removal of most pollutants in wetlands has been attributed primarily to bacterial

processes and thus gaining a better understanding of how microbial communities respond to changes in water quality is of the utmost importance (Faulwetter *et al.,* 2009).

Bacteria do not and cannot exist in isolation and are heavily influenced by interactions with plants (Stottmeister *et al.,* 2003; Bonfante and Anca, 2009). Within the wetland ecosystem, microbial communities can be considered individually by habitat type. Water-associated microbial communities can be isolated from either surface water or discharge effluent in a wetland system lacking aboveground standing water. Additionally, plant-associated microbial communities can be subdivided based on rhizo-compartment: 1) rhizosphere, 2) rhizoplane and 3) endosphere. The rhizosphere and rhizoplane communities are the most relevant to the microbial ecology influencing wetland remediation capabilities (e.g. Muratova *et al., 2003*). The rhizosphere can be defined as the narrow region of soil surrounding plant roots that is influenced by the growth, respiration and root secretions of the plant (Berendsen *et al.,* 2012). The rhizosphere microbial community is more diverse and more concentrated than that of the surrounding bulk soil due largely to organic deposits made by plant roots which act as a nutrient source (Baudoin *et al.,* 2003; Philippot *et al.,* 2013). The rhizoplane differs from the rhizosphere in that these microorganisms are attached directly to the roots of the plant via microbial biofilms (Edwards *et al.,* 2015). Microbial root-associated communities are actively determined by the plant (Haichar *et al.,* 2008; Hartmann *et al.,* 2009; Berendsen *et al,* 2012; Philippot *et al.,* 2013). For this reason, these communities have a species-specific composition that is distinct from the surrounding bulk soil (Costa *et al.,* 2006).

Information available on the effects of water quality on plant-associated wetland microbial communities is very lacking. Both natural and constructed wetlands rely on these communities for the effective remediation of contaminated wastewaters (e.g. Zhuang *et al.,*

2007). To design the most effective and robust constructed wetlands possible, a better understanding of the effects of anthropogenic activities on wetland-associated microbial communities is needed. Information available from the literature on this subject is limited. What has currently been established is primarily confined to analyses of rhizosphere or sediment microbial community compositions using molecular techniques, or the measurement of microbial community functional changes as assessed by carbon source utilization profiles or changes in quantities of functional genes associated with the bacterial metabolism of specific contaminants (e.g. Ahn *et al.,* 2007, Zhao *et al.,* 2010, Bowen *et al.*, 2011). None of the studies published to date, to the best of our knowledge, have looked at these two measurements of community change together to attain a more complete picture of the structure and function of microbial communities in the face of changing water quality parameters. Furthermore, most studies have explored this subject matter by singling out individual parameters associated with water quality (e.g. elevated C, N, P or pathogenic bacteria). Although this approach does reduce the number of variables in an experimental design, microbial communities in natural and constructed wetland systems will rarely ever experience conditions such as this, with elevation of only a single element of water quality. To gain a better understanding of how microbial communities will react to changes in water quality, treatments need to be designed to better reflect the realistic chemical and biological composition of wastewater and contaminated source waters. In addition, changes to the remedial capabilities of the microbial communities in the face of different water quality conditions must also be examined in order to fully understand how changing water quality could impact the ecosystem services provided by wetland communities.

To further our understanding of how changes to water quality can affect plant-bacteria community dynamics and their capacity to remediate contaminated water, we will be examining the following hypotheses:

1) Natural and constructed wetland systems highly impacted by anthropogenic activities will differ from less affected systems in both community composition, function and remediation capabilities (ability to remove contaminants and pollutants from water).
2) The microbial community composition of wetland plants will differ among species. Different plant species will harbour unique microbial communities that differ in their associated microbial community compositions, functionality and remediation capabilities.

In order to test these hypotheses we will determine the differences in structure, function and contaminant removal ability among wetland-associated microbial communities (water, rhizosphere, rhizoplane) for two different wetland plant species (*Phalaris arundinacea* and *Veronica anagallis-aquatica*) in lab-scale mesocosm systems subjected to water affected by contrasting degrees of anthropogenic impact.

5.2 Materials and Methods

5.2.1 Mesocosm Design and Set-up

Twelve wetland mesocosms were constructed, assembled and planted on September 19, 2016 using 20 gallon glass aquariums (Marineland®, Blacksburg, Virginia USA) to mimic the design of a subsurface flow wetland. Treatment water was stored in 5 gallon food application approved buckets (Home Depot®, Atlanta, Georgia USA) and distributed through a peristaltic pump system (Masterflex 12 channel peristaltic pump and with 2.76 mm santoprene tubing, Cole Parmer®, Montreal, Quebec CA) using ¼ inch black plastic drip irrigation tubing (Indoor

Farmer, Kitchener, Ontario CA). The bottom 10 inches of each tank were filled with 3/8 inch pea gravel (KING®, Burlington, Ontario CA) on top of which a 75:25 (v:v) mixture of silica sand (#20 grade high purity filter sand, Fairmount Minerals®, Wedron, Illinois USA) and Grand River sediment collected from the West Montrose sampling location (West Montrose, ON N0B 2S0 43.588219, -80.470979) on September 18, 2016 (Fig 5.1). River sediment was collected using sterile bags and stored at 4°C until use. The back 2 inches of each tank was filled from bottom to a depth of 14 inches with ¾ inch drainage gravel (KING®, Burlington, Ontario CA). Drainage from the mesocosms was facilitated by drilling holes at the bottom of each aquarium and inserting a 10 inch length of ¾ inch PVC pipe which was held in place using aquarium grade non-toxic silicone sealant (Marineland®, Blacksburg, Virginia USA). Multiple 1-2 mm wide slits were cut along the length of a larger piece of PVC pipe in 1 inch intervals (2 inch diameter, 14 inch height, Home Depot®, Atlanta, Georgia USA) which was then placed around the 3/4 inch outflow pipe and attached to the base of the aquarium using the aquarium sealant to prevent the outflow pipe from getting plugged with solid matter.

Once the mesocosms were assembled they were filled to capacity (8.42-8.67 L) with water obtained from the West Montrose sampling site along the Grand River. Each mesocosm was then planted with seeds from either *Phalaris arundinacea* (collected from the Grand River in 2015) or *Veronica spicata* (collected from the Grand River in 2016). Seedlings were germinated in the dark at room temperature (21±2°C) under sterile conditions on filter paper saturated with DI water for 96 hours before planting. A total of 32 seedlings were transferred to each tank in a roughly even distribution.

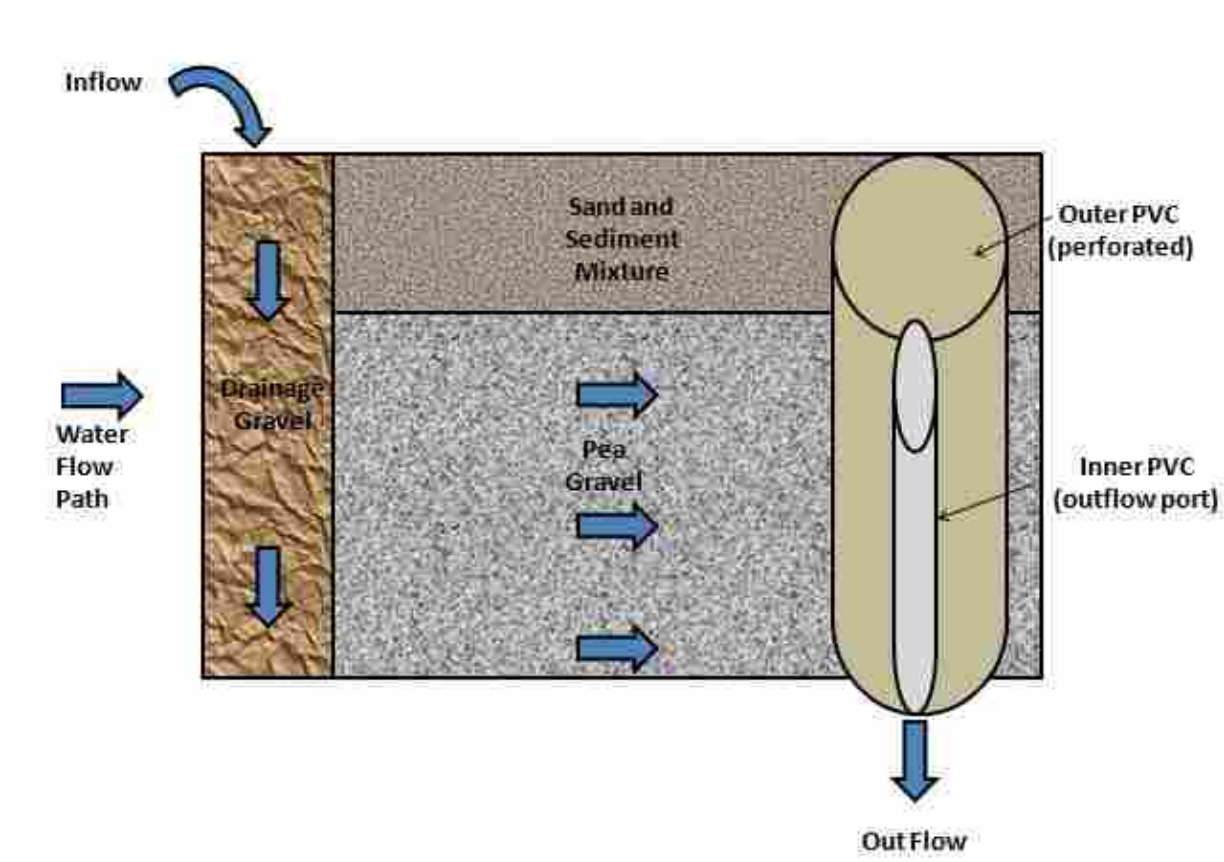

Figure 5.1 Horizontal subsurface flow constructed wetland based lab-scale mesocosm design. Water flow is indicated from the back to the front of the mesocosm.

5.2.2 Operating Conditions

The mesocosms were maintained under greenhouse conditions with a relative humidity and temperature of 40-60% and 24-28°C, respectively. All mesocosms were exposed to artificial illumination (100 W/m^2) with a 16 hour photoperiod. From the time of setup until October 26, 2016 water from West Montrose (collected from the river on a weekly basis) was added to the back of each tank manually (0.5 L/day) due to issues with the peristaltic pump operation. From this time onward all tanks were fed using the peristaltic pump system with a constant inflow rate of approximately 1.11-1.32 mL/min. Hydraulic retention time (HRT) for the mesocosms varied throughout the experiment due to changes in evapotranspiration volumes over the lifespan of the plants, however during the experimental time-period HRT was approximately 6 days. On October 28, 2016, two tanks were damaged and could not be repaired. The broken mesocosms were replaced by constructing replacement mesocosms from new materials and emptying and refilling all 12 mesocosm in the manner described previously. Seedlings were also replanted at this time. Due to poor growth of *V. spicata* seedlings up to this time point, the mixture of silica sand and West Montrose sediment was adjusted to a 50:50 (v:v) mixture, and additional Grand River sediment was added that had been collected on October 26, 2016. On December 12, 2016 another tank broke (*Veronica* #6) and a new tank was constructed at this time. Contents from the old tank, including established seedlings were transferred to the new tank. By December 20, 2016 seedlings in the mesocosms were established enough that it became apparent that plants growing in the 6 *Veronica* mesocosms were in fact not the seedlings that had been planted and instead were plants that had become established from the natural seed bank in the sediment used to build the mesocosms. By May 8, 2016 the *Veronica* treatments were dominated by *Veronica anagallis-aquatica* (70-95% cover). A small number of other plant species were also present in the tanks (representing no more than 5% cover total) including

Epilobium spp (WM *Veronica* #2 (2% cover), D *Veronica* #3 (5% cover)), *Verbena hastata (*D *Veronica* #1 (≤1% cover)), *Eupatorium altissimum* (D *Veronica* #1 (≤1% cover), D *Veronica* #2 (≤1% cover)), *Lythrum salicaria (*WM *Veronica* #2 (2% cover), WM *Veronica* #3 (3% cover)) and one unknown species belonging to the family Solanaceae (D *Veronica* #2 (2%cover)). On May 8, 2017 half of the *Phalaris arundinacea* mesocosms (3 of 6 total) and half of the *Veronica anagallis-aquatica* mesocosms were randomly chosen to receive water from our poor water quality site (Doon, Kitchener, ON 43.386376, -80.387547) (Fig 5.2 and 5.3). This site was determined previously to have had the poorest water quality of all the sites sampled and is classified as a poor water quality site by the Grand River Conservation Authority (Loomer and Cooke, 2011).

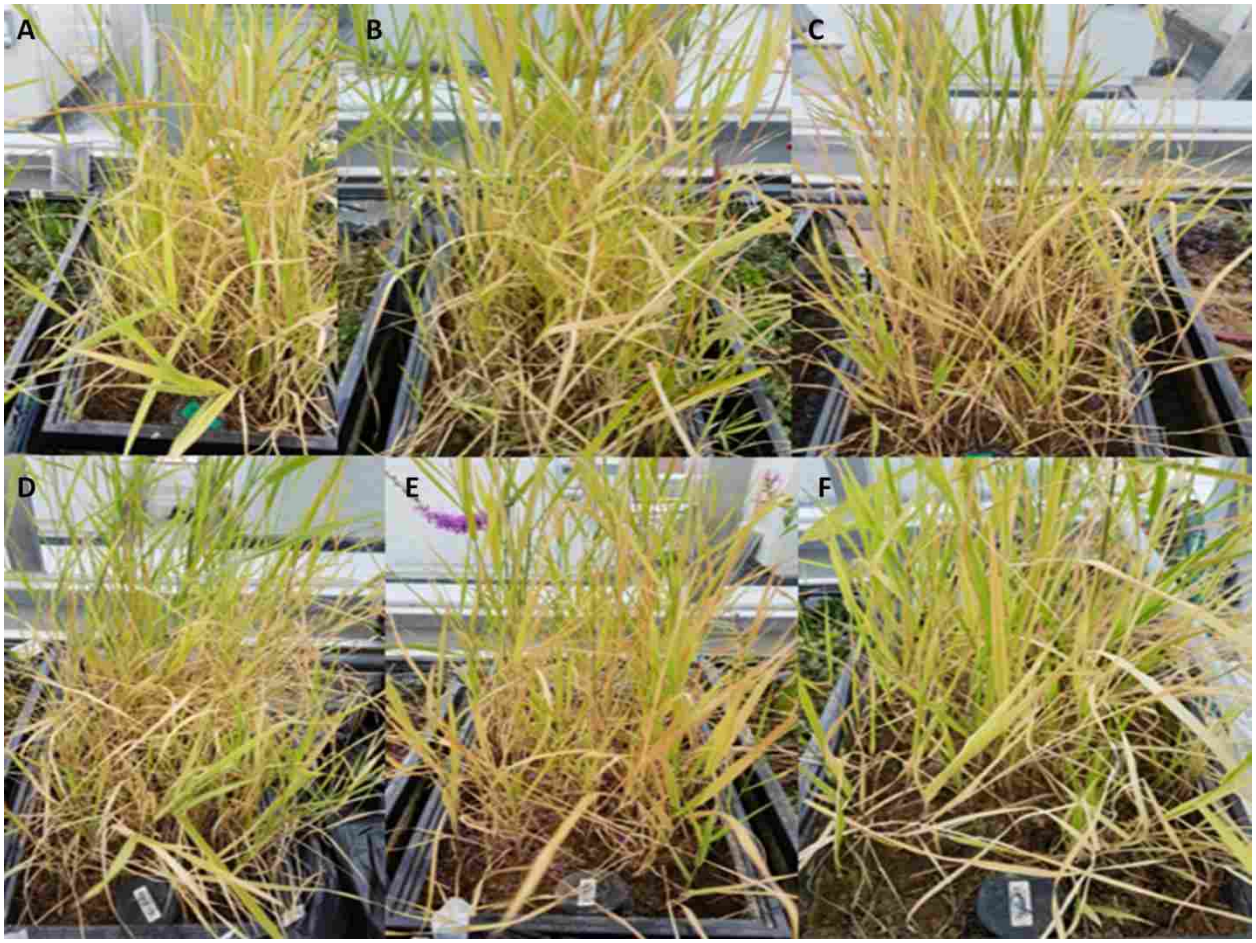

Figure 5.2 Mesocosms on May 8, 2017 (0DAE) planted with *Phalaris arundinacea.* Mesocosms A-C started receiving water from Doon (D) on this date and were subsequently referred to as D *Phalaris* #1 (A), #2 (B), and #3 (C). Mesocosms D-F continued to receive water from West Montrose (WM) throughout the rest of the experiment and were subsequently referred to as WM *Phalaris* #1 (D), #2 (E), and #3 (F).

Figure 5.3 Mesocosms on May 8, 2017 (0DAE) containing predominantly *Veronica anagallis-aquatica.* Mesocosms B, C and F started receiving water from Doon (D) on this date and were subsequently referred to as D *Veronica* #1 (B), #2 (C), and #3 (F). Mesocosms A, D and E continued to receive water from West Montrose (WM) throughout the rest of the experiment and were subsequently referred to as WM *Veronica* #1 (A), #2 (D), and #3 (E).

5.2.3 Sample Collection

Bacterial communities were assessed from interstitial water, inflow water from both the West Montrose and Doon sites as well as *P. arundinacea* and *V. anagallis-aquatica* rhizosphere and rhizoplane material as described in Chapter 2. Interstitial water samples were collected by placing a sterile glass bottle beneath the outflow port of each mesocosm and collecting the water as it drained from the tank. The surface of each mesocosm was divided into nine equal sized quadrants and rhizosphere and rhizoplane samples were collected from a different quadrant during each sampling event which was chosen randomly using a random number generator. Samples of water (inflow and outflow) and rhizosphere material were collected from the mesocosms monthly from December 2016 to May 2017 (experimental start-up) to monitor mesocosm development and bacterial community establishment so that the experiment could commence once the bacterial community in the mesocosms became stable. Following experimental start-up on May 8, 2017 when half of the wetland mesocosms began to receive Doon water, samples were obtained on 0, 14, 28, 49, 70 and 91 days after exposure (DAE). Rhizoplane samples were obtained on 0, 49 and 91 DAE only due to the invasive nature of removing large parts of the plant root systems. All samples were fully processed within 24 hours of collection and stored at 4 °C when not in use.

5.3 Results

5.3.1 Structural Profiles of Water, Rhizosphere and Rhizoplane-Associated Microbial Communities from Mesocosms Receiving High and Low Water Quality Treatments

At 0 DAE cluster analysis of microbial community structural profiles obtained from bacterial DNA isolated from inflow and outflow (interstitial) water, as well as rhizosphere and rhizoplane material, showed distinct structural profiles based on sample type. Rhizosphere microbial community structural profiles exhibited the highest degree of similarity among samples (82.7% similarity) (Fig 5.4). Rhizoplane microbial communities likewise showed a high degree of similarity among experimental replicates and treatments (78.9% similarity). Interstitial water samples exhibited 63.5 % similarity among experimental replicates and treatments, while inflow water communities showed only 48.6 % structural similarities to the rest of the microbial community sample types. Rhizoplane and water microbial communities were more closely related to each other (63.5% similarity) than to rhizosphere microbial communities (55.6% similarity). At 0 DAE all experimental replicates were receiving water from West Montrose (high water quality site). Experimental replicates marked to receive water from Doon (poor water quality site) did not exhibit structural similarity among each other within the rhizosphere and interstitial water microbial communities (as observed by clustering). Some clustering among the West Montrose and Doon treatment replicates was observed for the rhizoplane microbial community samples (clusters of 4 West Montrose and 4 Doon replicates) exhibiting 89.8% and 91.8% structural similarly, respectively. Overall, microbial community structural similarity within a sample type, by plant species, was not observed.

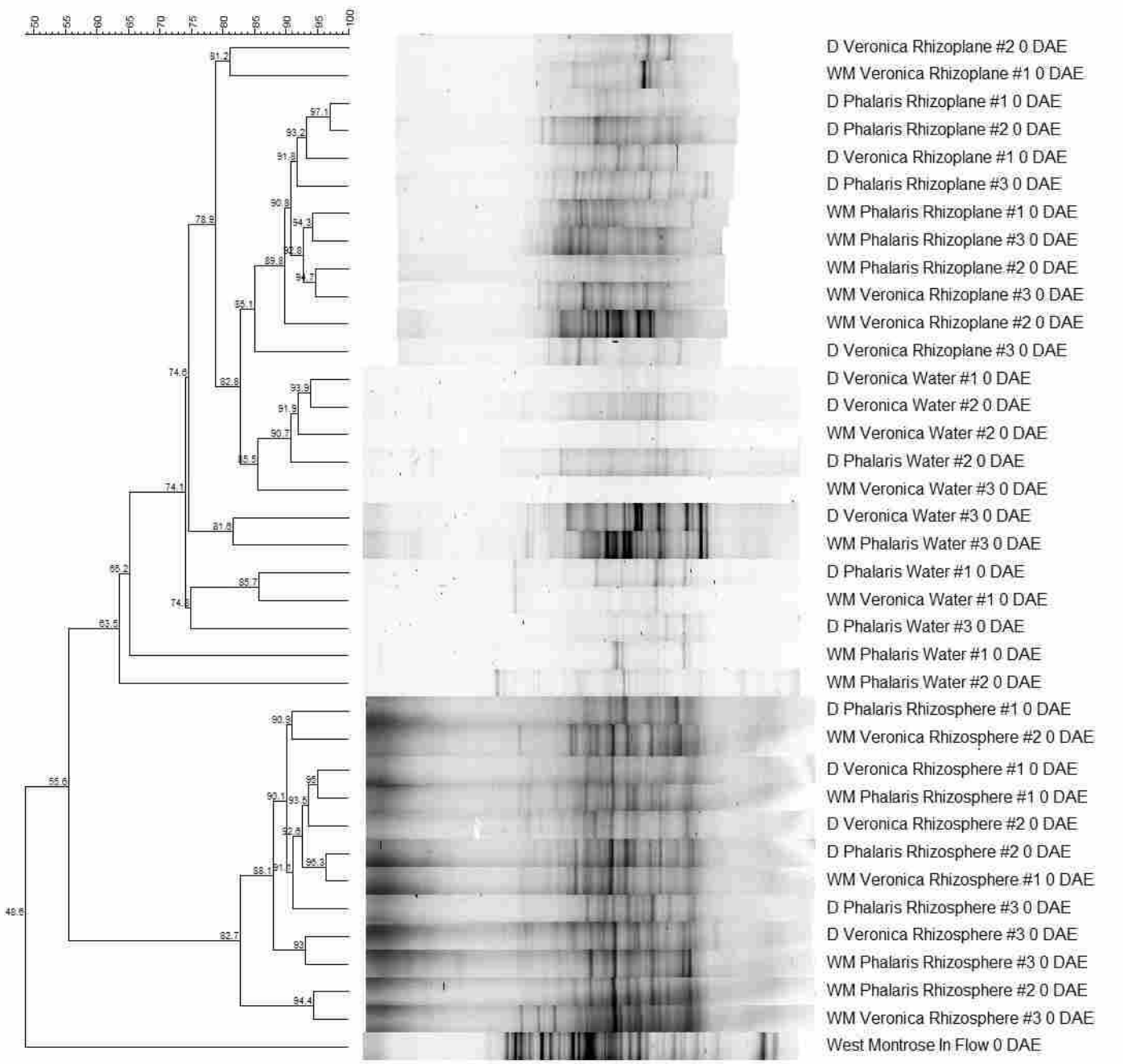

Figure 5.4 Hierarchical cluster analysis results of all DGGE profiles obtained from microbial DNA extracted at 0 DAE (all treatments receiving West Montrose water) demonstrated graphically as a UPGMA dendrogram. Mesocosms receiving high water quality inflow are indicated with a WM (West Montrose) and mesocosms marked to be receiving low water quality inflow are indicated with a D (Doon). Mesocosm replicates per treatment type are indicated with numbers 1-3. Percent similarity values are represented by the numbers above cluster branches.

Designated mesocosms had been exposed to Doon water (poor water quality site) for 14 days. West Montrose and Doon inflow water showed highly similar microbial community structural profiles (86.3% similarity) by 14 DAE. Microbial community structures from inflow water samples were very different from that of either the interstitial or rhizosphere microbial communities (26.3 % similarity) (Fig 5.5). The interstitial water microbial community structures were more similar to that of the rhizosphere microbial community than to the inflow water microbial community. All rhizosphere and interstitial water microbial communities exhibited 82.6 % and 65.4% structural similarity to each other, respectively. These similarity values are comparable to those observed at 0 DAE. Clustering based on water quality treatments was observed for some experimental replicates within the rhizosphere and interstitial water communities (approximately 3-4 replicates of 6). Structural similarity among communities associated with the same plant species was not observed.

At 28 DAE, inflow water from Doon and West Montrose was associated with microbial communities exhibiting similar structural profiles (91.1 % similarity) (Fig 5.6). This was consistent with previous observations at 0 and 14 DAE. Rhizosphere and interstitial water microbial community structural profiles exhibited 85.3% and 41.1% similarity, respectively. Unlike observations made at 14 DAE, inflow microbial communities were more structurally similar to rhizosphere communities than to interstitial water communities, with 71.1% similarity and 41.4% similarity, respectively. Relationships among structural community profiles receiving the same water quality treatment were strong and showed a high degree of similarity within each community type (interstitial water and rhizosphere). West Montrose water treated communities exhibited more structural similarity among experimental replicates than communities receiving Doon water. Interstitial water microbial communities receiving West Montrose water exhibited

81.5 % structural similarity compared to Doon treated microbial communities which only exhibited 41.1 % similarity. Comparatively, rhizosphere communities receiving West Montrose water exhibited 89.6% structural similarity compared to 85.3% structural similarity among communities receiving Doon water.

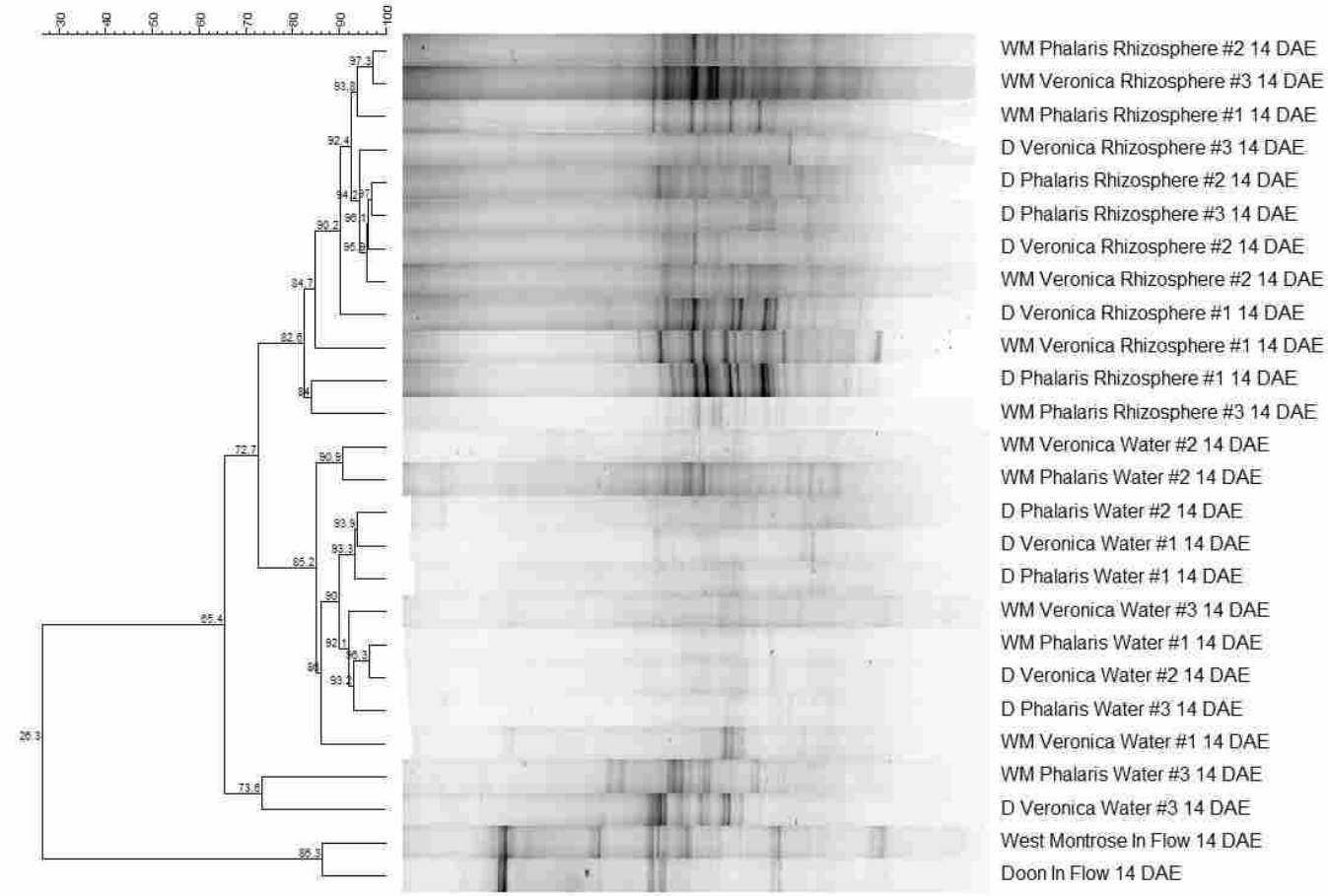

Figure 5.5 Hierarchical cluster analysis results of all DGGE profiles obtained from microbial DNA extracted at 14 DAE demonstrated graphically as a UPGMA dendrogram. Mesocosms receiving high water quality inflow are indicated with a WM (West Montrose) and mesocosms marked to be receiving low water quality inflow are indicated with a D (Doon). Mesocosm replicates per treatment type are indicated with numbers 1-3. Percent similarity values are represented by the numbers above cluster branches.

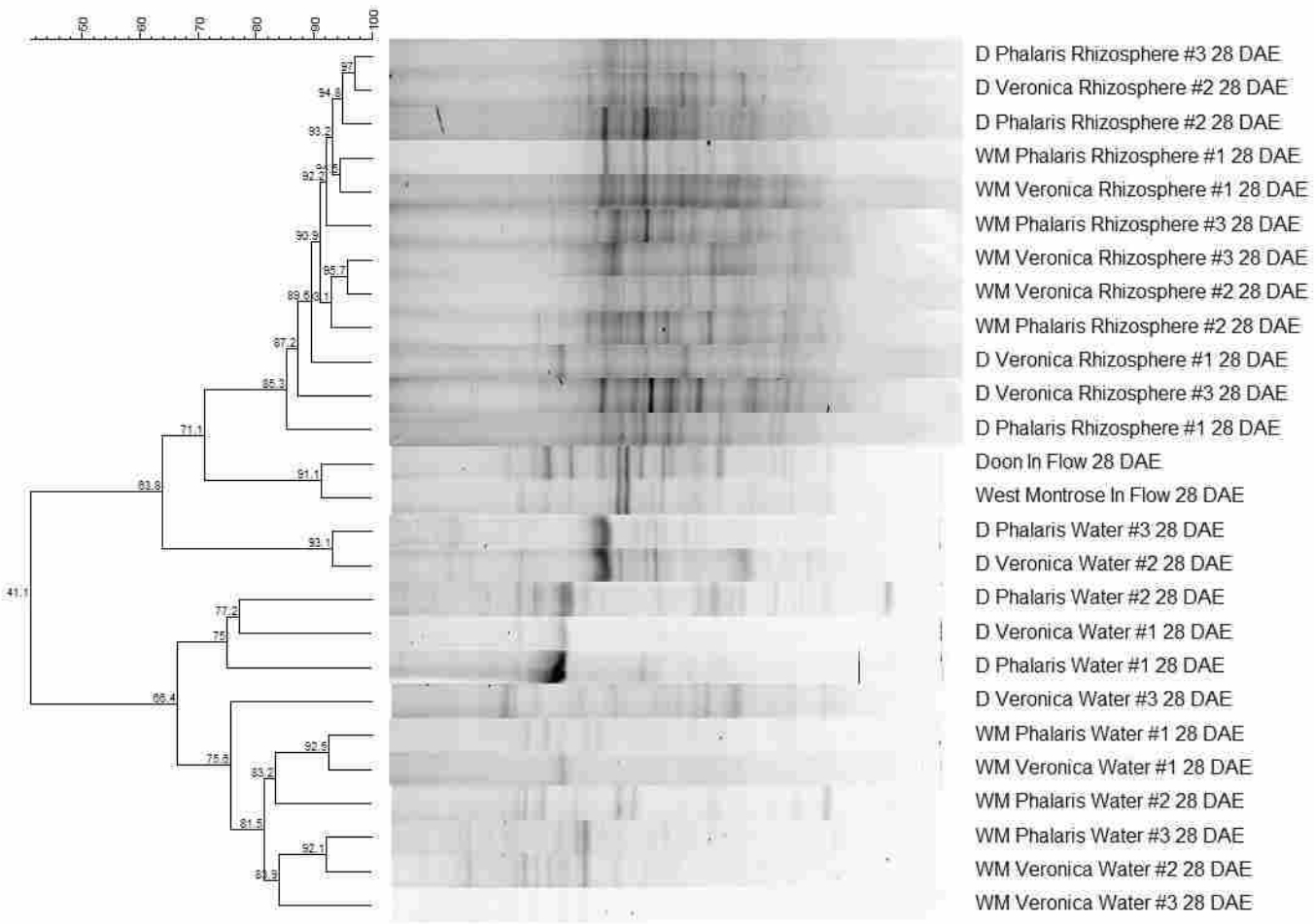

Figure 5.6 Hierarchical cluster analysis results of all DGGE profiles obtained from microbial DNA extracted at 28 DAE demonstrated graphically as a UPGMA dendrogram. Mesocosms receiving high water quality inflow are indicated with a WM (West Montrose) and mesocosms marked to be receiving low water quality inflow are indicated with a D (Doon). Mesocosm replicates per treatment type are indicated with numbers 1-3. Percent similarity values are represented by the numbers above cluster branches.

At 49 DAE West Montrose and Doon inflow water exhibited less structural similarity than observed at previous time points (76.4% similarity) (Fig 5.7). Rhizosphere and interstitial water microbial community structural profiles exhibited the highest degree of similarity by sample type, with 88.2% and 88% similarity, respectively. Rhizoplane microbial communities exhibited less similarity by sample type with 86.6% structural similarity. Inflow water samples exhibited the greatest degree of structural similarity with the microbial rhizosphere community (83.5 % similarity) as observed previously, while similarity was only 68.9% and 52.5% between rhizoplane and interstitial water communities, respectively. Within each community type, clustering based on water quality treatment was observed. Rhizoplane and rhizosphere microbial communities exhibited the highest degree of clustering among replicates receiving the same water quality treatment. Similar to observations made at 28 DAE, greater structural similarity was observed among communities receiving West Montrose water compared to Doon water for both rhizosphere and rhizoplane communities. Treatments receiving West Montrose water exhibited 90.2 and 90.7% structural similarity, respectively for rhizosphere and rhizoplane communities. Rhizosphere and rhizoplane communities receiving Doon water exhibited 88.2 and 86.6% structural similarity, respectively. The opposite was true for interstitial water communities, which exhibited 52.5 and 66% structural similarity for treatments receiving West Montrose and Doon inflow water, respectively.

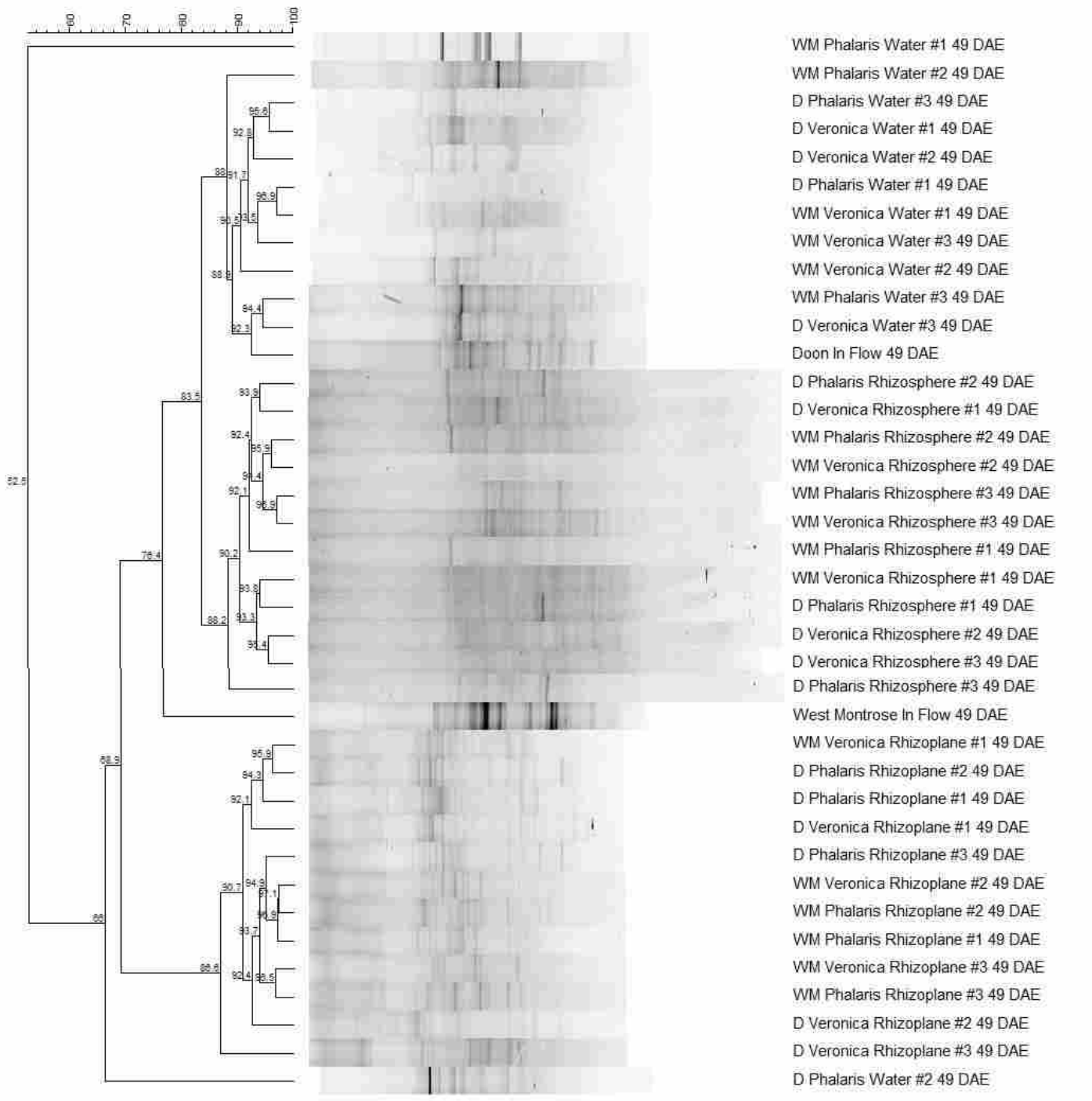

Figure 5.7 Hierarchical cluster analysis results of all DGGE profiles obtained from microbial DNA extracted at 49 DAE demonstrated graphically as a UPGMA dendrogram. Mesocosms receiving high water quality inflow are indicated with a WM (West Montrose) and mesocosms marked to be receiving low water quality inflow are indicated with a D (Doon). Mesocosm replicates per treatment type are indicated with numbers 1-3. Percent similarity values are represented by the numbers above cluster branches.

At 72 DAE the structure of West Montrose and Doon inflow communities were markedly different (32.6% similarity) and did not cluster together. The Doon inflow microbial community was most similar to rhizosphere community structural profiles, while the West Montrose inflow community was most similar to interstitial water microbial community structural profiles (Fig 5.8). A high degree of structural similarity was observed within the rhizosphere microbial community (86%) while only 32.6% was observed among interstitial water microbial communities. However, a single outlier that was also observed at 49 DAE (D *Phalaris* water #2) increases community structure similarity to 66.9% among interstitial water community profiles when excluded. Similar to observations at previous time points, clustering of structural community profiles was observed within water quality treatments but not within plant species treatments. West Montrose treatments exhibited more similarity within a sample type as compared to samples taken from mesocosms receiving Doon water. Rhizosphere communities receiving either West Montrose or Doon water exhibited 88.1% and 86 % similarity, respectively. Interstitial water microbial communities receiving West Montrose or Doon water exhibited 74.5 and 66.9% similarity, respectively (excluding outlier).

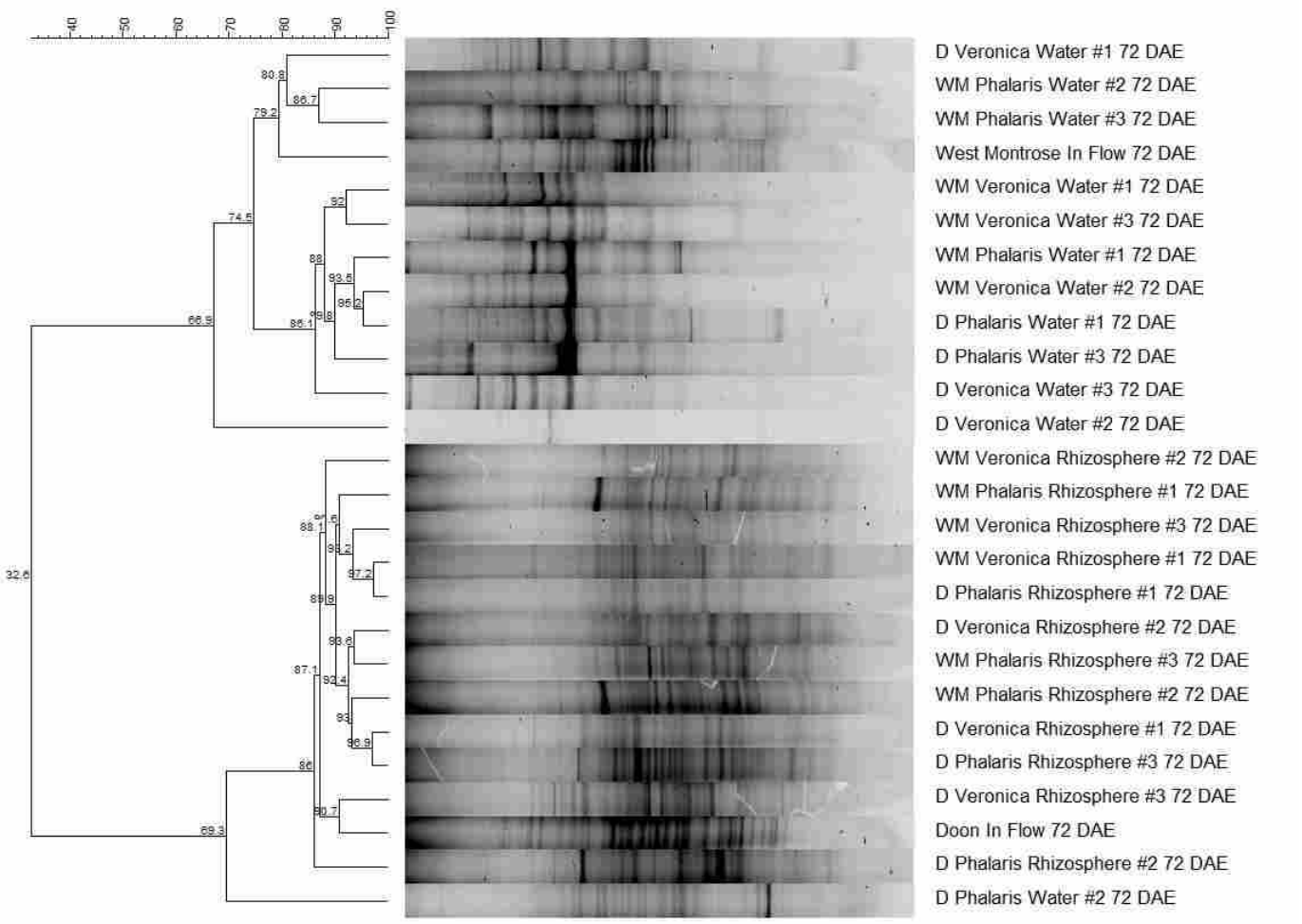

Figure 5.8 Hierarchical cluster analysis results of all DGGE profiles obtained from microbial DNA extracted at 72 DAE demonstrated graphically as a UPGMA dendrogram. Mesocosms receiving high water quality inflow are indicated with a WM (West Montrose) and mesocosms marked to be receiving low water quality inflow are indicated with a D (Doon). Mesocosm replicates per treatment type are indicated with numbers 1-3. Percent similarity values between clusters are indicated by the numbersabove branches.

By 91 DAE the structural community profiles of each community type showed increased variability compared to previous sampling time points (Fig 5.9). Several outliers were present in both the rhizoplane and interstitial water microbial community groupings (3 each). There was also more total variation among the samples of all community types when compared to other time points. Similar to earlier sampling time points, microbial community structures of inflow water communities in West Montrose and Doon water exhibited the strongest similarity compared to other sample types (69% similarity). Inflow water community structures were also most similar to rhizoplane community structures (47% structural similarity, excluding outliers). Rhizosphere community profiles exhibited the highest degree of similarity among replicates within the same sample type (84.1%), followed by rhizoplane (74.2% similarity excluding outliers, 26% including outliers) and interstitial water community profiles (46.5% similarity excluding outliers, 43.1 % including outliers). Clustering of structural community profiles within sample types by water quality treatment was observed at 91 DAE; however, this was to a lesser extent than observed previously (28-70 DAE). Within the rhizosphere community both water quality treatment groups exhibited 84.1% similarity, although treatments receiving Doon water showed a trend towards higher structural similarity than the West Montrose treated community; 5 of the 6 Doon treatments were contained within a cluster (89.4% similarity) compared to only 4 of 6 of the West Montrose treatments (92.2% similarity). Within the rhizoplane community, West Montrose water-treated microbial communities exhibited a higher degree of structural similarity (74.2% similarity among 5 of 6 treatments) than Doon water-treated microbial communities (only 2 replicates exhibited greater than 78% similarity). Interstitial water microbial communities exhibited the highest degree of variability and only exhibited limited structural similarity among samples from the same water quality treatment. Interestingly, for the first time

during the experimental period, the influence of plant species the associated microbial community structural profiles became apparent. Evidence for this included one cluster of *P. arundinacea* rhizoplane structural profiles (78.7% structural similarity) and one cluster of *V. anagallis-aquatica* microbial community profiles (67.3% similarity), which included both water and rhizoplane microbial communities.

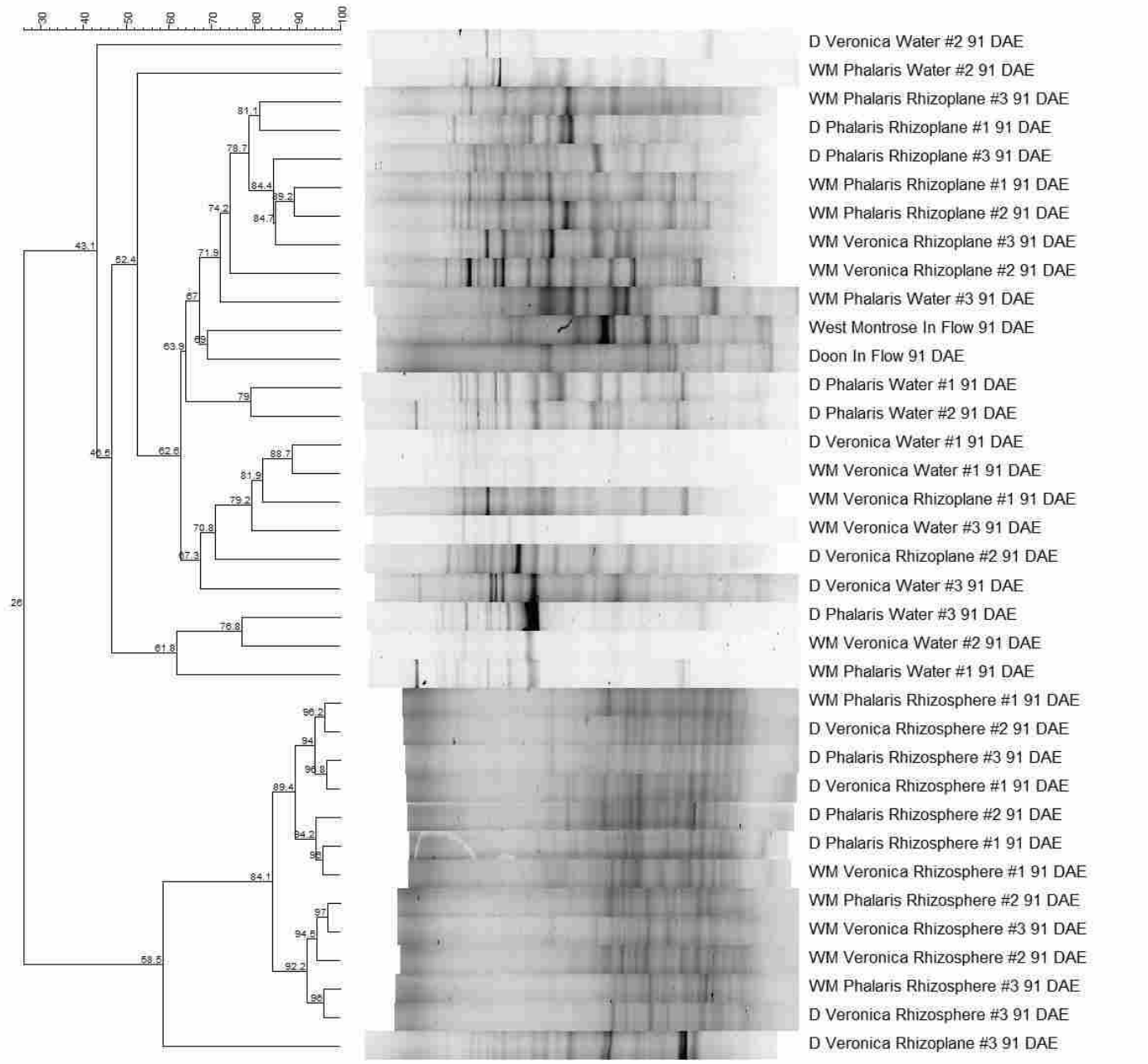

Figure 5.9 Hierarchical cluster analysis results of all DGGE profiles obtained from microbial DNA extracted at 91 DAE demonstrated graphically as a UPGMA dendrogram. Mesocosms receiving high water quality inflow are indicated with a WM (West Montrose) and mesocosms marked to be receiving low water quality inflow are indicated with a D (Doon). Mesocosm replicates per treatment type are indicated with numbers 1-3. Percent similarity values between clusters are indicated by the numbersabove branches.

For the majority of the sampling events, within treatment and sample type community structural similarity was highest in the rhizosphere and the rhizoplane and lowest in the interstitial water microbial communities (Table 5.1).Within the same sample type and treatment, structural community similarity seemed to steadily increase within the Doon treated communities until sampling at 72 DAE when structural similarity among interstitial water communities began to decline. Sampling at 91 DAE exhibited a large decrease in structural similarity within the rhizoplane and water-associated microbial communities.

Table 5.1 Summary of Within Group Percent Similarity for Microbial Community Structural Profiles as Determined by PCR-DGGE-Based Hierarchical Cluster Analysis

			0 DAE	14 DAE	28 DAE	49 DAE	72 DAE	91 DAE
Rhizoplane		West Montrose	89.8%	N/A	N/A	90.7%	N/A	74.2%*(1)
		Doon	85.1%	N/A	N/A	86.6%	N/A	26%
Rhizosphere		West Montrose	82.7%	82.6%	90.9%	90.2%	88.1%	84.1%
		Doon	88.1%	82.6%	85.3%	88.2%	86%	89.4%*(1)
Water	**Inflow**	West Montrose	N/A**	86.3%	91.1%	76.4%	32.6%	69%
		Doon						
	Interstitial (outflow)	West Montrose	63.5%	65.4%	81.5%	88%*(1)	74.5%	46.5%*(2)
		Doon	74.1%	65.4%	66.4%	88.9%*(1)	66.9%*(1)	62.6%(1)

*Percent similarity value excludes outliers and the numbers of outliers excluded are indicated in the brackets

** Percent similarity between inflow water communities represents the percent of structural similarity between the river water microbial communities collected from West Montrose and Doon

5.3.2 Functional Carbon Source Utilization Profiles Associated with Microbial Communities from Mesocosms Receiving High and Low Water Quality Treatments

5.3.2.1Principal Component Analysis of Carbon Source Utilization by Rhizoplane, Rhizosphere and Water-Associated Microbial Communities from Mesocosms

Principal component analysis (PCA) was performed on Biolog^{TM} EcoPlate OD_{590} readings for all samples after 96 hrs of incubation. PC1 vs. PC2 plots from all mesocosm samples at 0 DAE (Fig 5.10 A-C) shows that all three sample types (rhizoplane, rhizosphere, and water) exhibited no clear differences by water quality treatment, prior to the addition of the poor water quality treatment (Doon). Within the rhizoplane, by 49 DAE (Fig 5.10 D), carbon source utilization profiles for both plant species were unique among the high (West Montrose) and poor (Doon) water quality treatments. Doon *V. anagallis-aquatica* and West Montrose *V. anagallis-aquatica* treatments were primarily separated along the PC1 axis (28.43% variance), while West Montrose *P. arundinacea* and Doon *P. arundinacea* functional community profiles were separated along the PC2 axis (19.00% variance) (Fig 5.10 D). These differences became more exaggerated by 91 DAE (Fig 5.10 G), exhibiting greater differentiation among water quality treatments for both plant species. *V. anagallis-aquatica* treatments continued to be separated along the PC1 axis (28.27% variance) while *P. arundinacea* water quality treatments were separated by the PC2 axis (15.56% variance) (Fig 5.10 G). Rhizoplane communities associated with *P. arundinacea* at 49 DAE were differentiated by the usage of carbon sources containing nitrogen (amino acids and amines) as well as polymeric compounds (Tween 80) and several carboxylic acids (ketobutyric acid) in the poor water quality treatment, while the high water quarter treatment was associated primarily with the usage of carbohydrates and carboxylic acids (itaconic acid and γ-hydroxybutyric acid). By 91 DAE the water quality treatments were still

differentiated by the use of nitrogen containing carbon sources (poor water quality treatment) and several carboxylic acids, while the high water quality treatment was discriminated by the use

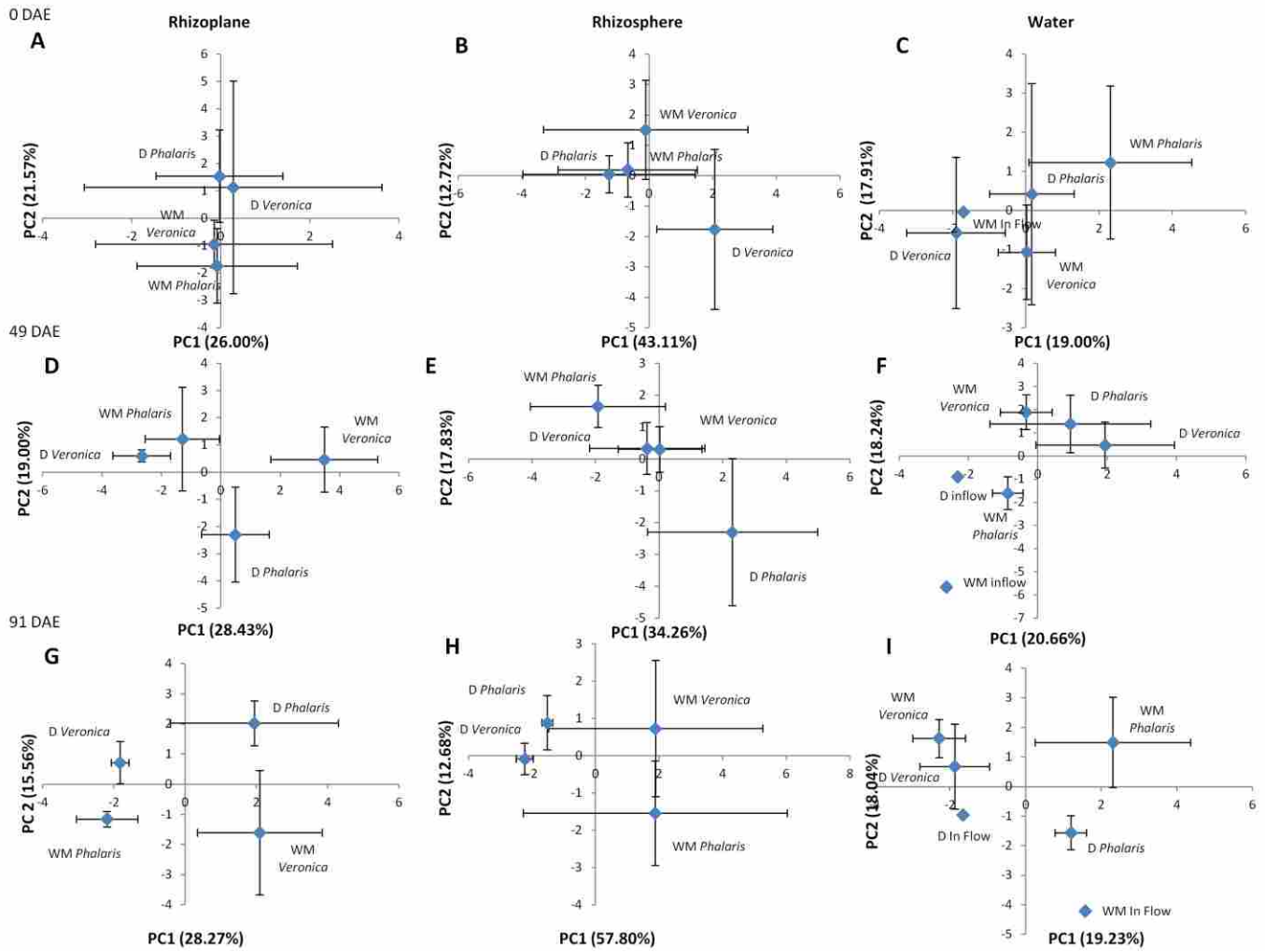

Figure 5.10 PC1 vs. PC2 plots of Biolog^{TM} carbon source utilization profiles generated from microbial communities in the rhizoplane (A, D, G), rhizosphere (B, E, D) and interstitial/inflow water (C, F, I) at 0 (A-C), 49 (D-F) and 91 (G-I) DAE. Mesocosms receiving high water quality inflow are indicated with a WM (West Montrose) and mesocosms receiving low water quality inflow are indicated with a D (Doon). Values are means of 3 experimental replicates and three technical replicates plus standard error for each treatment. Values for in flow water represent the average of a single experimental replicate with triplicate technical replicates only.

of simple carbohydrates only. Microbial rhizoplane communities from the poor water quality treatment associated with *V. anagallis-aquatica* were discriminated from the high water quality treatment primarily through differential usage of several polymers, carbohydrates and carboxylic acids at both time points (e.g. cyclodextrine, lactose, glucosaminic acid) while the high water quality treatment was differentiated by the use of several polymers, carbohydrates, carboxylic acids and amino acids (e.g. Tween 40, 4-hydroxybenzoic acid, arginine, serine, putrescine) (Table A8).

Within the rhizosphere (Fig 5.10 E) no clear differentiation between carbon source utilization profiles associated with *V. anagallis-aquatica* water quality treatments were observed by 49 DAE. However, carbon source utilization profiles associated with *P. arundinacea* receiving high and low water quality treatments were distinct and primarily separated along the PC2 axis (17.83% variance). By 91 DAE differences among water quality treatments increased (Fig 5.10 H). The carbon source utilization profiles associated with *V. anagallis-aquatica* became apparent at this time and were separated along the PC1 axis (57.80% variance). Carbon source utilization profiles associated with *P. arundinacea* rhizosphere communities remained distinct between water quality treatments and were separated primarily along the PC2 axis (12.68% variance). Differentiation between high and low water quality treatments in the rhizosphere of *P. arundinacea* at 49 DAE was due to differential use of multiple carbon sources. The poor water quality treatment was differentiated by usage of a polymer, carbohydrate and amine while the high water quality treatment was differentiated by usage of a carbohydrate and several carboxylic acids (Table A9). By 91 DAE the compounds resulting in differences between water quality treatments changed and the poor water quality treatment was differentiated by usage of carbohydrates and carboxylic acids while the high water quality treatment was using

differentiated by usage of a polymer and amino acid. In the rhizosphere of *V. anagallis-aquatica* the high and low water quality treatments were differentiated by usage of many different compounds from all different carbon source types with no clear patterns emerging.

Interstitial and inflow water communities were initially somewhat differentiated between plant species before treatment began and inflow carbon source utilization profiles were indistinct from interstitial communities (Fig 5.10 C). After the start of water quality treatments, by 49 DAE (Fig. 4F), *V. anagallis-aquatica* water quality treatments did not exhibit distinct carbon source utilization profiles. *P. arundinacea* associated communities were distinct primarily along the PC2 axis (18.24% variance) (Fig 5.10 F). Differences among treatments increased by 91 DAE, however interstitial water communities from *V. anagallis-aquatica* treatments remained undifferentiated. *P. arundinacea* associated interstitial water communities exhibited further differentiation along the PC2 axis (18.04% variance) (Fig 5.10 I). At 49 DAE the poor water quality treatment associated with *P. arundinacea* was differentiated by the usage of several carboxylic acids and the amino acid threonine, while the high water quality treatment was differentiated by usage of a polymer, a carbohydrate and several carboxylic and amino acids. The differentiating compounds change by 91 DAE, and the poor water quality treatment was associated with the usage of several polymers and amino acids as well as the carboxylic acid γ-hydroxybutyric acid (Table A10). The high water quality treatment was largely differentiated by usage of pyruvic acid methyl ester and D-galacturonic acid.

5.3.2.2 Percent Utilization of Different Biolog™ Carbon Source Types by Rhizoplane, Rhizosphere and Water-Associated Microbial Communities from Mesocosms

Within the rhizoplane the relative usage of the four different classes of carbon sources present in Biolog™ EcoPlates (polymers, carbohydrates, carboxylic and acetic acids and nitrogenous compounds) was similar among the four treatment groups (Fig 5.11A). For all treatments, relative usage of carbohydrates (10 of 31 carbon sources) was highest having between 28 and 31% of total usage, followed by nitrogen containing carbon sources (8 of 31 carbon sources) at 26-29% of total usage, then carboxylic and acetic acids (9 of 31 carbon sources) with between 23 and 25% of total usage, and finally polymers (4 of 31 carbon sources) which represented 19-21% of total carbon source utilization. More variability between treatments was present within the rhizosphere (Fig 5.11B). Rhizosphere communities associated with high water quality treated *P. arundinacea* mesocosms exhibited more usage of carbohydrate based carbon sources, while the poor water quality treatment used a greater percentage of nitrogenous carbon sources. The rhizosphere community associated with *V. anagallis-aquatica* receiving the high water quality treatment used a higher percentage of carboxylic/acetic acids and nitrogenous carbon sources compared to the poor water quality treatment which exhibited stronger utilization of carbohydrates. Both rhizosphere and rhizoplane communities exhibited a relatively high preference for polymers (19-21% total usage) considering these carbon sources represented only 13% of the carbon sources available on the Biolog™ EcoPlate. Interstitial and inflow water communities exhibited the greatest amount of variability in relative carbon source usage both among treatments and among time periods (Fig 5.11C). *P. arundinacea* associated communities receiving high water quality inflow had a higher relative usage of carboxylic and acetic acids, while the poor water quality treatment showed a preference for carbohydrates. Communities associated with *V. anagallis-aquatica* receiving high water quality inflow had the highest usage

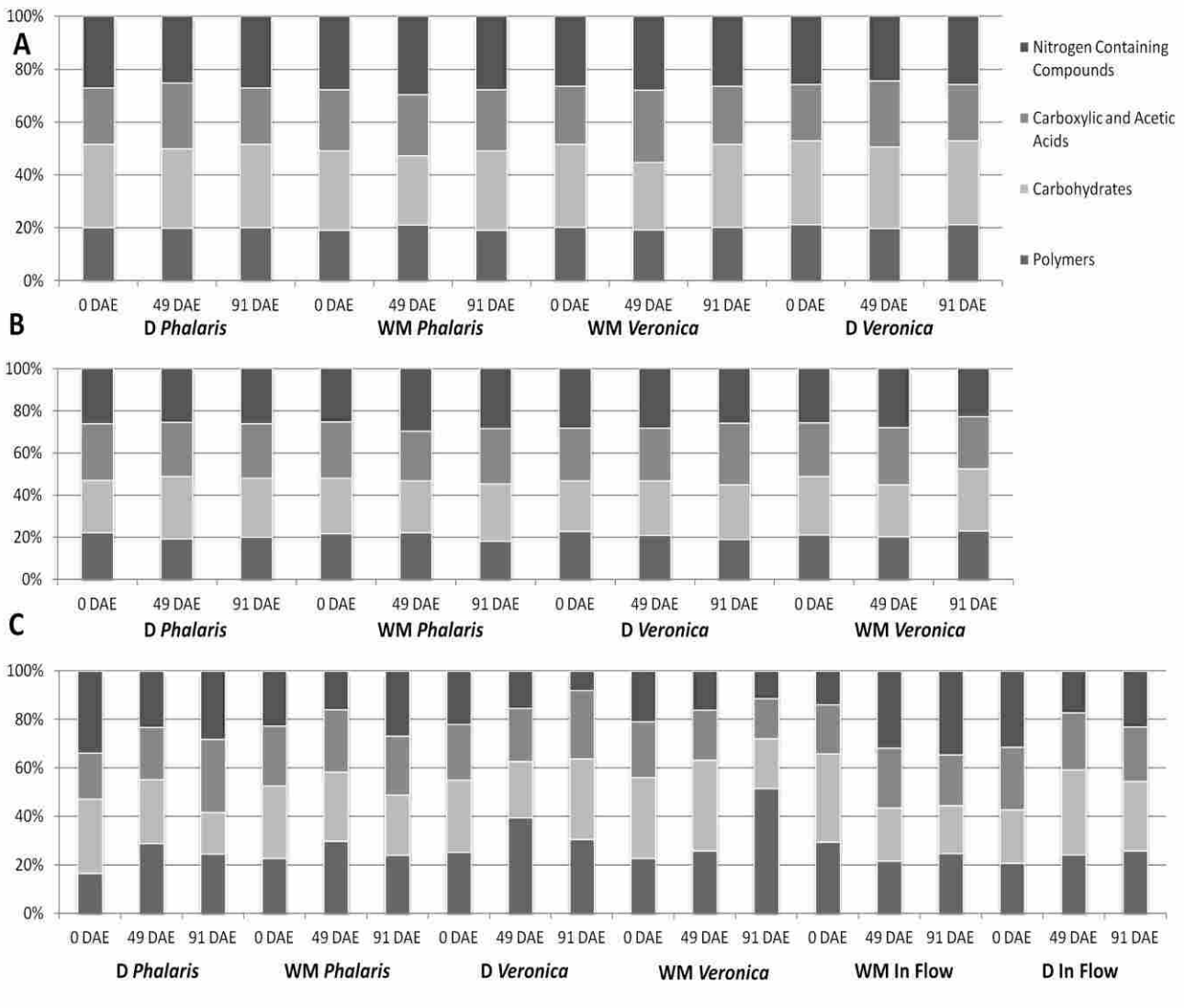

Figure 5.11 Percentage of total utilization for different carbon source types by microbial communities in the rhizoplane (A), rhizosphere (B) and interstitial/inflow water (C) calculated from Biolog^{TM} EcoPlate absorbance readings after 96h of incubation. Communities exposed to high water quality are indicated with a WM (West Montrose) and with a D (Doon) for poor water quality. Each bar represents average values for 3 experimental replicates and 3 technical replicates, or 1 experimental replicate and 3 technical replicates for inflow communities.

of carbohydrates while the poor water quality treatment showed a preference for polymers. Compared to *P. arundinacea* associated communities, *V. anagallis-aquatica* communities had higher usage of carbohydrates and a much lower usage of nitrogenous compounds. Inflow water communities had a more balanced distribution of carbon source usage among the groups and the primary difference between the two inflow source water communities was higher usage of carbohydrates in the poor water quality source and a greater usage of nitrogen containing compounds in the high water quality source, especially during the later sampling events.

5.3.2.3 Metabolic Diversity

Differences in metabolic diversity or metabolic potential, as measured using the Shannon Diversity Index on carbon source utilization readings revealed differences among treatments and community types. Within the rhizoplane (Fig 5.12 A) metabolic diversity was consistently higher in the *P. arundinacea* high water quality treatment compared to *V. anagallis-aquatica*. The rhizosphere did not exhibit any consistent differences in metabolic diversity among treatments (Fig 5.12 B). Interstitial water communities exhibited reoccurring differences between the poor water quality treatments, with *P. arundinacea* exhibiting greater diversity compared to *V. anagallis-aquatica* (Fig. 5.12C). The poor water quality inflow community tended to have higher metabolic diversity compared to the high water quality source at most sampling events.

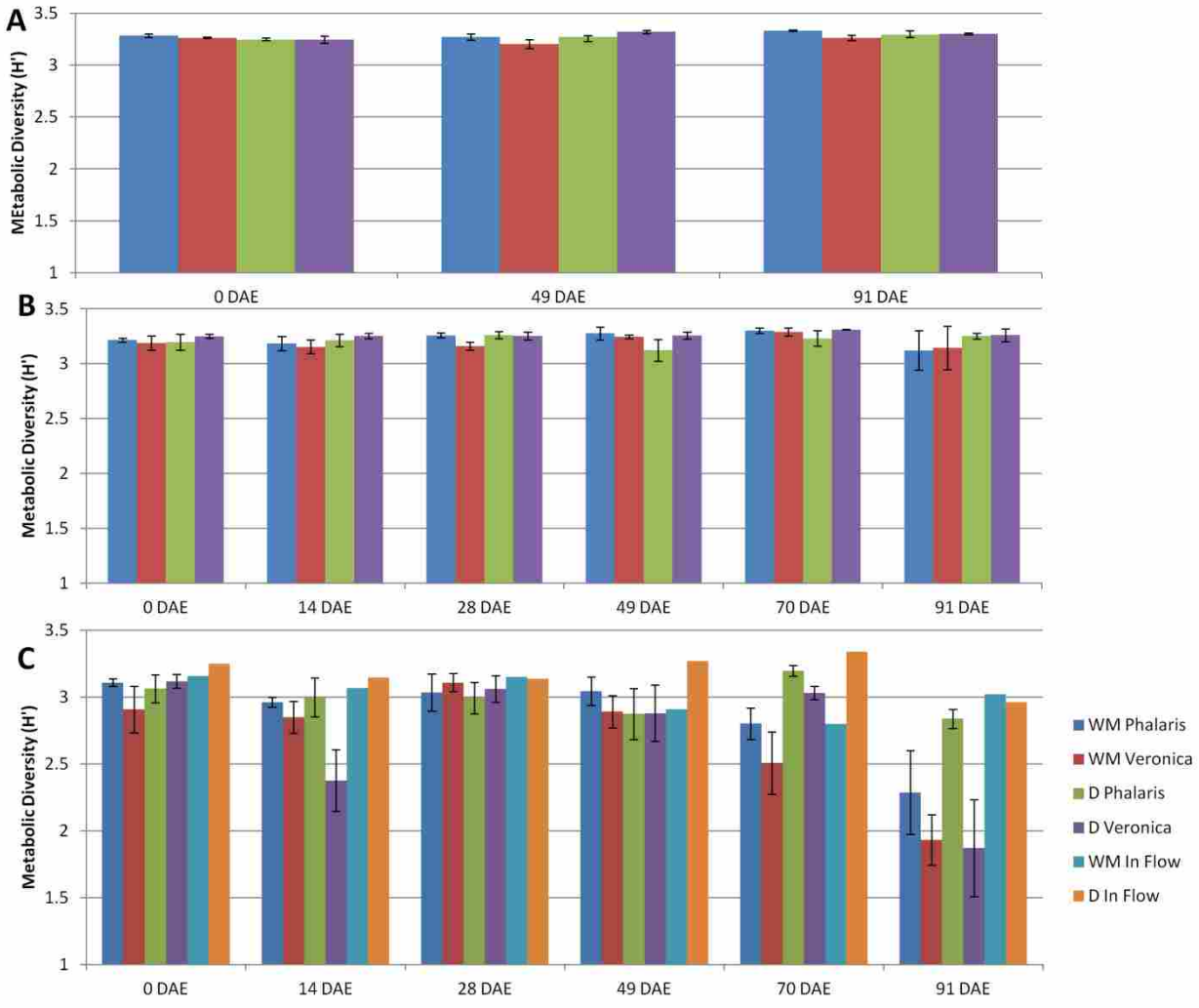

Figure 5.12 Metabolic diversity of microbial communities in mesocosm rhizoplane (A), rhizosphere (B) and interstitial/inflow water (C) as determined by Biolog^{TM} EcoPlate carbon source usage following 96h of incubation. Communities exposed to high water quality are indicated with a WM (West Montrose) and with a D (Doon) for poor water quality. Values represent the average of 3 experimental replicates and 3 technical replicates with the exception of inflow samples which represent the average of 3 technical replicates. Error bars represent standard error from experimental replicates only.

5.3.3 Water Chemistry and Water Quality Variables Associated with Mesocosms Receiving either High or Low Water Quality Treatments

5.3.3.1 Differences in Water Chemistry between West Montrose and Doon Grand River Locations

Average unionized ammonia concentrations differed by almost two-fold between the two sites across sampling events (0.13 ± 0.05 mg/L in West Montrose and 0.24 ± 0.04 mg/L in Doon, P=0.14, Student's T-test) and both sites consistently exhibited concentrations of unionized ammonia above the recommendations for surface waters provided by the provincial water quality objectives (PWQO) (0.0165 mg/L, Loomer and Cooke). Similarly, average nitrite concentrations measured in water from the Doon site (0.12 ± 0.03 mg/L) were greater than double those measured from West Montrose water (0.05 ± 0.01 mg/L, P=0.09). Only the Doon site typically exhibited nitrite concentrations above PWQO (0.06 mg/L, Loomer and Cooke, 2011). Average nitrate concentrations were also higher in Doon water (10.9 ± 0.76 mg/L) compared to West Montrose water (7.9 ± 1.5 mg/L, P=0.14), however, the difference between sites was relatively small. Nitrate concentrations at both sites were above the target value set by PWQO (2.93 mg/L, Loomer and Cooke, 2011) during most sampling events. Sites-specific differences were least significant for total reactive phosphorus concentrations. Doon exhibited slightly higher concentrations of TRP (0.15 ± 0.04 mg/L) compared to West Montrose (0.1 ± 0.03 mg/L, P=0.37), which were both considerably higher than the PWQO (0.03 mg/L, Loomer and Cooke, 2011). There was significant variability in fecal coliform bacteria recovered from the different water quality sites among sampling events, but on average more fecal coliforms were recovered from the poor water quality site (Doon) ($4 \times 10^5 \pm 3.4 \times 10^5$ CFU/mL) than from high water quality site (West Montrose) ($7 \times 10^3 \pm 5 \times 10^3$ CFU/mL) (P=0.24).

5.3.3.2 Nitrate

Quantities of nitrate detected in outflow water showed an increasing trend over the course of the experiment for all treatments (Fig 5.13). Additionally, levels of nitrate in outflow water were similar between Doon and West Montrose-treated mesocosms, as well as between mesocosms planted with *P. arundinacea* and *V. anagallis-aquatica*. Both outflow and inflow water nitrate concentrations were typically above the provincial water quality objectives for nitrate in surface water sources, especially beyond 49 DAE (Loomer and Cooke, 2011). The mesocosms removed nitrate from the system during most of the sampling time points, however, there was a large degree of variability in the amount of nitrate detected in the outflow as a percentage of the inflow concentration (between 17 and 109%). The average removal efficiency for nitrate over all sampling events ranged from 65% to 34% (35% and 66% of inflow detected in outflow) among treatments. Overall, the *V. anagallis-aquatica* mesocosms receiving Doon water had a higher nitrate removal efficiency than the *V. anagallis-aquatica* mesocosm receiving West Montrose water. Similarly, the *P. arundinacea* mesocosms receiving Doon water also had higher average nitrate removal efficiency than the *P. arundinacea* mesocosms receiving West Montrose water. Nitrate removal efficiencies between plant species treatments within a water quality treatment were not different.

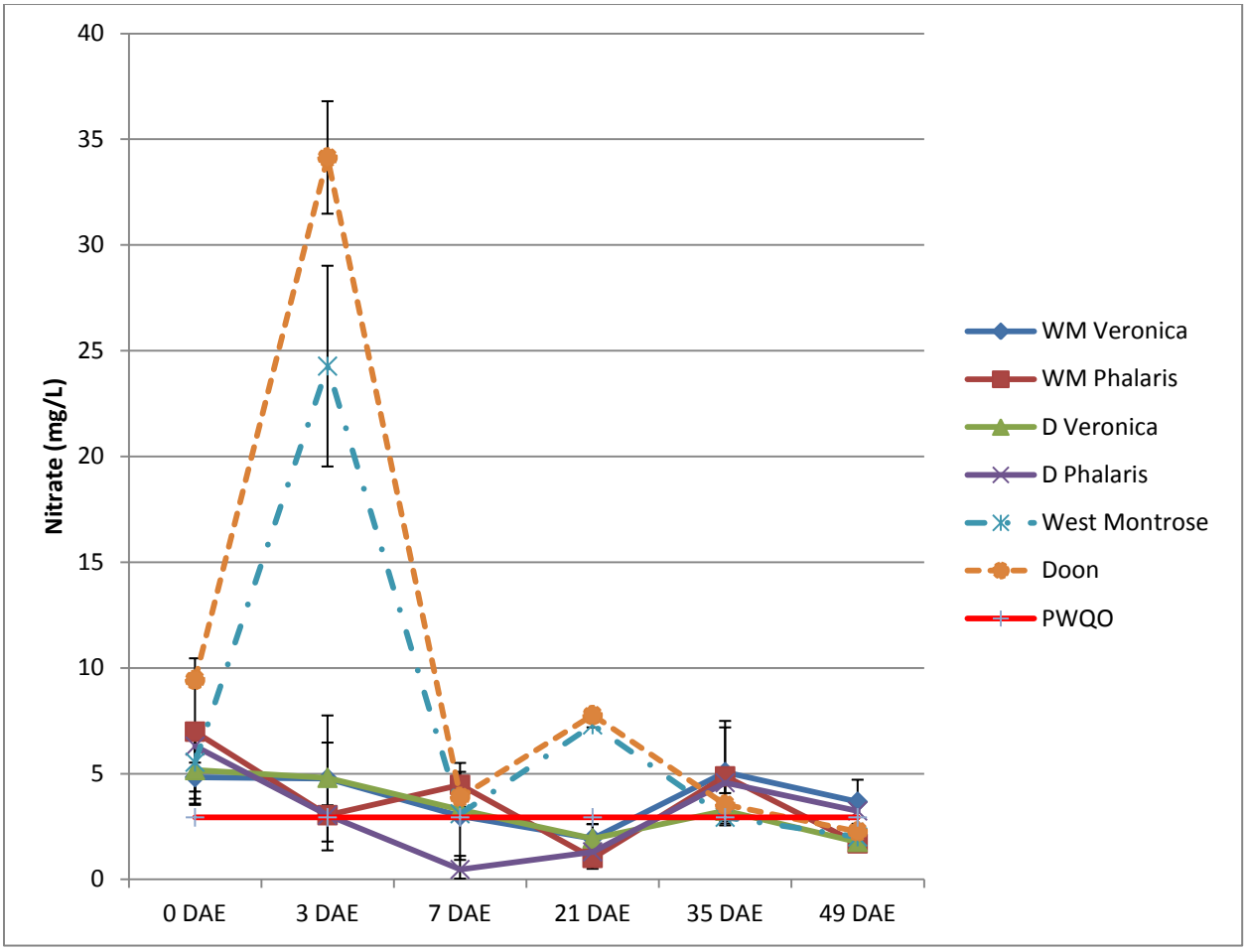

Figure 5.13 Nitrate quantification (mg/L) in mesocosm outflow water and inflow water (West Montrose (WM) and Doon (D)) using HACH® water test kits. The Ontario provincial water quality objective (PWQO) for nitrate in surface water sources is indicated in red. Inflow water is designated with a dashed line while outflow water is indicated with a solid line. Each data point is the average of three experimental replicates for outflow water samples and one experimental replicate for inflow samples. Error bars are representative of standard error.

5.3.3.3 Nitrite

Nitrite concentrations showed similar trends to that of nitrate, with inflow water concentrations tending to be higher than outflow water concentrations (Fig 5.14). As well, nitrite concentrations in Doon water were higher than in West Montrose water across all sampling events. Differences in outflow water nitrite concentrations were not observed among treatments and outflow values for nitrite were consistently below that of the provincial water quality standard for nitrite in surface water sources. The percentage of nitrite detected in the inflow water that was measured in the outflow water of mesocosms was not consistent throughout the experimental timeline, with between 0 and 97 percent of the nitrite entering the system being detected in the collected outflow water. The average amount of nitrite being removed by the mesocosms over the course of the experiment was between 64 and 73% (36 and 27% of inflow detected in outflow). No significant differences were observed among treatments.

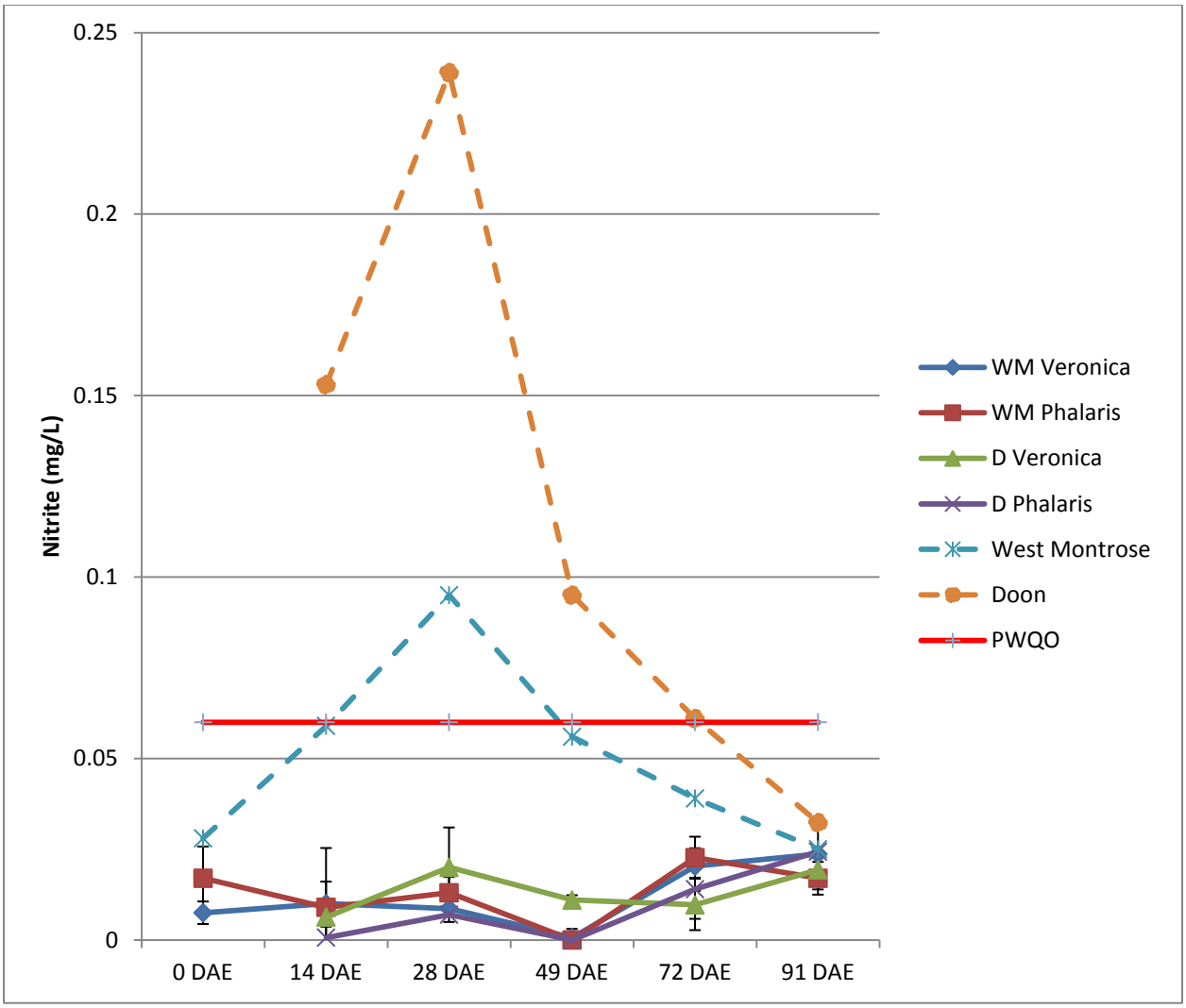

Figure 5.14 Nitrite quantification (mg/L) in mesocosm outflow water and inflow water (West Montrose (WM) and Doon (D)) using HACH® water test kits. The Ontario provincial water quality objective (PWQO) for nitrite in surface water sources is indicated in red. Inflow water is designated with a dashed line while outflow water is indicated with a solid line. Each data point is the average of three experimental replicates for outflow water samples and one experimental replicate for inflow samples. Error bars are representative of standard error.

5.3.3.4 Ammonia

Unionized ammonia concentrations were similar between inflow and out flow water samples (Fig 5.15). Doon water tended to contain higher levels of unionized ammonia compared to West Montrose water on most sampling events. Differences in outflow water ammonia concentrations were not observed between the different treatment types and both inflow and outflow ammonia concentrations exceeded the provincial water quality guidelines for unionized ammonia in source waters for most sampling time points. The percentage of ammonia in the inflow water that was present in the outflow water of mesocosms (removal efficiency) was variable across time points and between treatments. The average ammonia removal efficiency during the experiment was considerably lower for West Montrose treatments compared to the Doon treatments. Mesocosms receiving Doon water removed an average of approximately four times more ammonia throughout the course of the experiment than mesocosms being fed with West Montrose water. Within water quality treatments, *V. anagallis-aquatica* and *P. arundinacea* ammonia removal efficiencies were comparable.

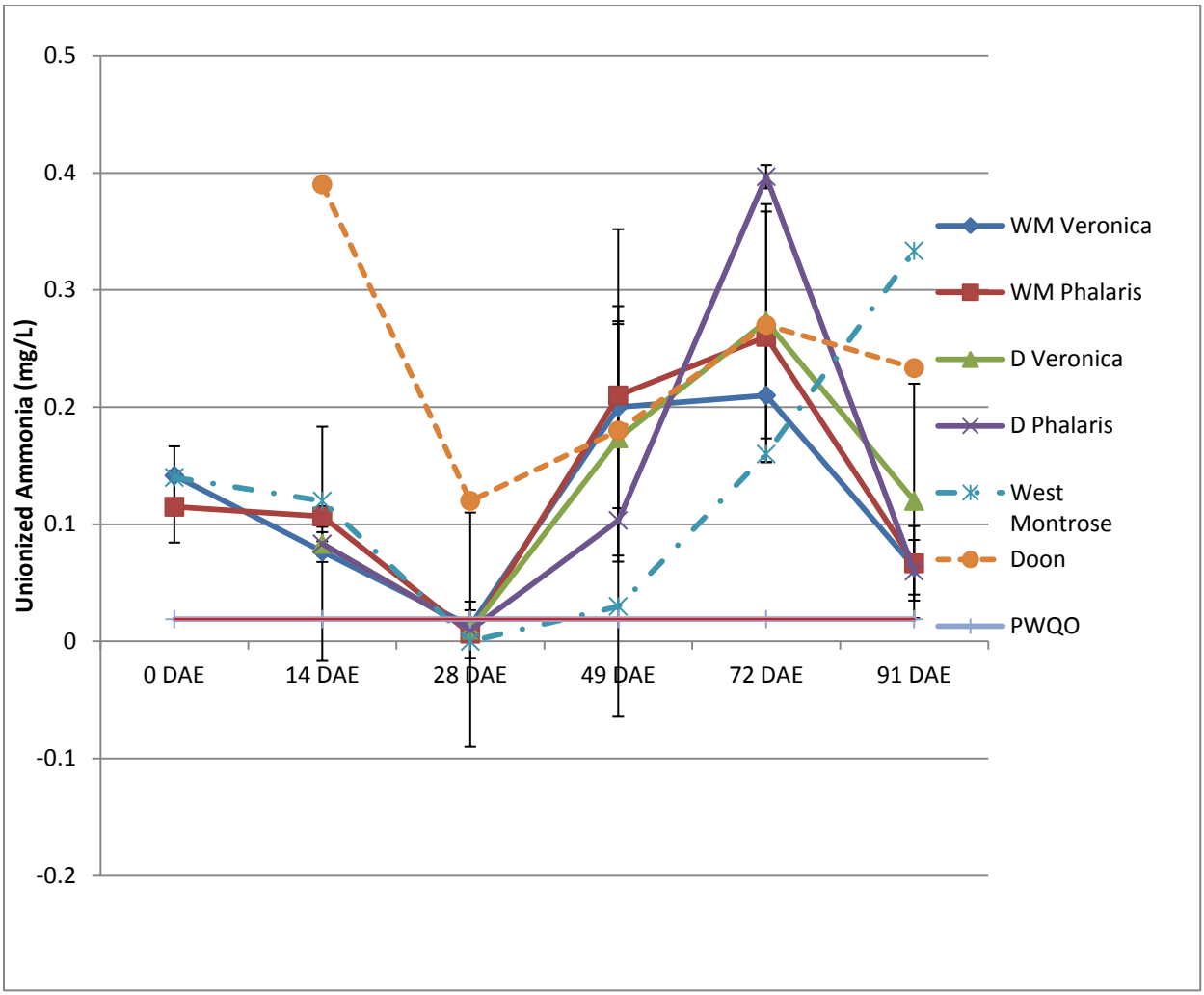

Figure 5.15 Unionized ammonia quantification (mg/L) in mesocosm outflow water and inflow water (West Montrose (WM) and Doon (D)) using HACH® water test kits. The Ontario provincial water quality objectives (PWQO) for nitrite in surface water sources is indicated in red. Inflow water is designated with a dashed line while outflow water is indicated with a solid line. Each data point is the average of three experimental replicates for outflow water samples and one experimental replicate for inflow samples. Error bars are representative of standard error.

5.3.3.5 Total Reactive Phosphorus

The concentration of total reactive phosphorus in Doon and West Montrose inflow water were similar to each other throughout the sampling period and were both either equivalent to or less than the concentration of total reactive phosphorus present in outflow water across sampling events (Fig 5.16). The concentration of total reactive phosphorus in mesocosm outflow water was highly variable across sampling time points. Additionally, there were large fluctuations in outflow total reactive phosphorus concentrations between mesocosm treatments at most time points, particularly at 14 and72 DAE. These variations coincide with time points when inflow concentrations of total reactive phosphorus in the West Montrose and Doon source waters were significantly lower than that of what was measured in the outflow water. Total reactive phosphorus concentrations measured from *P. arundinacea* mesocosms treated with Doon water showed an increasing trend over the course of the experiment, however, all other treatments did not exhibit this trend and fluctuated from one sampling time point to another. By observing the concentration of total reactive phosphorus in mesocosm outflow water as a percentage of the inflow concentration it is apparent that the amount of reactive phosphorus entering the mesocosms was consistently lower than the outflow concentrations for the majority of sampling time points. The average amount of reactive phosphorus leaving the system ranged from two to four times the concentration of total reactive phosphorus measured in the inflow water. The percentage of TRP measured in inflow water, present in mesocosm outflow water was not significantly different between treatment types.

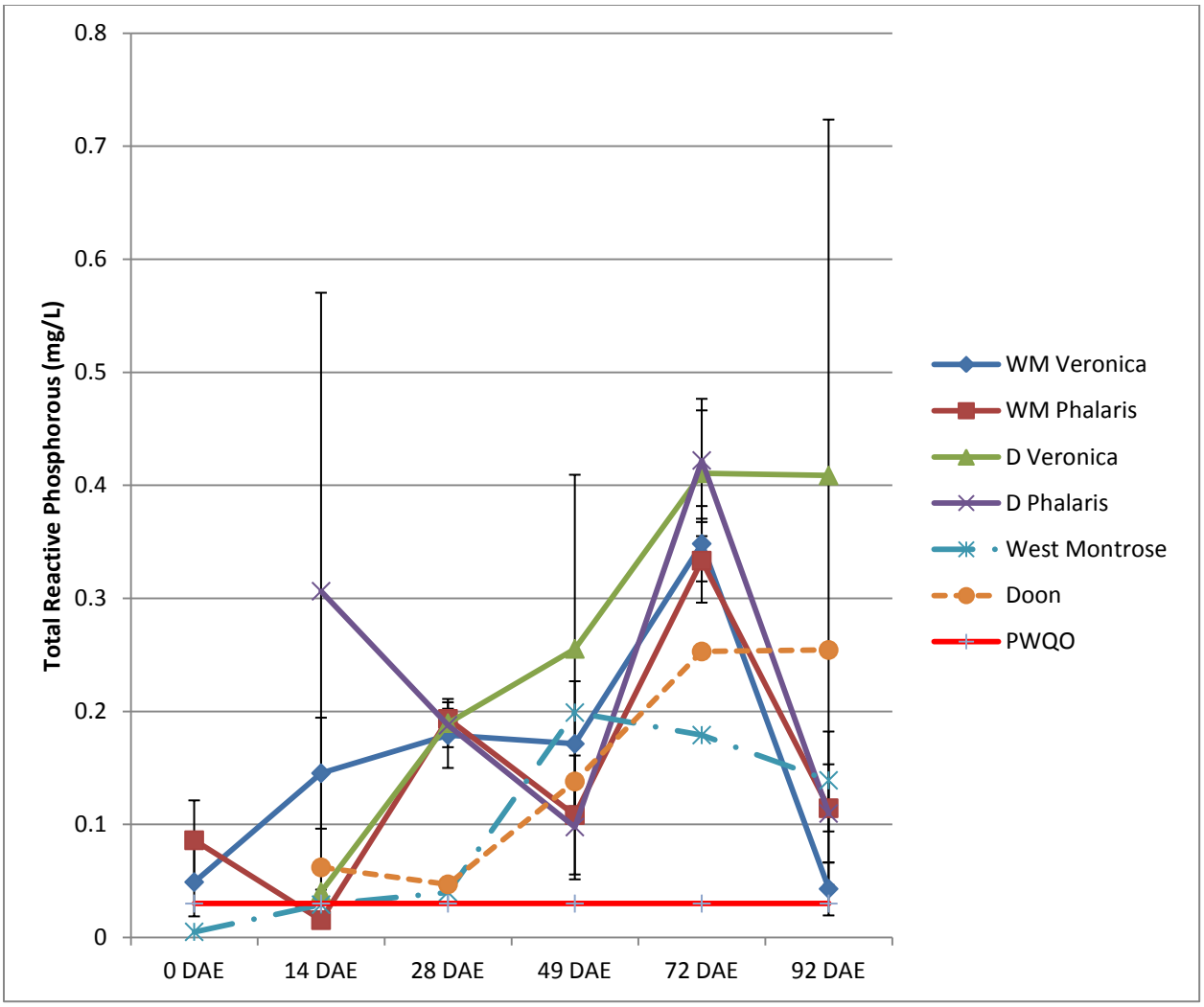

Figure 5.16 Total reactive phosphorus quantification (mg/L) in mesocosm outflow water and inflow water (West Montrose (WM) and Doon (D)) using HACH® water test kits. The Ontario provincial water quality objective (PWQO) for total reactive phosphorus in surface water sources is indicated in red. Inflow water is designated with a dashed line while outflow water is indicated with a solid line. Each data point is the average of three experimental replicates for outflow water samples and one experimental replicate for inflow samples. Error bars are representative of standard error.

5.3.3.6 Fecal Coliforms (Culture-Based)

Doon inflow water consistently exhibited higher numbers of fecal coliforms than West Montrose inflow water as measured by the membrane fecal coliform method (Fig 5.17). Mesocosm outflow water from the different treatments did not exhibit significant differences in fecal coliform presence over most of the sampling period. However, at 49 DAE outflow water from West Montrose-treated *P. arundinacea* mesocosms contained fewer fecal coliforms than outflow water from West Montrose-treated *V. anagallis-aquatica* mesocosms. Alternatively by 91 DAE the opposite trend was observed where outflow water from West Montrose treated *V. anagallis-aquatica* contained higher numbers of fecal coliforms than outflow water from West Montrose treated *P. arundinacea* mesocosms. As well, *V. anagallis-aquatica* mesocosms receiving water from Doon had a higher fecal coliform load in outflow water compared to *V. anagallis-aquatica* mesocosms receiving West Montrose inflow water. If we observe mesocosm outflow water fecal coliform concentrations in relation to inflow concentration, it is apparent that the mesocosms (both *V. anagallis-aquatica* and *P. arundinacea*) receiving Doon water removed significantly more fecal coliforms than the mesocosms receiving West Montrose water throughout the sampling period. There were no differences in removal efficiencies observed between plant species treatments within a water quality treatment. Additionally, removal efficiencies for fecal coliforms varied greatly among sampling time points and tended to increase over time. The amount of fecal coliforms detected in the outflow water of mesocosms ranged from below detection limits to several orders of magnitude above concentrations measured from the inflow water.

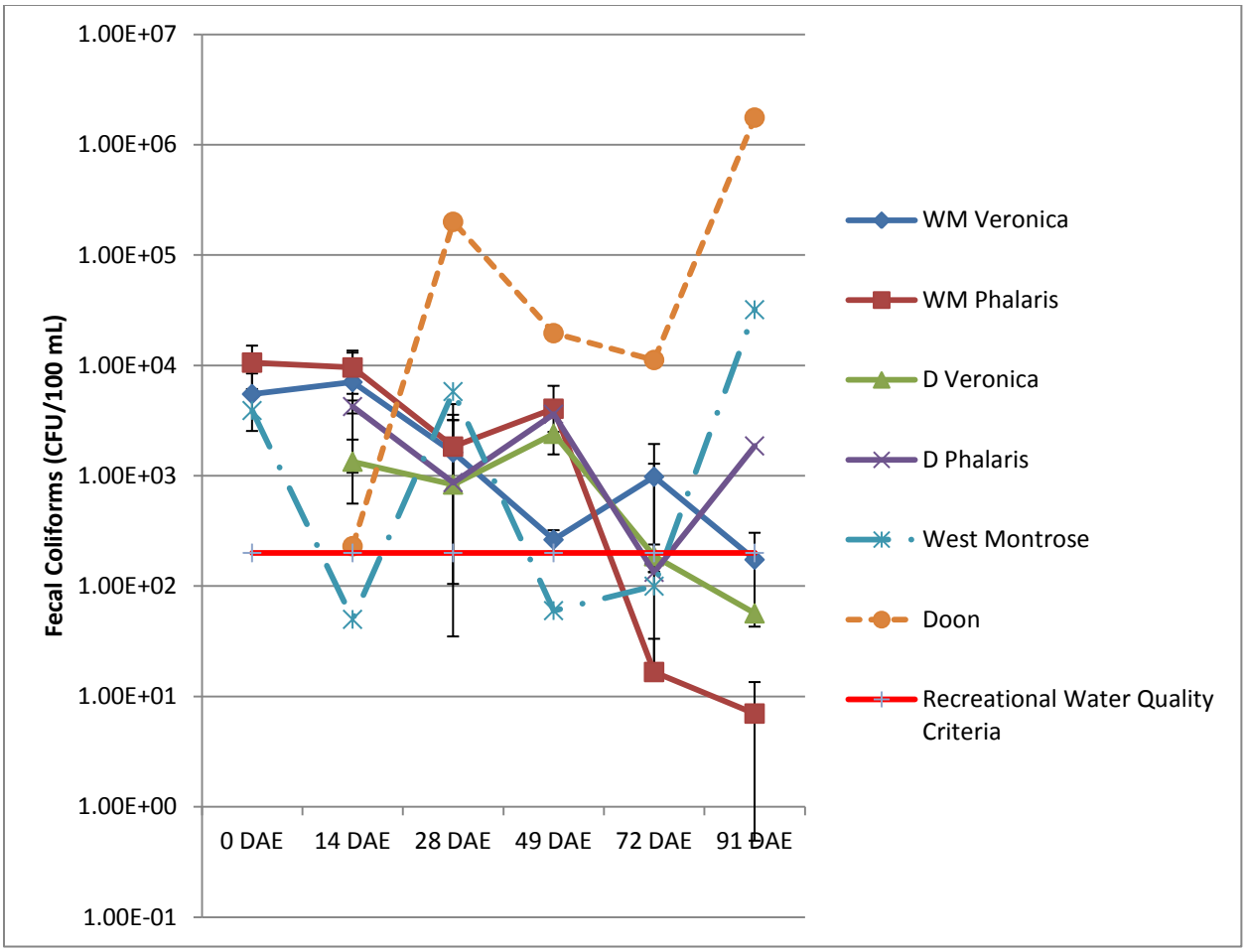

Figure 5.17 Fecal coliforms (CFU/100 mL) present in mesocosm outflow water and inflow water (West Montrose (WM) and Doon (D)) as determined by the membrane fecal coliform method (m-FC). The Canadian recreational water quality guideline for fecal coliforms in surface water sources is 200 CFU/100 mL. Inflow water is designated with a dashed line while outflow water is indicated with a solid line. Each data point is the average of three experimental replicates for outflow water samples and one experimental replicate for inflow samples. Error bars are representative of standard error.

5.3.4 Molecular Quantification of Bacterial Pathogens in Mesocosm Communities

The molecular detection of pathogenic microorganisms indicated that there were differences among treatments and among community types. Of the three organisms investigated, *E. coli* was consistently the most abundant within the rhizoplane (Fig 5.18 A). Within both the *P. arundinacea* and *V. anagallis-aquatica* rhizoplane communities, *E. coli* was more abundant within the poor water quality treatments. *Enterococcus* and *Salmonella* were present in equal quantities among all treatments (Fig 5.18 D and G). Within the rhizosphere, *E. coli* showed a strong trend in *P. arundinacea* treatments, with numbers dramatically declining in the poor water quality treatment over the course of the experiment and remaining stable in the high water quality treatment (Fig 5.18 B). *V. anagallis-aquatica* retained similar quantities of *E. coli* within the rhizosphere of both water quality treatments. Similar to observations from the rhizoplane, numbers of *Enterococcus* and *Salmonella* did not differ consistently between the treatments (Fig 5.18 E and H). The numbers of *E. coli* within interstitial water communities was greater in the high water quality treatments for both plant species despite higher incoming quantities within the poor water quality treatment during most time points (Fig 5.18 C). Differences were not detected among the treatments for *Enterococcus* or *Salmonella* (Fig 5.18 F and I).

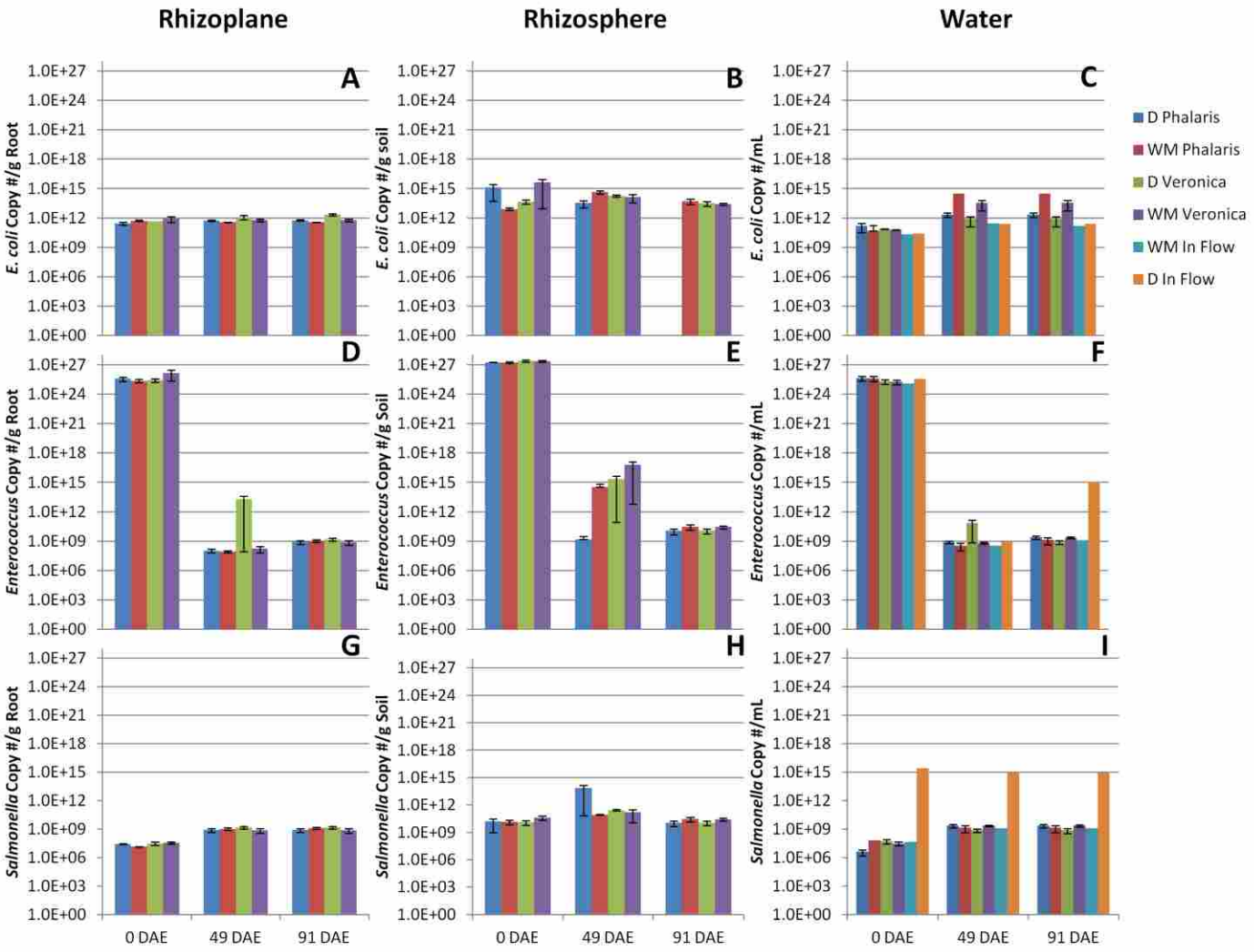

Figure 5.18 Average abundance of fecal associated pathogenic microorganisms (*Eschericia coli* A-C, *Enterococcus* D-F and *Salmonella* G-I) detected in the rhizoplane (A, D, G), rhizosphere (B, E, D) and interstitial/in flow water (C, F, I) at 0, 49 and 91 DAE. Mesocosms receiving high water quality inflow are indicated with a WM (West Montrose) and mesocosms receiving low water quality inflow are indicated with a D (Doon). Values are means of 3 replicate samples plus standard error for each treatment. Values for inflow water represent a single sample.

5.4 Discussion

The two hypotheses being tested by this experiment were that 1) constructed wetland systems impacted by anthropogenic activities (e.g. receiving poor water quality effluent from highly impacted sites) would have a unique microbial community structure and function which would be reflected by differences in capacity for remediation and 2) microbial communities associated with different plant species would be unique in structure, function and remediation capabilities. To test these hypotheses two field sites were chosen with contrasting water qualities (West Montrose and Doon) as determined by designations from the Grand River Conservation Authority (GRCA) and previous water quality testing done in the field. These contrasting water quality sources were exposed to mesocosms planted with either *P. arundinacea* or *V. anagallis-aquatica* for 13 weeks in order to determine whether contrasting water quality exposures resulted in changes to microbial community structures, functional community profiles, inorganic nutrient removal capacities or microbial pathogen removal efficiency.

In order to answer the question if water quality affected the structure of wetland plant associated microbial communities, structural profiles were examined for rhizosphere, rhizoplane, outflow water and inflow water microbial communities using DGGE and cluster analysis (Fig 5.4 to 5.9). Structural community profiles consistently clustered based on community type during all time points, indicating that the greatest indicator of microbial community structural similarity is where the community was taken from in relation to the wetland. It is difficult to place this finding in the context of the literature surrounding wetland microbial communities as the majority of published studies focus on a single community type within the wetland and do not compare community structures among different plant species for multiple community types. Smalla *et al.,* (2001) is one of the few studies to address this topic, by examining microbial

community structural similarity between bulk soil and rhizosphere soil associated with three different plant species. It is worthy of note that this study used both soil adhering to roots and root material itself to define the rhizosphere microbial community, which we would have defined in this study as three distinct communities: rhizosphere (adhering soil), rhizoplane (root attached biofilm) and endosphere (microbial community housed within the root epidermis).Despite this difference in experimental design, Smalla *et al.* (2001) found that regardless of which plant species the rhizosphere community was associated with, microbial community structure was more similar among rhizosphere communities than between rhizosphere samples and bulk soil within a treatment. Similarly, Edwards *et al.* (2015) examined the structure of microbial communities associated with the bulk soil, rhizosphere, rhizoplane and endosphere of six cultivated rice varieties (*Oryza* spp.). They found that community structures were separable by rhizo-compartment, despite genotypic differences in rice varieties and differences in soil type across three different rice fields. Structural differences by community type included variations in the abundance of different phyla, species diversity and richness. These findings reflect the trends observed, with rhizo-compartment being the greatest factor influencing community structure, and differences in water quality treatment and plant species being secondary or tertiary factors influencing structural similarity. This indicates that the spatial relationship to plant roots changes microbial communities from their non-plant impacted initial state (bulk soil) in similar ways regardless of plant species in these instances. This is interesting to note, as there can be large differences among plant species in terms of root exudation profiles, root structure, mycorrhizal status etc. However, it appears that the overall association with plant roots has a greater degree of similar environmental pressures (despite plant species specific physiological differences) driving changes in bulk soil community structure during plant root development. If the differences

among plant species were more significant than their similarities, microbial community structures should resemble more closely that of the parent bulk soil microbial community structure than the rhizosphere community structure of a different species of plant.

Within a microbial community type, we observed a clear effect of water quality treatment for both *P. arundinacea* and *V. anagallis-aquatica* as early as 28 DAE to the different water quality types (Fig 5.6). Originally, the similarity among plant associated communities receiving West Montrose water was greater than among those communities receiving Doon water, which can be explained by the fact that all mesocosms had been receiving West Montrose water for 8 months prior to the start of the experiment (Table 5.1). As well, by 91 DAE the similarity among Doon treated communities had become more similar than those of West Montrose treated communities, indicating that the influence of the water quality treatment likely increased with the duration of exposure (Fig 5.9). As the water quality treatments used for each mesocosm exposure were routinely collected from the source and not sterilized prior to treatment, the simplest explanation for the differences observed between West Montrose and Doon treated mesocosm community structures is that each mesocosm was continuously being re-colonized by the bacteria present in the incoming source water, so obviously if the incoming communities were different, this would lead to differences in the wetland communities of each treatment. However, we found that at almost every sampling time point, the highest degree of similarity within a microbial community type occurred between the incoming water communities from West Montrose and Doon which clustered together at 60% of the sampling events exhibiting 62-91% similarity in community structure (Table 5.1). This is not completely surprising as both treatments are part of the Grand River and share a common upstream source. Thus, the most likely explanation is that differences in microbial community structure by water quality treatment are more so a result of

differences in the physicochemical characteristics of the different water quality types interacting with the conditions in each mesocosm, rather than a difference in introduced inoculums. In a study by Kirchman *et al.* (2004) where bacterial activity and community structure were compared for six sites within the Hudson River system in New York, significant differences were observed in structure and function of microbial communities from different sites with unique chemical compositions, particularly in terms of measurements of dissolved organic matter. In order to determine the cause of these differences, sterile water from different sites was inoculated with the microbial communities of another site. They found that water source was more important than bacterial inoculum characteristics in determining microbial community structure and ectoenzyme activity. Similarly, Van der Gucht *et al.* (2007) examined the structure of microbial communities across several water systems including lakes and interconnected ponds and they found that local environmental factors including water depth, pH, nitrogen concentration, bacterial density, vegetation presence and the densities or biomass of *Bosmina* (water fleas), heterotrophic nanoflagellates and ciliates were more important in determining community structure than spatial factors (e.g. sharing an inoculum source due to being very close together in interconnected bodies of water).

The effect of plant species on microbial community structure was not clearly demonstrated in our experiment. The literature has many examples of plant species dependent effects on the structure of rhizosphere and rhizoplane microbial communities (e.g. Marschner 2001; Smalla *et al.*, 2001; Wieland *et al.*, 2001; Costa *et al.*, 2006; Berg and Smalla, 2009). Our study did not identify a clear consistent distinction among community structural profiles associated with each plant species, within root-associated microbial communities. However, by 91 DAE some clustering between microbial community structural profiles of the same plant

species was observed, although this was restricted to the rhizoplane (Fig 5.9). One possible explanation for the lack of a clear plant species specific effect on microbial community structure is the length of the study. Plants in this study were grown from seeds and developed within the mesocosms over an 11 month period with the experimental period occurring from month 9-11. Smalla *et al.,* (2001) found that the effect of plant species on rhizosphere community differentiation from bulk soil was only minimal at the one year sampling point, while species specific differences were much more significant after two years of plant growth and microbial community development. Alternatively, Wieland *et al.* (2001) found that plant development (age) was a significant factor in determining microbial community structure between 2 and 6 weeks in clover, bean and alfalfa, after which soil type and plant species became more significant in determining microbial community structure in the rhizosphere and rhizoplane. Plant species was more important than soil type in determining community structure for the rhizoplane, while in the rhizosphere soil type was more important than plant species in determining community composition. We can infer that due to the rhizoplane being directly attached to the plant root, it would experience the greatest degree of plant influence, which explains why our findings and the findings of Wieland *et al.* (2001) found a greater influence of plant species on microbial community structure in the rhizoplane. Additionally, the lack of a plant species effect observed for the other microbial community types could be explained by the fact that water treatment was a more significant driver of community structure than plant species in rhizosphere and outflow water communities, just as Wieland *et al.* (2001) found that soil type was the most prominent driver of community structure in the rhizosphere and bulk soil (our experimental design controlled for soil type). Thus the influence of the plant species on these community structures was overridden by the impacts of water composition in these cases.

To determine if water quality affected the function of wetland plant associated microbial communities, community level physiological profiles were examined for rhizoplane, rhizosphere, outflow water and inflow water microbial communities using Biolog^{TM} EcoPlates to examine the use of various carbon sources over the experiment by each community type (Fig 5.10). Early analyses of the data (data not shown) lead us to assess each community type separately to better observe treatment specific patterns in carbon utilization profiles, as analysis of all samples together yielded community type specific clustering (as observed in the structural community profiles) making more in depth analysis of treatment specific trends difficult to decipher. Within the water communities, treatment specific differences in carbon source utilization profiles appeard by sampling at 49 DAE in *P. arundinacea* but did not appear at all for the *V. anagallis-aquatica* associated communities. A plant species effect in carbon source utilization profiles between the two species is discernible by 91 DAE in water-associated microbial communities. The rhizoplane exhibited the most distinct carbon source utilization profiles associated with the different water quality treatments, which were established in both plant species associated communities by 49 DAE. Interestingly, there is no clear trend observed by plant species at any time point within the CLPP data. Finally, rhizosphere microbial communities associated with *P. arundinacea* were distinct between high and low water quality treatments by 49 DAE, however those associated with *V. anagallis-aquatica* were not different by this time (Fig 5.10). By 91 DAE the rhizosphere communities associated with *V. anagallis-aquatica* only started to show some differentiation by water quality treatment. Carbon source utilization profiles associated with rhizosphere microbial communities did not cluster by plant species at any time point.

Using carbon source utilization profiles from Biolog^{TM} EcoPlate absorbance measurements, we determined the metabolic diversity of each microbial community by treatment

(Fig 5.12). These measurements helped to explain some of the differences in CLPP trends observed by principal component analysis. Outflow water microbial communities showed differences in metabolic diversity among the different plant species and water quality treatments. While diversity measurements remained similar for *V. anagallis-aquatica*-associated communities between water quality treatments, metabolic diversity measurements were higher in *P. arundinacea* communities receiving Doon water compared to those receiving West Montrose water. Rhizoplane communities also showed a water quality dependent effect, with Doon treated *V. anagallis-aquatica* communities having higher values for metabolic diversity compared to those receiving West Montrose water. As we also observed in the PCA of Biolog^{TM} Ecoplate carbon source utilization for this plant species, rhizosphere communities did not exhibit differences in metabolic diversity by water quality or plant species treatment. These results indicate that the effect of water quality and plant species on microbial community function measured by CLPP are different depending on the microbial community type and also depending on plant species the community is associated with. The rhizosphere was less affected by water quality treatment compared with rhizoplane or outflow water microbial communities. Theoretically, proximity to the plant roots should increase plant species related effects of carbon usage on microbial community function (possibly subsequently decreasing any water quality effects), but the rhizoplane community exhibited a water quality effect while the rhizosphere did not. As such, there must be an additional factor influencing carbon source utilization patterns for microbial communities in the rhizosphere beyond that of water treatment and plant species. Unsurprisingly, there is significant evidence from the literature to suggest that the microbial functional profile of a rhizosphere or rhizoplane community is affected by the plant species it is associated with (e.g. Stephan *et al.*, 2000, Lupwayi *et al.*, 2004, Li *et al.*, 2011). Compared to the

rhizosphere, rhizoplane communities of both *P. arundinacea* and *V. anagallis-aquatica* exhibited greater overall carbon metabolism measurements (AWCD) (data not shown) and higher metabolic diversity, a trend which was also reported for microbial rhizoplane communities associated with barley (*Hordeum vulgare*), wheat (*Triticum aestivum*) and canola (*Brassica rapa*) (Lupwayi *et al.*, 2004). It is possible that greater proximity to plant roots, or some other factor associated to microbial lifestyle changes associated with rhizoplane attachment causes increased metabolic diversity and total carbon metabolism, resulting in the community experiencing greater functional changes in response to altered abiotic conditions associated with different water quality treatments. Additionally, the differences in how these communities respond metabolically to changes in water quality may also be related to where the community is obtaining the majority of its carbon. The composition and concentration of dissolved organic matter can vary greatly among aquatic systems (Cotner *et al.* 1997, Mulholland *et al.* 2001, Cole *et al.* 2002) and these differences have been shown to influence microbial enzyme activities at the community level (Kirchman *et al.*, 2004). It follows that outflow water microbial communities should experience changes in functional diversity related to different water quality exposures. Soil organic carbon quantity and quality have been shown to affect microbial metabolism and carbon source utilization patterns (Bossio and Scow, 1995, Wang *et al.*, 2003, Grayston *et al.*, 2001). As such, it is possible that rhizosphere microbial communities are obtaining carbon primarily from the soil as well as from the plant which is why we see limited effects from changes in water quality on community metabolic characteristics.

Differences in functional community changes occurring in response to the different water quality treatments were observed between the microbial communities associated different plant species (*P. arundinacea* and *V. anagallis-aquatica*). Carbon source utilization profiles

established by microbial communities associated with *P. arundinacea* were affected to a much greater extent by changes in water quality than *V. anagallis-aquatica* associated communities (specifically in rhizosphere and outflow water communities) (Fig 5.10). Previous studies have found that plant species can differ in the amount and types of carbon that they allocate to microbial biomass (Grayston *et al.* 1998; Bardgett *et al.*, 1999; Berg and Smalla, 2009, Ladygina and Hedlund, 2010) and differ in how changes in environmental factors may influence carbon allocation characteristics (Grayston *et al.*, 1996). These differences in plant species specific carbon allocation patterns have been hypothesized to influence microbial community functional characteristics (Grayston *et al.*, 1996). One possibility is that *P. arundinacea* is more physiologically affected by the water quality treatments than *V. anagallis-aquatica*, resulting in the changes observed in root-associated microbial communities. Differences among plant species in associated microbial community responses to water quality may have implications for constructed wetland design if these functional community shifts also affect microbial community metabolic capabilities related to constructed wetland remediation potential.

Metabolic characteristics of a microbial community will be influenced by the structure of that community, as not all microorganisms possess the same metabolic capabilities, thus community composition will influence metabolic potential. Several studies have found that changes in microbial community structure are also mirrored by changes in microbial community function, as measured by carbon source utilization profiles using Biolog^{TM} plates or by changes in activity levels for certain microbial enzymes or processes (e.g. Ravit *et al.*, 2003, Mentzer *et al.*, 2006, Ahn *et al.*, 2007) however this trend is not absolute (e.g. Hadwin *et al.*, 2006). In both the structural and functional microbial community profiles, community changes associated with

the different water quality treatments emerged between 14 and 28 DAE (Fig 5.5, 5.6 and 5.10). Carbon utilization profiles also exhibited trends related to association with different plant species beginning around the same time point. Plant species specific effects on microbial community structure did not appear until 91 DAE, at which time the association was still much weaker than what was observed in the functional profiles. This discrepancy may be attributed to the methodology used to capture the structural and functional microbial community profiles. Structural differences in microbial communities were assessed using microbial DNA, which is not necessarily associated with living, metabolically active microorganisms. DNA can remain in the environment after cell death for a considerable amount of time (several days to 3 weeks) (Josephson *et al.,* 1993, Masters *et al.,* 1994, Griffiths *et al.,* 2000). Additionally, the Biolog™ method requires metabolically active organisms in the plate inoculum under incubation conditions, which are appreciably different from the environment they were obtained from. As such, the metabolic capabilities measured under these conditions will ultimately not completely reflect the community's capabilities in its original environment. Changes in microbial metabolism may also occur without structural community changes as has been noted previously in the literature (e.g. Truu *et al.,* 2009). Consequentially, capturing community characteristics from both a structural and functional perspective is important in obtaining a complete and detailed picture of how microbial communities respond to changes in environmental conditions.

The poor water quality treatment from Doon exhibited higher average levels of all inorganic nutrients measured (nitrate, nitrite, ammonia and total reactive phosphorus) compared to West Montrose water over the six sampling events (Fig 5.13-5.16). However, each site demonstrated considerable temporal variability in inorganic nutrient concentrations, leading to large fluctuations in measurements from one sampling event to the next. Overall, the differences

between the sites were not statistically significant, likely due to the high level of variability and small sample size. Statistically significant differences between water quality parameters at these sites have been reported previously by the Grand River Conservation Authority (Loomer and Cooke, 2011). In the report released by the GRCA in 2011 (Loomer and Cooke) analyzing water quality parameters in the Grand River from 2003 to 2008, the Doon site exhibited significantly higher levels of all water quality parameters in relation to the West Montrose site. This lends credence to the notion that the differences between sites may have been statistically significant with a greater number of samples. However, while the relationship between the sites were similar to what was previously reported by Loomer and Cooke (2011), our actual measurements of inorganic nutrient concentrations were considerably higher than those reported by the GRCA from 2003 to 2008. Our measurements for unionized ammonia, total nitrates (nitrate and nitrite) and total reactive phosphorus were on average three times higher than the median values reported by Loomer and Cooke (2011) for the West Montrose site. For the Doon site, there was more variation in how much each water quality parameter changed between studies, but the increase in levels of inorganic nitrogen was much higher than the increase for phosphorus. Some of the differences between reported values could be due to differences in methodology, however, most of our measurements were taken during the spring and summer months which are also correlated with higher inorganic nutrient concentrations due to nonpoint source impacts associated with rain events (Carpenter *et al.*, 1998). Rapid urbanization and rising populations have most likely resulted in increased inorganic nutrient loads entering the Grand River Watershed leading to the higher recorded values for water quality indicators reported in this study (Sato *et al.*, 2013, Morris 2015). West Montrose may be experiencing this change to a greater extent due to urban expansion and population growth, which is occurring in this once

sparsely populated area, to a greater extent than in the already highly developed city center where our Doon site is located (Morris 2015). Thus, the difference between these two sites in terms of water quality is likely diminished now from what was observed historically.

Outflow values of the inorganic nutrients measured in this study did not differ significantly among the treatment types at any time point and also showed a large amount of variability from one sampling event to the next (Fig 5.13-5.16). The only treatment specific differences noted were observed in the removal efficiency of unionized ammonia between the West Montrose and Doon treatments (Fig 5.15). The percentage of ammonia from the inflow water detected in the outflow water was lower on average in *P. arundinacea* and *V. anagallis-aquatica* containing mesocosms receiving Doon influent than in mesocosms receiving West Montrose water. This is indicative that despite differences in incoming nutrient loads between water quality treatments, the mesocosm systems were able to moderate the amount of nutrients leaving the system. In some cases this resulted in an increase in the amount of inorganic nutrients present in the outflow compared to the inflow water, typically when inflow concentrations dropped. While under conditions of increased nutrient loading we tended to observe lower outflow concentrations compared to the inflow. While inorganic nitrogen levels were normally reduced from inflow to outflow water, phosphorus levels showed a trend towards higher outflow levels compared to inflow levels (Fig 5.16). This is possibly due to the fact that the amount of total reactive phosphorus entering the system was not sufficient to meet the needs of the plant and microbial community in the mesocosm resulting in an increase in microbial, fungal and plant mediated processes to generate higher levels of phosphorus available to meet metabolic needs. Although total reactive phosphorus concentrations from mesocosm influents were high compared to the PWQO, other studies have shown that these concentrations are actually about one order of

magnitude lower than what is typical for constructed wetland influents (Ahn and Mitsch, 2002). Ahn *et al.* (2007) found that when microcosms were exposed to effluents with either 0.5 mg/L (low P) or 2.4 mg/L (high P), the low phosphorus treatment actually resulted in a release of phosphorus from the system for microcosms containing *Schoenoplectus tabernaemontai*. Outflow concentrations of phosphorus were not significantly different between the high and low P treatments and despite a 5-fold increase in incoming phosphorus, phosphorus bound to microcosm soil only increase by 2-fold between treatments, indicating that the biotic community was responsible for the majority of phosphorus removal. Under conditions of limiting phosphorus availability some bacteria are known to produce extracellular phosphatases and other exoenzymes used to degrade phosphorus containing compounds (Harder and Dijkhuizen 1983). Similarly, mycorrhizal fungi are well known for their ability to mobilize phosphorus under conditions of low availability with exoenzyme production (Read and Perez-Moreno 2003). Similar findings have been reported for conditions of limiting inorganic nitrogen (Harder and Dijkhuizen 1983, Read and Perez-Moreno 2003).

Despite evidence of unique microbial community structures and metabolic functions associated with the different water quality treatments, we failed to observe significant differences between the communities in terms of their ability to ameliorate inorganic nutrient loads. This could be due to the fact that the inorganic nutrient loads present in the water quality treatments were not sufficiently different to stimulate these types of physiological changes in the associated microbial communities. Alternatively, differences in remediation capabilities among treatments may be absent due to the types of water quality indicators being monitored. While inorganic forms of phosphorus and nitrogen can be extremely damaging to aquatic ecosystems when present in excess, these compounds can be limiting nutrients in microbial communities and are

required by all microorganisms for survival (Harder and Dijkhuizen, 1983; Schimel and Weintraub 2003. In other related studies that found changes in the remediation ability of microbial communities exposed to different effluent levels, the remediation ability of the community was being monitored with respect to a compound that was not widely used or degraded by the general population and/or was selectively toxic to a significant portion of the resident microorganisms (Atlas and Bartha, 1992; MacNaughton *et al.,* 1999; Nyman, 1999; Bachoon *et al.,* 2001; Logan *et al.* 2005; Yergeau *et al.,* 2012; Bartlett 2016). Thus, these exposure treatments placed a stronger selective pressure on the community to be able to handle the contaminant load, compared to the pressures associated with our study.

Similar to the observed trends with inorganic nutrient loads, Doon site water consistently exhibited higher numbers of culturable fecal coliforms compared to the West Montrose site, by approximately two orders of magnitude (Fig 5.17). Molecular detection of *E. coli* and *Enterococcus* did not differ between sites; however, detection of *Salmonella* spp. was also consistently higher at Doon (Fig 5.18). With all treatment types, culturable levels of fecal coliforms were greater in outflow water compared to inflow water during the majority of sampling events and this occurred to a greater extent in the West Montrose site (high water quality). Thus, it appears that these organisms were being retained within the mesocosms and increasing in numbers which were then being shed through the mesocosm effluent over time. Our molecular data supports this, as we observed a substantial increase in the quantity of genetic material detected from *Salmonella* spp. (rhizoplane and rhizosphere) and *E. coli* (rhizoplane only) of *P. arundinacea* and *V anagallis-aquatica* over the course of the experiment. The traditional view of these organisms is that they are unable to proliferate under non-host environmental conditions and are thus reliable indicators of fecal contamination and public

health risk (U.S. Environmental Protection Agency 2000). However, it is becoming increasingly apparent that many of these organisms are capable of not only persisting under non-host conditions, but of actively replicating as well which has been referred to as "naturalization" (e.g. Winfield and Groisman, 2003; Anderson *et al.,* 2005; Ishii and Sadowsky, 2008). The rhizosphere specifically has been identified as a potential reservoir for opportunistic human pathogens (Berg *et al.,* 2005). *Salmonella* and *E. coli* specifically have been found to persist in association with plants under various environmental conditions (Warriner and Namvar, 2010). Ibekwe *et al.,* (2004) found that the presence of rye and alfalfa roots in soil allowed *E. coli* 0157:H7 to persist for longer periods of time and reach higher densities than in unplanted plots. Similarly, in a study by Ongeng *et al.* (2011), persistence of *E. coli* O157:H7 and *Salmonella enterica* was enhanced within the rhizosphere of cabbage plants (*Brassica oleracea*) compared to the bulk soil. Klerks *et al.* (2007) found that known virulence genes were up regulated in *S. enterica* exposed to root exudates from lettuce cultivars which were associated with host cell attachment and colonization. As such, Holden *et al.,* (2009) postulated that many of the same physiological traits that allow these organisms to colonize the gastro-intestinal tract of host organisms are also involved in making them successful colonizers of the rhizosphere.

The detection of fecal coliforms and bacterial pathogens in wetland associated bacterial communities exhibited the greatest distinction between mesocosm treatments compared to other methods of examining community remediation ability (Fig 5.17 and 5.18). In particular, *V. anagallis-aquatica* was observed to harbor different loads of *E. coli* within the outflow water community treated with West Montrose water. Similarly, *E. coli* and *Enterococcus* spp. were completely absent from the rhizosphere of *V. anagallis-aquatica* plants receiving Doon effluent, while both were still detectable within the West Montrose treatments. Treatment specific

differences associated with *P. arundinacea* were limited to the detection of *Enterococcus* spp., which were more prevalent in the rhizosphere of the West Montrose treatment compared to the Doon treatment. However, this trend was reversed in the rhizoplane, where higher quantities of genetic material were detected in the Doon treatment compared to the West Montrose treatment. The general trend seems to be that these organisms are better able to survive in the West Montrose treatment, despite similar or higher incoming inocula from the Doon treatment inflow water and that this trend is observed to a greater extent in communities associated with *V. anagallis-aquatica.* *V. anagallis-aquatica* also exhibited a more profound change in functional community measurements in response to altered water quality, which may indicate that this plant species is more sensitive to changes in environmental conditions, leading to altered microbial community-associated characteristics. The literature has established that the plant microbiome can change in response to abiotic stressors such as salinity, temperature, pH and toxicity (Mendes *et al.*, 2013). This is likely the result of a combination of factors, such as physiological changes occurring in the plant in response to environmental circumstances (e.g. changes in root exudation profiles and carbon allocation) and adaptation to ambient environmental conditions (e.g. Badri and Vivanco, 2009). How a plant responds to environmental stress will differ among individual plant species and cultivars (e.g. Chapin III *et al.*,1993). As to why these organisms tended to do better when exposed to high water quality effluent under these circumstances, it is possible that they are better competitors under conditions of more limiting nutrient availability. All the mesocosms were originally being exposed to West Montrose water and were seeded with soil from West Montrose. Thus, the communities were adapted to these conditions before being exposed to the Doon water so the change of water treatment may have acted as a stressor and altered microbial community dynamics making these organisms poor competitors under the

altered water quality conditions in conjunction with the *V. anagallis-aquatica* root environment. Furthermore, while we observed the complete elimination of *E. coli* and *Enterococcus* spp. from the rhizosphere of some *V. anagallis-aquatica* mesocosms by 91 DAE, they were still detectable within the rhizoplane community. Proximity to the root or attachment to root surfaces (instead of soil surfaces) may be an important factor in the survival and proliferation of these organisms within plant root-associated wetland environments.

5.5 Conclusions

Water quality treatments were shown to affect the structure and function of microbial communities associated with *P. arundinacea* and *V. anagallis-aquatica.* However, *P. arundinacea* associated communities were affected to a greater extent. Different community types varied in how they responded to changes in water quality. For example, community level changes were detected to a greater extent within the rhizoplane than in the rhizosphere. All three types of microbial pathogens were detected in all community types however, only *Salmonella* and *E. coli* exhibited signs of naturalization within the wetland plant-associated microbial communities. *E. coli* quantities in rhizoplane, rhizosphere and mesocosm outflow water were influenced by the different water quality treatments. These findings indicate that wetland-associated microbial communities will differ in their response to changes in environmental conditions depending on the community type and the associated plant species. As such, we cannot assume that changes, or lack thereof, occurring in one microbial community type will be reflective of the microbial communities within the wetland, as a whole. In order to determine potential impacts of environmental factors on wetland-associated microbial communities, as many community types, associated with a variety of different plant species, should be studied.

5.6 References

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Chapter 6

Microbial Community Changes Associated with Short Term Phosphorus Loading in Lab-Scale Wetland Mesocosms

6.1 Introduction

The health of aquatic ecosystems can be severely compromised by the presence of excess phosphorus. Available inorganic phosphorus concentrations as low as 5 µg/L have been associated with cyanobacterial algae blooms and eutrophication in some aquatic ecosystems. However, eutrophication is typically associated with levels of inorganic phosphorus between 0.05 and 0.1 mg/L (Smith, 2003; Shaw *et al.*, 2009). Wetlands (natural, restored or constructed) can provide a means of natural protection for downstream aquatic systems from nutrient pollution, including elevated phosphorus concentrations. Sources of phosphorus entering natural environments can be described as either point source, mostly encompassing waste water treatment plant (WWTP) effluents, and non-point source, which is dominated by surface agricultural runoff. Point source pollution from WWTP effluent may contribute on average 50% of the total phosphorus entering aquatic ecosystems, while agricultural sources can account for approximately 40%, depending on the aquatic system (Mainstone and Parr, 2002).

The primary mechanisms by which wetlands can remediate source waters contaminated with high levels of inorganic phosphorus involves physical, chemical and biological processes. Microorganisms are typically responsible for removing between 60 and 91% of total phosphorus from aquatic ecosystems, including streams and wetlands (Elwood *et al.*, 1981; Newbold *et al.*, 1983; Lee *et al.*, 1975; Sloey *et al.*, 1978). Microbially-mediated phosphorus retention occurs through assimilation. Microorganisms require only minimal amounts of phosphorus for survival

but can store phosphorus extensively under conditions of surplus environmental phosphorus (Gächter and Meyer, 1993; Kulaev *et al.*, 2005). Although this mechanism of phosphorus removal may seem transitory, as the phosphorus will be released back into the environment when the microorganisms die and decompose, most of what is released back into the environment is refractory organic compounds biologically unavailable for processes involved in eutrophication (Gächter and Mares, 1985; Gächter *et al.*, 1988). Plants can also assimilate phosphorus, which is converted into plant biomass, although there is an upper limit to the amount of phosphorus that can be stored by specific plants (Stottmeister *et al.*, 2003; Vymazal, 2007). The average amount of phosphorus contained within the biomass of a large number of different plant species was calculated to be about 0.15 to 1.05% of the total plant mass, which only equates to approximately 5% of the average phosphorus load in WWTP effluent (McJannet *et al.*, 1995). However, plants have another important role which involves their ability to support and foster the growth of microbial communities in association with their root systems. Plant roots create important niche environments that cultivate unique microbial communities. This facilitates several bacterial processes, including the rapid cycling of nutrients, which increases the effectiveness of water remediation processes (Stottmeister *et al.*, 2003; Vymazal, 2007). The area of soil influenced by plant roots can be sub-divided into two unique microbial communities: the rhizosphere and the rhizoplane. The rhizosphere is the area of soil surrounding plant roots that is actively influenced by plant growth, respiration and secretions, while the rhizoplane is the microbial biofilm attached directly to plant roots (Berendsen *et al.*, 2012).

There is little information presently available in the literature with regards to how phosphorus loading affects the structure and function of the microbial communities present in wetlands. We wanted to address this question and investigate how previous microbial

community adaptation to varying water qualities (and degrees of anthropogenic impact) affected the response of microbial communities to phosphorus loading. Some other studies in the literature have found that previous exposure to different types of effluent may enhance the remediation capabilities of certain types of microbial wetland-associated communities (e.g. Hallberg and Johnson, 2005; Logan *et al.* 2005; Huijie *et al.*, 2011).Water quality can be defined by a diverse list of characteristics including inorganic nutrient concentrations, microbial pathogen presence, heavy metal contamination and micropollutant contamination (e.g. pesticides, petroleum hydrocarbons etc.) (Meybeck *et al.*, 1989). We chose to focus our water quality definitions on nutrient pollution (inorganic nitrogen and phosphorus) and the presence of pathogenic microorganisms associated with fecal contamination, which are the two biggest water quality concerns in the Grand River (ON, Canada). Our research question was addressed using lab-scale wetland mesocosms which had been exposed previously to water from either a high water quality site (West Montrose, ON N0B 2S0 43.588219, -80.470979) or a poor water quality site (Doon, Kitchener, ON 43.386376, -80.387547) for 13 weeks prior to the start of the experiment, which were planted with either *Phalaris arundinacea* or *Veronica anagallis-aquatica*, two species of wetland plants abundant in the Grand River Watershed. Phosphorus loading was simulated by adding 5 mg/L of inorganic phosphorus as P_2O_5, the form of inorganic phosphorus most commonly found in fertilizers. We hypothesized that the poor water quality-adapted mesocosms would be more resistant to perturbations, related to structural and functional community changes, caused by the phosphorus loading and perform better at removing inorganic phosphorus from the incoming water.

6.2 Materials and Methods

6.2.1 Phosphorus Exposure and Sample Collection

Mesocosm construction, design and operating conditions were carried out as described in section 5.2. Mesocosms were held under conditions described in the previous section for 91 days before being exposed to the phosphorus loading phase, which consisted of 5 mg/L of P_2O_5 (Sigma Aldrich, Oakville, ON, CA). The phosphorus was added to the buckets of either West Montrose or Doon water (depending on which water quality treatment was being used) and mixed by stirring continuously. The water was then added to each mesocosm over a 2 h period at a volume of 8.5 L per mesocosm (average approximate mesocosm capacity, mesocosm retention time is approximately 6 days as stated in section 5.2). Once the phosphorus exposure was completed, previous operating conditions described earlier were resumed. Mesocosm rhizosphere material was sampled at 0, 7, 21, 35 and 49 days after exposure (DAE). Rhizoplane material was sampled less frequently on 0, 21 and 49 DAE due to the invasive nature of removing portions of the plant root system which potentially impacts hydrologic flow regimes and disrupts the establishment of microbial communities within the mesocosm. Outflow (interstitial) and Inflow water was sampled on 0, 3, 7, 21, 35 and 49 DAE. Microbial community structural analysis was performed using methods described previously for denaturing gel gradient electrophoresis of extracted microbial community DNA subjected to PCR using primers to target the V3 region of the 16S ribosomal DNA in Bacteria. Functional community profiles of microbial communities were established using the Biolog^{TM} EcoPlate protocol for analysing carbon substrate utilization as previously described. Water quality of source water and mesocosm outflow water was determined using HACH® reagents to determine water concentrations of inorganic nitrogen (nitrate, nitrite and ammonia) and phosphorus and the

membrane fecal coliform method to estimate fecal contamination and potential human pathogen presence as previously described (Chapter 2).

6.3 Results

6.3.1 Structural Profiles of Rhizoplane, Rhizosphere and Water-Associated Microbial Communities Before and After Short-Term Phosphorus Loading

6.3.1.1 Cluster Analysis of Microbial Community Structural Profiles

Pre-treatment (0 DAE) the structural community profiles of water, rhizosphere and rhizoplane microbial communities showed some variability by sample type, but with most structural profiles of the same sample type clustering together (Fig 6.1). Several outliers were present in both the rhizoplane and interstitial (outflow) water microbial community structural profile groupings (3 each). Microbial community structures of inflow water communities associated with West Montrose and Doon water exhibited strong similarity to each other, compared to other sample types (69% similarity). As well, inflow water microbial communities were more structurally similar to rhizoplane communities compared to other community types (47% structural similarity, excluding outliers). Inflow water community structural profiles were more distantly related to the community structure of rhizosphere-associated microbial communities (26% similarity). Rhizosphere community profiles exhibited the highest degree of similarity among replicates (84.1%), followed by the rhizoplane community (74.2% similarity excluding outliers, 26% including outliers) and the interstitial water community (46.5% similarity excluding outliers, 43.1 % including outliers). Clustering of structural community profiles within community types by water quality treatment was observed. Within the rhizosphere community both water quality treatment groups exhibited 84.1% similarity, although treatments receiving Doon water showed a trend towards higher structural similarity than West

Montrose-treated communities; 5 of the 6 Doon treatments were contained within a cluster (89.4% similarity) compared to only 4 of 6 of the West Montrose treatments (92.2% similarity). Within the rhizoplane, West Montrose-treated microbial communities exhibited a higher degree of structural similarity (74.2% similarity among 5 of 6 treatments) compared to Doon-treated microbial communities (only 2 replicates exhibited greater than 78% similarity). Interstitial water microbial communities exhibited the highest degree of variability and only demonstrated minimal structural similarity by water quality treatment. Some evidence for the influence of plant species on microbial community structural profiles was apparent during this sampling event. One cluster of *P. arundinacea* rhizoplane structural profiles (78.7% structural similarity) and one cluster of *V. anagallis-aquatica* microbial community profiles (67.3% similarity), including both water and rhizoplane microbial communities, suggested some plant species-specific effects on microbial community structures among the different community types.

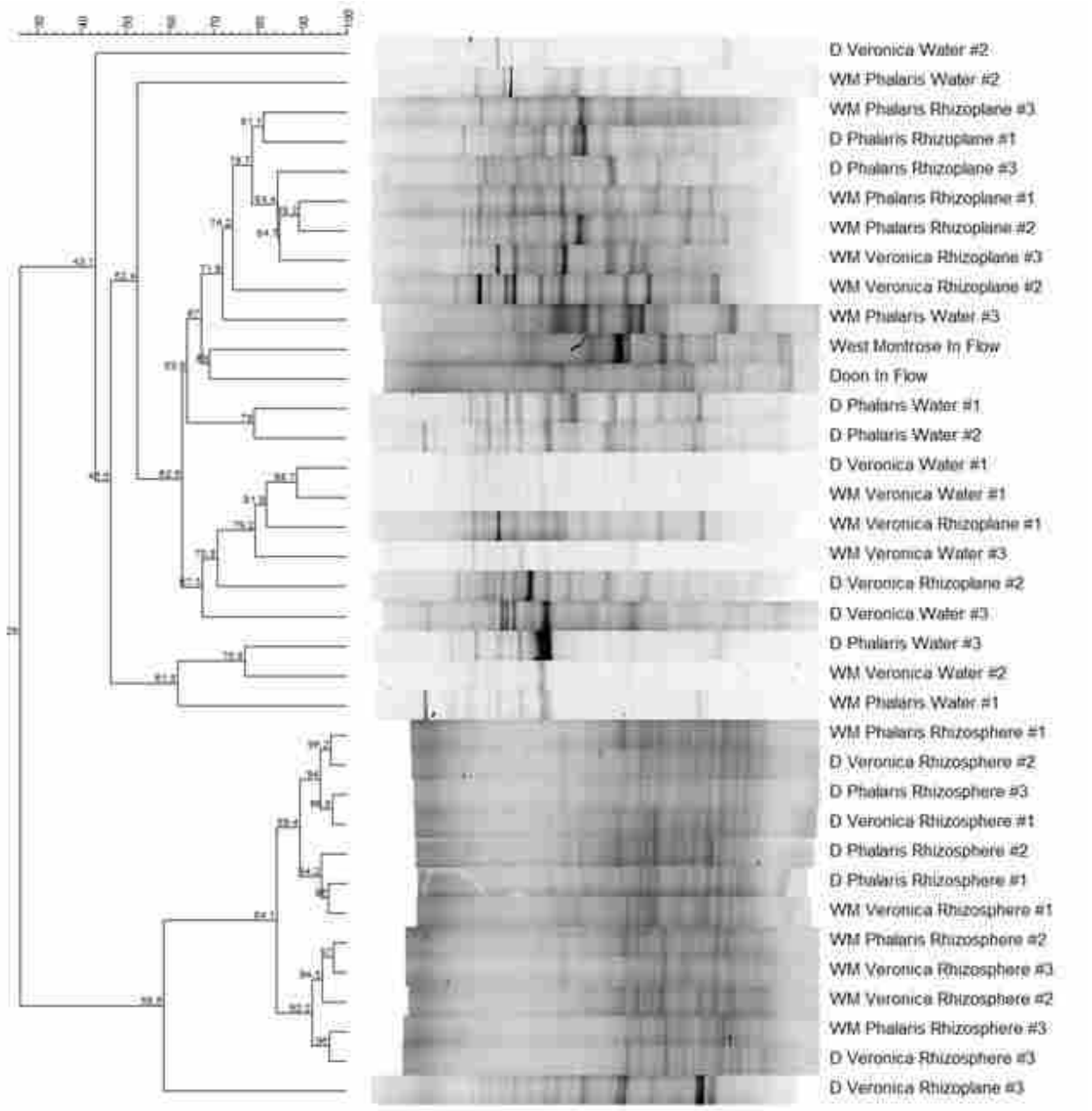

Figure 6.1 Hierarchical cluster analysis results of all DGGE profiles obtained from microbial DNA extracted at 0 DAE to 5 mg/L of phosphorus at P_2O_5 demonstrated graphically as a UPGMA dendrogram. Mesocosms receiving high water quality inflow are indicated with a WM (West Montrose) and mesocosms marked to be receiving low water quality inflow are indicated with a D (Doon). Mesocosm replicates per treatment type are indicated with numbers 1-3. Inflow water samples are designated by the water source name followed by the identifier "in flow". Percent similarity between clusters is indicated by the number above branches.

Structural similarity among the different community types (rhizoplane, rhizosphere, water) increased dramatically by 7 DAE compared to those from the pre-treatment sampling event. The highest degree of structural similarity within sample types was between inflow water communities, with Doon and West Montrose inflow water communities sharing 84% structural similarity (Fig 6.2). Inflow water microbial communities were the most structurally similar to microbial interstitial water communities (78% similarity) and shared 65% similarity to rhizosphere microbial communities (rhizoplane microbial communities were not sampled during this sampling event). Rhizosphere microbial community structures exhibited 74% structural similarity, which was a decrease in similarity compared to the previous sampling event (84% similarity). Structural profiles were grouped into two separate clusters; the first one with 85% structural similarity contained five of the West Montrose treatments and one of the Doon treatments, while the second cluster exhibited 90% structural similarity and contained five of the Doon treatments and one of the West Montrose treatments. An increase in structural similarity among rhizosphere communities receiving either West Montrose or Doon inflow water was observed compared to sampling at 0DAE. Inflow water samples were more structurally similar to each other than at the previous sampling event (78% similarity vs. 43% similarity). Outflow water communities receiving Doon water exhibited 78% structural similarity as did communities receiving West Montrose water. Interstitial water communities clustered into four smaller sub-groupings each containing a mixture of both West Montrose and Doon *P. arundinacea* and *V. anagallis-aquatica* interstitial water community structural profiles. This pattern of community structure clustering was unique from the previous time point which generated cluster patterns indicating structural similarity based on the associated plant species.

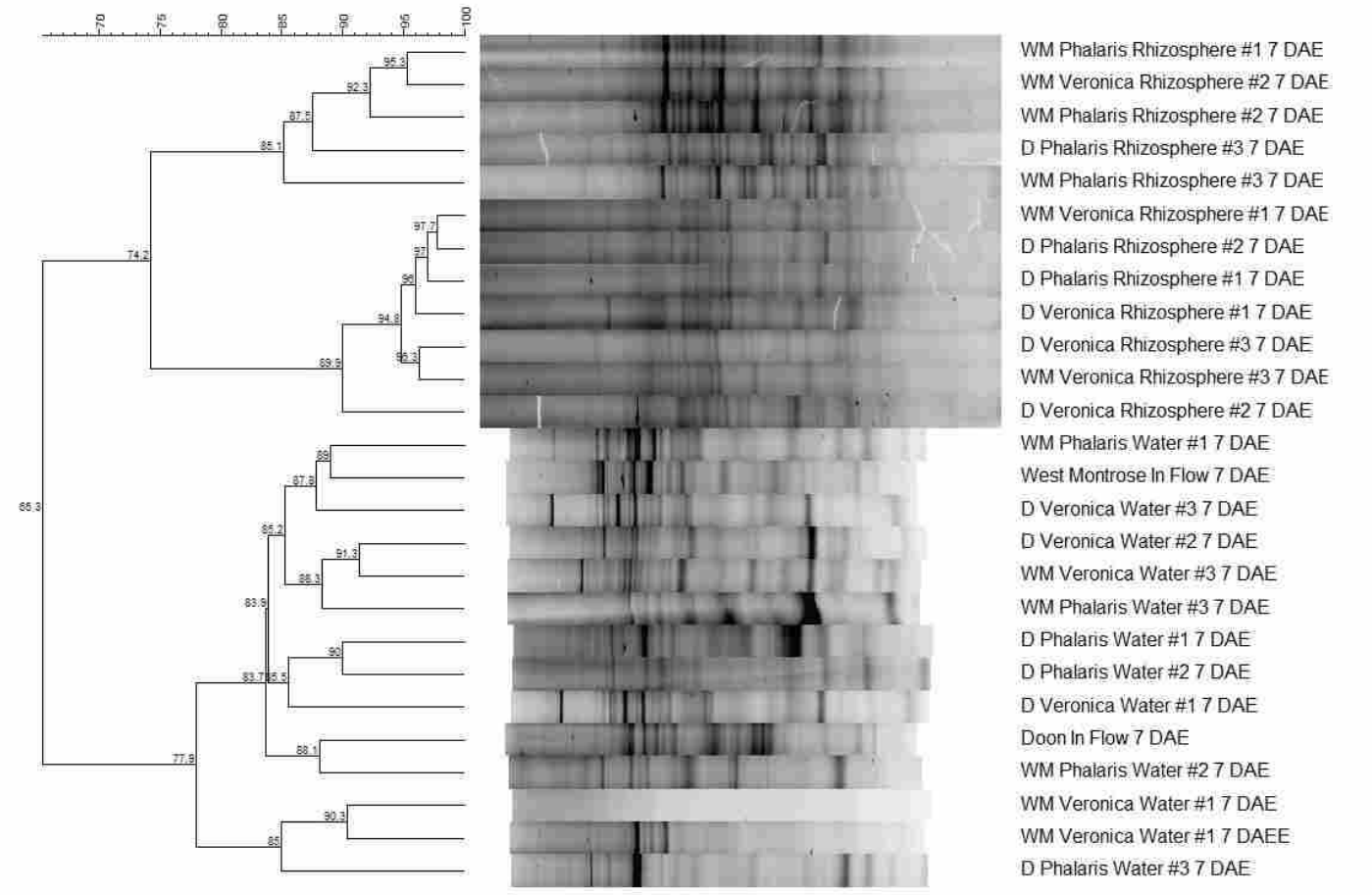

Figure 6.2 Hierarchical cluster analysis results of all DGGE profiles obtained from microbial DNA extracted at 7 DAE to 5 mg/L of phosphorus at P_2O_5 demonstrated graphically as a UPGMA dendrogram. Mesocosms receiving high water quality inflow are indicated with a WM (West Montrose) and mesocosms marked to be receiving low water quality inflow are indicated with a D (Doon). Mesocosm replicates per treatment type are indicated with numbers 1-3. Inflow water samples are designated by the water source name followed by the identifier "inflow". Percent similarity between clusters is indicated by the number above branches.

At 21 DAE to 5 mg/L of added phosphorus as P_2O_5, structural similarity within and among sample types decreased substantially compared to those obtained on 7 DAE. By 21 DAE structural similarities among and between sample types decreased to values comparable with those obtained on 0 DAE. Inflow water samples from West Montrose and Doon only shared 2% structural similarity, which was much lower than the similarity between these source water communities measured on previous sampling events (Fig 6.3). The West Montrose inflow water community structure was most similar to other water microbial communities from West Montrose interstitial water samples, while the Doon inflow water sample was more structurally related to rhizosphere microbial community structures. Interstitial water community structures shared 35% structural similarity with rhizoplane community structures, and both community types shared 2% structural similarity with rhizosphere-associated microbial communities. Rhizosphere microbial communities had the greatest amount of within sample type structural similarity (60% similarity). Community structural profiles clustered into two main sub-groupings, the first with 64 % similarity, contained the profiles of primarily West Montrose-treated microbial communities (4 West Montrose, 1 Doon), while the second cluster contained primarily Doon-treated structural profiles (5 Doon, 2 West Montrose). Interstitial water microbial community structures clustered intermittently with rhizoplane community structures and shared only 35% structural similarity. Structural profiles from interstitial water communities were grouped into five smaller clusters. One of these clusters contained structural profiles from West Montrose *V. anagallis-aquatica*-associated microbial communities. Another of these clusters containing all *P. arundinacea*-associated communities from both Doon and West Montrose- treated mesocosms. The remaining interstitial water microbial community structural profiles clustered separately and formed sub-groupings consisting of a mixture of structural

community profiles associated with all treatment types. Rhizoplane microbial community structures also showed only 35% structural similarity with samples of the same community type. Structural community profiles associated with the rhizosphere clustered into three larger groupings. The first subgroup of rhizoplane community profiles was composed of five *V. anagallis-aquatica* associated rhizoplane communities and one *P. arundinacea* associated rhizoplane community from both West Montrose and Doon treatments (63% similarity). Within the larger cluster there were pairs grouped together by water quality treatment. The second cluster contained four *P. arundinacea* associated communities (3 Doon and 1 West Montrose, with 67% similarity). The remaining rhizoplane-associated structural community profiles clustered as a pair with 68% similarity and consisted of structural profiles from different water quality and plant species treatment types. Structural similarity among rhizoplane-associated communities from the same plant species treatment showed an increase compared to observations made at 0 DAE, showing a shift towards plant species exerting a greater influence over community structure than water quality treatment type, which was observed previously.

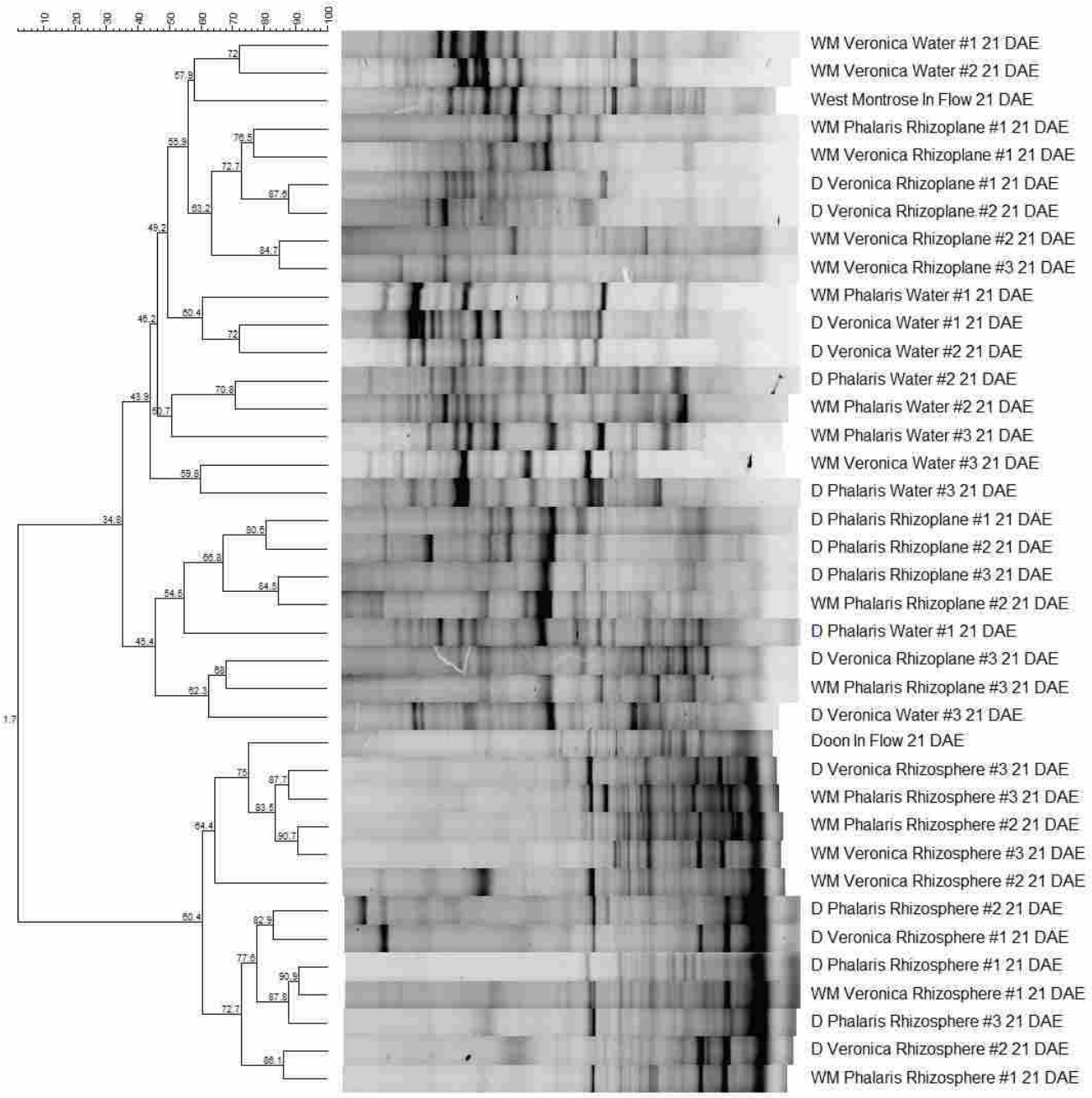

Figure 6.3 Hierarchical cluster analysis results of all DGGE profiles obtained from microbial DNA extracted at 21 DAE to 5 mg/L of phosphorus at P_2O_5 demonstrated graphically as a UPGMA dendrogram. Mesocosms receiving high water quality inflow are indicated with a WM (West Montrose) and mesocosms marked to be receiving low water quality inflow are indicated with a D (Doon). Mesocosm replicates per treatment type are indicated with numbers 1-3. Inflow water samples are designated by the water source name followed by the identifier "in flow". Percent similarity between clusters is indicated by the number above branches.

At 35 DAE structural similarity values within and between microbial communities of different community types was similar to observations made at 21 DAE. The highest degree of within-community type structural similarity was observed for inflow microbial communities with West Montrose and Doon microbial inflow communities sharing 68% structural similarity (Fig 6. 4). Inflow water communities shared the highest degree of structural similarity with interstitial water microbial communities (13% similarity, 28% similarity excluding outliers). Interstitial water and inflow water microbial communities only shared 13 % similarity (19% excluding outliers) with microbial rhizosphere communities. Rhizosphere-associated microbial community structural profiles shared the second highest amount of within community type structural similarity (44%). Microbial rhizosphere-associated communities receiving Doon water exhibited higher structural similarity (82%) than West Montrose-treated microbial communities (44% similarity). This trend was also observed at 0 DAE but not during any of the previous sampling events (7, 21 DAE). Rhizosphere structural community profiles formed clusters consisting of one large grouping containing 10 of the 12 structural profiles and formed secondary groupings related to water quality treatments. This community type had two outliers that did not cluster with the rest of the rhizosphere-associated microbial community structural profiles, which consisted of one replicate from the Doon *P. arundinacea* treatment and one from the West Montrose *V. anagallis- aquatica* treatment. Interstitial water microbial communities only shared 31% structural similarity (28% excluding outliers), and formed two primary clusters, one with 41% similarity containing primarily Doon-treated interstitial water communities, while the second grouping shared 44% structural similarity and contained primarily West Montrose-treated water communities.

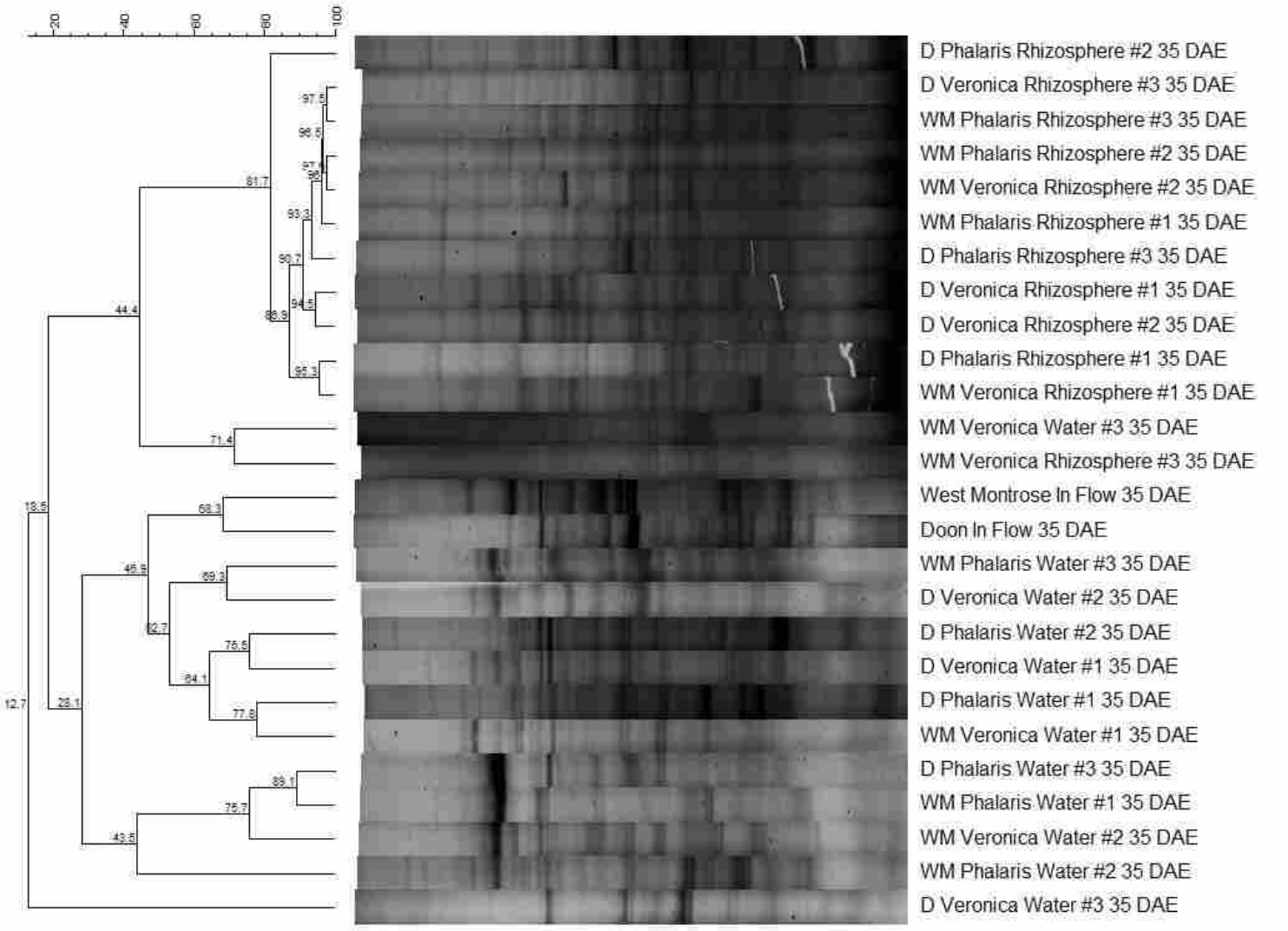

Figure 6.4 Hierarchical cluster analysis results of all of the DGGE profiles obtained from microbial DNA extracted at 35 DAE to 5 mg/L of phosphorus at P_2O_5 demonstrated graphically as a UPGMA dendrogram. Mesocosms receiving high water quality inflow are indicated with a WM (West Montrose) and mesocosms marked to be receiving low water quality inflow are indicated with a D (Doon). Mesocosm replicates per treatment type are indicated with numbers 1-3. Inflow water samples are designated by the water source name followed by the identifier "in flow". Percent similarity between clusters is indicated by the number above branches.

At 49 DAE strong clustering was observed among structural profiles of the same sample type. Rhizosphere-associated microbial communities exhibited the highest degree of within sample type similarity (58%), followed by the inflow water communities (55%), interstitial water communities (55%) and then the rhizoplane microbial communities (49%) (Fig 6.5). Interstitial and inflow water microbial communities clustered together and were the most similar to rhizoplane-associated microbial community structural profiles (38% similarity). Water-associated microbial communities shared 24% structural similarity with rhizosphere microbial communities. Within the rhizosphere-associated microbial community structural profiles, similar to observations made at 0 and 35 DAE, Doon microbial community structures showed a higher degree of similarity compared to West Montrose-treated communities. Structural profiles of rhizosphere microbial communities formed two secondary clusters, one with 81% structural similarity (3 West Montrose communities, 1 Doon community) and a second with 82% structural similarity (5 Doon communities, 3 West Montrose communities). Interstitial water microbial communities exhibited clustering patterns related to water quality treatment type. Similar to previous observations made at other time points, rhizoplane community structures showed clustering based on both plant species treatment as well as water quality treatment. Structural community profiles from rhizoplane-associated microbial communities were clustered into two sub-groupings. The first larger grouping (75% similarity) contained eight structural profiles that clustered into smaller tertiary groupings based on the associated plant species and then water quality treatment (more loosely). Within these groupings, *V. anagallis-aquatica*-associated microbial communities were more closely related structurally than *P. arundinacea*- associated communities. The second smaller sub-grouping (64% similarity) contained primarily West

Montrose-treated rhizoplane-associated microbial communities and a mix of plant species treatments.

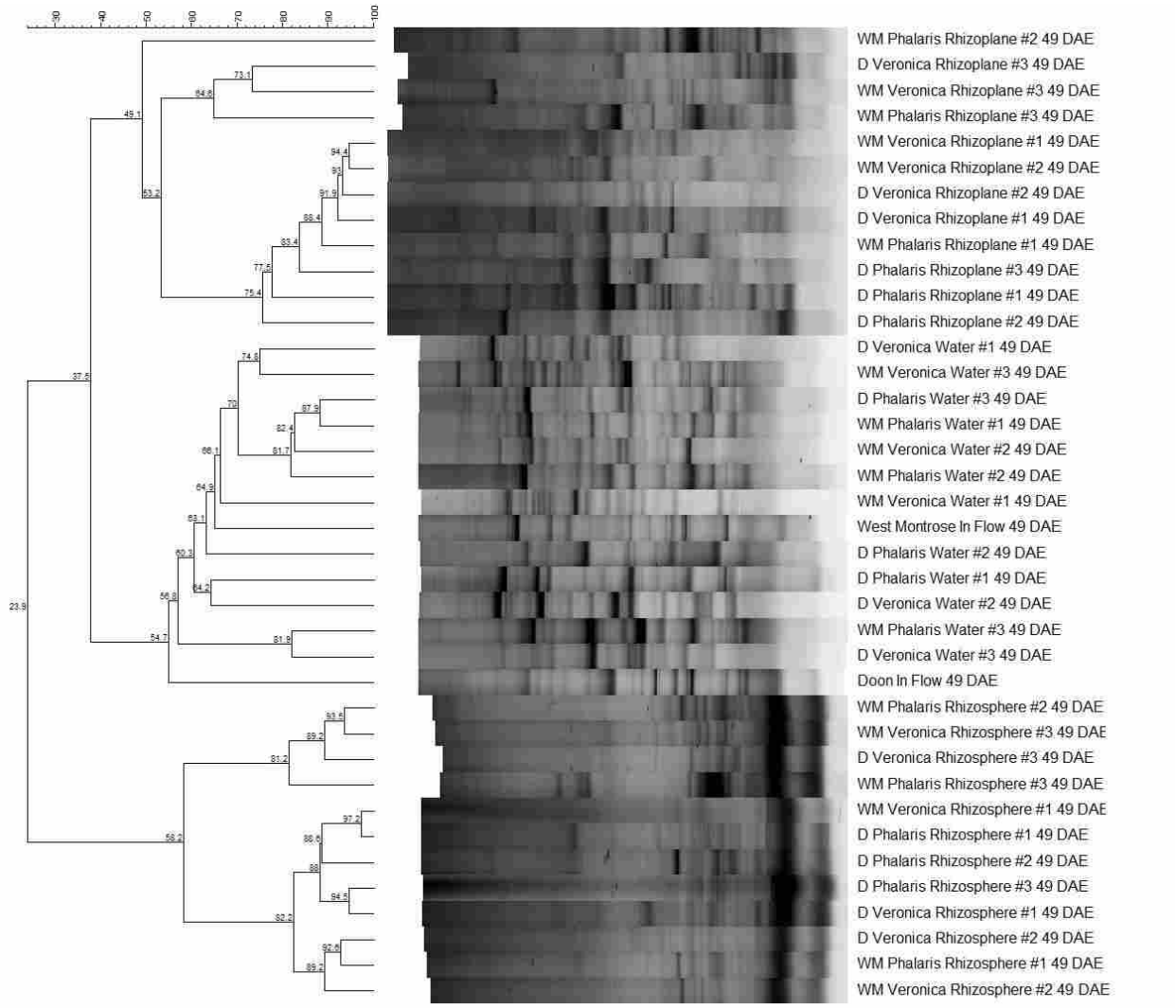

Figure 6.5 Hierarchical cluster analysis results of all of the DGGE profiles obtained from microbial DNA extracted at 49 DAE to 5 mg/L of phosphorus at P_2O_5 demonstrated graphically as a UPGMA dendrogram. Mesocosms receiving high water quality inflow are indicated with a WM (West Montrose) and mesocosms marked to be receiving low water quality inflow are indicated with a D (Doon). Mesocosm replicates per treatment type are indicated with numbers 1-3. Inflow water samples are designated by the water source name followed by the identifier "in flow". Percent similarity between clusters is indicated by the number above branches.

An increase in structural similarity among mesocosm interstitial water microbial communities was observed one week after phosphorous loading (Table 6.1). This increase was followed by a decline in within-group similarity for both water quality treatments. Within the rhizosphere, structural similarity among microbial communities showed a fairly steady decline for the West Montrose treatment group, while the Doon treatment group remained more stable with fairly small fluctuations in within-treatment structural similarity between sampling events. Interestingly, structural similarity trends in rhizoplane microbial communities differed by water quality treatment. Rhizoplane-associated microbial communities receiving West Montrose water showed a trend of decreasing within- group similarity after phosphorus exposure, while the Doon-treated rhizoplane communities increased in similarity.

Table 6.1 Summary of Within Group Percent Similarity for Microbial Community Structural Profiles as Determined by PCR-DGGE-Based Hierarchical Cluster Analysis after Mesocosm Phosphorus Loading

			0 DAE	7 DAE	21 DAE	35 DAE	49 DAE
Rhizoplane		West Montrose	74.2%*(1)	N/A	63.2%*(2)	N/A	49.1%
		Doon	26%	N/A	45.4%*(2)	N/A	63.2%
Rhizosphere		West Montrose	84.1%	74.2%	60.4%	44.4%	58.2%
		Doon	89.4%*(1)	89.9%*(1)	72.7%(1)	81.7%	82.2%*(1)
Water	**Inflow**	West Montrose	69%	83.7%	1.7%	68.3%	54.7%
		Doon					
	Interstitial (outflow)	West Montrose	46.5%*(2)	77.9%	43.9%	28.1%(1)	56.8%
		Doon	62.6%(1)	77.9%	34.8%	46.9%*(2)	56.8%

*Percent similarity value excludes outliers and the numbers of outliers excluded are indicated in the brackets

6.3.1.2 Structural Diversity

At 0 DAE rhizoplane communities showed similar levels of structural diversity, which did not change significantly by 21 DAE to 5 mg/L of added phosphorus (Fig 6.6 A). All treatment groups exhibited a large decrease in structural diversity by 49 DAE, as compared to pre-treatment values. Most treatment groups showed a steady decreasing trend over the course of the experiment with reductions in diversity at both 21 and 49 DAE.

Within-rhizosphere microbial communities at 0 DAE, structural diversity among treatment groups differed between the West Montrose *P. arundinacea* and the Doon *P. arundinacea* treatments. Doon-treated rhizosphere microbial communities exhibited higher values for structural diversity (Fig 6.6 B). All other treatment groups had similar structural diversity values at 0 DAE. Microbial communities associated with the West Montrose *P. arundinacea* treatment exhibited an increase in structural diversity at 7DAE, while all other groups maintained similar diversity values. By 21 DAE both Doon treatment groups showed a decrease in structural diversity which was not observed in the West Montrose-treated microbial communities. All treatment groups showed a decrease in structural diversity by 49 DAE.

The structural diversity of microbial communities associated with water (interstitial/outflow and inflow) exhibited changes over the course of the experiment (Fig 6.6 C). At 0 DAE there was no difference among treatment types in community structural diversity for interstitial microbial communities and these values were similar to those measured from inflow water microbial communities. At 7 DAE to 5 mg/L of added phosphorus both Doon-treated microbial communities showed increased structural diversity which was maintained through 21 DAE and then decreased back to pre-treatment levels by 35 and 49 DAE. This increase was not

observed among the West Montrose-treated microbial communities, where structural diversity levels were similar to pre-treatment values throughout the experiment.

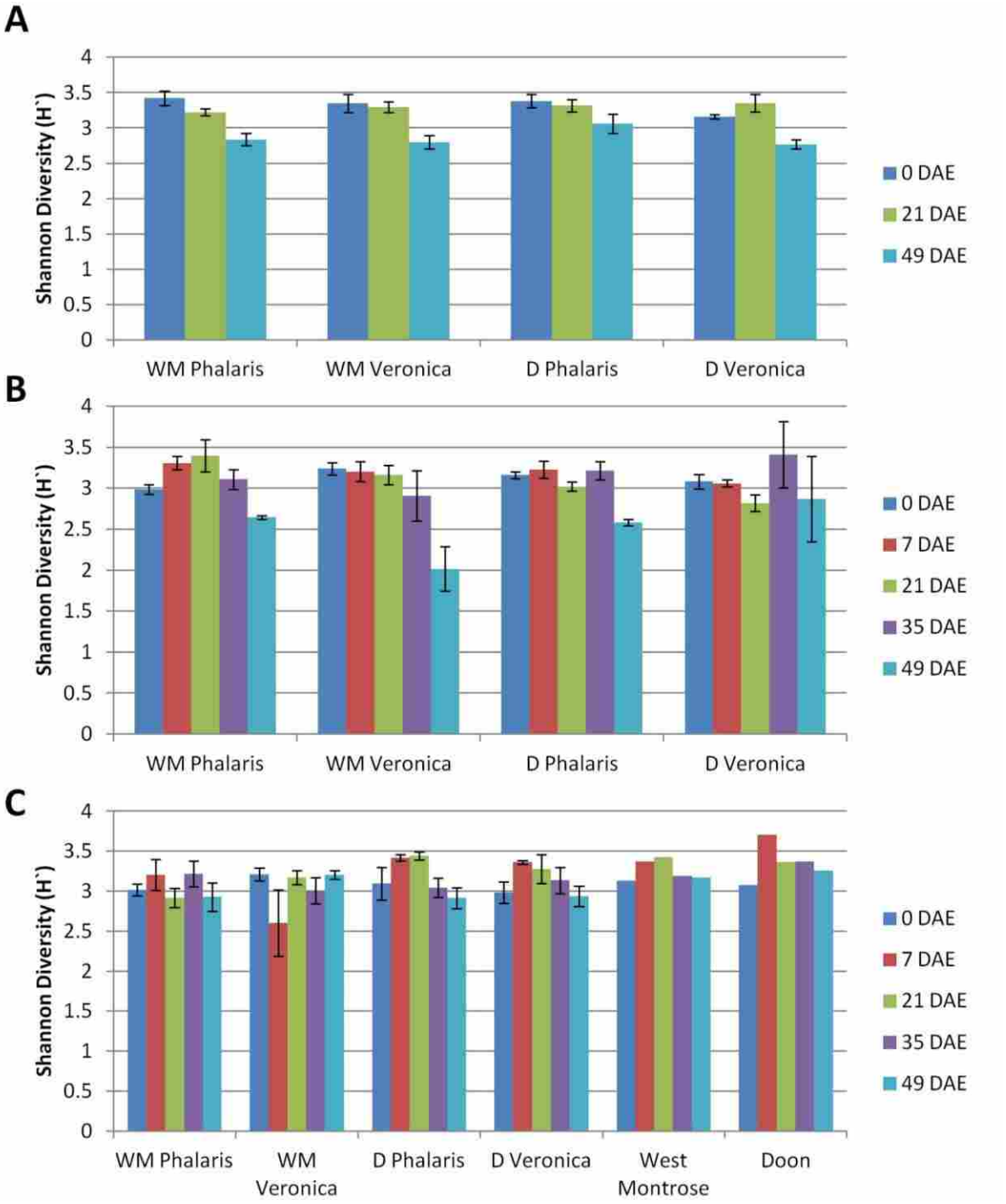

Figure 6.6 Structural diversity of microbial communities in mesocosm (A)rhizoplane samples, (B) rhizosphere samples and (C) water samples (calculated from DGGE-analysis using GelCompar®II software). Error bars are representative of standard error for 3 experimental replicates (n=3). Inflow water samples do not have experimental replicates (n=1).

6.3.2 Functional Carbon Source Utilization Profiles Associated with Microbial Communities from Mesocosms Receiving High and Low Water Quality Treatments Before and After Short Term Phosphorus Loading

6.3.2.1 1Principal Component Analysis of Carbon Source Utilization by Rhizoplane, Rhizosphere and Water-Associated Microbial Communities from Mesocosms Before and After Phosphorus Loading

Functional profiles of microbial rhizoplane communities determined by carbon source utilization on Biolog™ EcoPlates exhibited distinct profiles for each different treatment type before phosphorus loading (Fig 6.7 A). By 21 DAE to phosphorus, carbon source utilization profiles of rhizoplane communities had shifted dramatically (Fig 6.7 D). At this time, only the Doon *P. arundinacea* treatment-associated microbial communities remained distinct, while the carbon source utilization profiles associated with the rhizoplane in each of the other treatments clustered together. By 49 DAE unique carbon source utilization profiles for each treatment community were re-established, however, at this point the relationship between the different treatment types had changed (Fig 6.7G). Prior to exposure, carbon source utilization profiles for the different water quality treatments (West Montrose vs. Doon) were separated along the PC2 axis, while at 49 DAE, the water quality treatments became separated along the PC1 axis. This indicates that water quality treatment represented a greater component of the variability among community carbon source utilization profiles post-treatment compared to pre-treatment (56.64% variability vs. 15.56% variability) (Table A11).

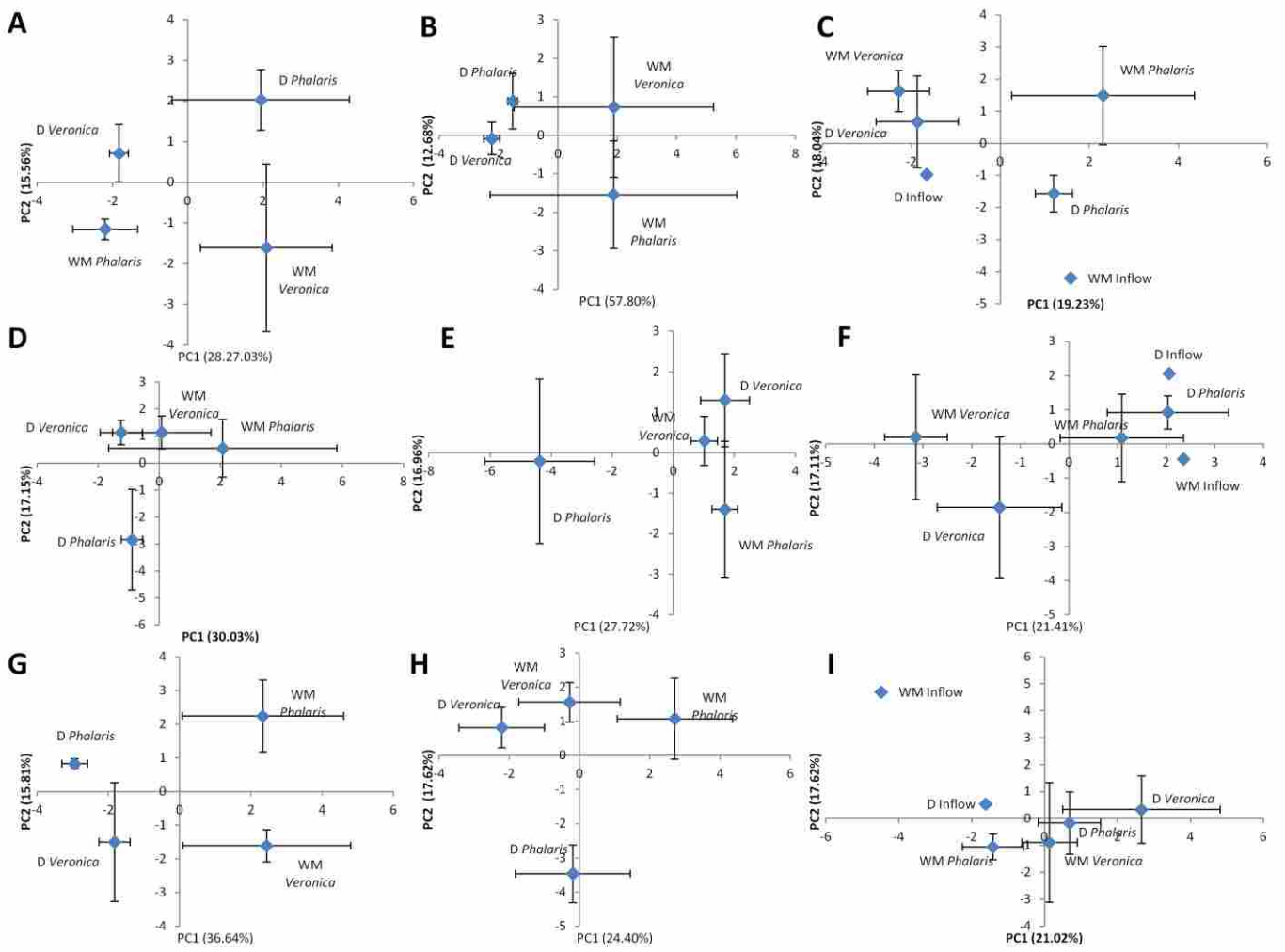

Figure 6.7 PC1 vs. PC2 plots of Biolog^{TM} carbon source utilization profiles generated from microbial communities in the rhizoplane (A, D, G), rhizosphere (B, E, D) and interstitial/in flow water (C, F, I) at 0 (A-C), 49 (D-F) and 91 (G-I) DAE. Mesocosms receiving high water quality inflow are indicated with a WM (West Montrose) and mesocosms receiving low water quality inflow are indicated with a D (Doon). Values are means of 3 experimental replicates and three technical replicates plus standard error for each treatment. Values for inflow water represent the average of a single experimental replicate with triplicate technical replicates only.

Changes observed in the carbon source utilization profiles associated with rhizosphere communities after short-term phosphorus loading were similar to what we observed in rhizoplane-associated microbial communities. Pre-exposure, unique carbon source utilization profiles were associated with each treatment-specific rhizosphere community (Fig 6.7 B). By 21 DAE the only communities that retained unique carbon source utilization profiles were the rhizosphere communities associated with the Doon *P. arundinacea* treatment (Fig 6.7 E).By 49 DAE the *V. anagallis-aquatica* treatments remained undifferentiated (although they were slightly more distinct than observations made at 21 DAE) (Fig 6.7 H). Microbial communities associated with the West Montrose *P. arundinacea* treatment also started to differentiate from the other *V. anagallis-aquatica* treatments by 49 DAE. However, the carbon source utilization profiles associated with these microbial communities still retained similarities with the *V. anagallis-aquatica*-associated microbial communities, especially with those communities receiving West Montrose water (Table A12).

Water associated microbial communities exhibited carbon source utilization profiles that were distinct by water quality treatment in *P. arundinacea* planted mesocosms pre-exposure to added phosphorus (Fig 6.7 C). However, differences between the West Montrose and Doon treated mesocosms planted with *V. anagallis-aquatica* were not observed at this time. Plant species-specific differences were observed in carbon source utilization profiles and were separated along the PC1 axis. By 21 DAE, carbon source utilization profiles from interstitial water microbial communities showed a loss of differentiation among the *P. arundinacea* associated water quality treatments (Fig 6.7 F). By 49 DAE, further loss of differentiation between the communities associated with the different treatment types occurred, with a large

degree of overlap occurring among all four treatment-associated microbial communities (Fig. 6.7 I) (Table A13).

6.3.2.2 Percent Utilization of Different Biolog™ Carbon Source Types by Rhizoplane, Rhizosphere and Water-Associated Microbial Communities from Mesocosms Before and After Phosphorus Loading

Some of the differences observed in the principal component analyses of carbon source utilization profiles pre-and post-exposure to phosphorus can be explained by changes in the relative usage of different carbon sources types (Fig 6.8). Biolog™ EcoPlate carbon sources can be subdivided into the following classifications: polymers (n=4), carbohydrates (n=10), carboxylic and acetic acids (n=9) and nitrogenous carbon sources such as amino acids, amines and amides (n=8). Rhizoplane microbial communities exhibited the most dramatic shift in the relative usage of the different carbon source types post-exposure (Fig 6.8 A,D,G,J). Polymer and carbohydrate usage decreased after phosphorus exposure in all rhizoplane microbial communities with the exception of the Doon *P. arundinacea* associated community (Fig 6.8 A and D). Alternatively, all treatments exhibited an increase in usage of carboxylic and acetic acids post-exposure, again with the exception of the Doon *P. arundinacea* rhizoplane community (Fig 6.8 G). Note that in the principal component analyses, the Doon *P. arundinacea* treatment-associated microbial communities were the only ones that did not show a loss of differentiation after receiving the phosphorus treatment, while all the other treatments clustered together (Fig 6.7 D). There was no change in percent usage of nitrogen containing carbon sources by any community type following phosphorus loading (Fig 6.8 J).

In the rhizosphere, changes to carbon source usage were less consistent across treatments (Fig 6.8 B, E, H and K). Polymer usage showed a small decrease in Doon *V. anagallis-aquatica*-associated treatments post-exposure; while the Doon *P. arundinacea*-associated rhizoplane

communities exhibited a small increase (Fig 6.8 B). Carbohydrate usage did not change consistently across any of the treatments, while carboxylic and acetic acid usage decreased but only in the Doon *P. arundinacea*-associated microbial communities (Fig 6.8 E and H). Both West Montrose-associated rhizosphere communities exhibited an increase in nitrogenous carbon source usage, but not until 49 DAE, which likely means that this increase was the result of something else occurring in the mesocosms or inflow water treatments instead of the phosphorus exposure (Fig 6.8 K). Inflow water microbial community profiles also show an increase in the usage of nitrogenous carbon sources at 49 DAE which is probably the explanation for this observation in the rhizosphere.

Water-associated microbial communities responded with more variability to the phosphorus treatment than the other community types. Polymer usage decreased 7 DAE in the Doon *V. anagallis-aquatica*-associated interstitial water communities, but not in any other treatment type (Fig 6.8 C). Relative usage of carbohydrates and carboxylic and acetic acids did not change post-exposure, again with the exception of the Doon *V. anagallis-aquatica*-associated treatment which exhibited an increase in carbohydrate usage at 21 DAE and an increasing trend in carboxylic and acetic acid usage throughout the experimental period (Fig 6.8 F and I). With respect to the usage of nitrogenous carbon sources, trends differed by plant species. Both *P. arundinacea*-associated water communities showed a decrease in usage post-exposure, which returned to baseline by the end of the experiment. Conversely, both *V. anagallis-aquatica* treatments showed an increase in the usage of nitrogenous carbon sources post-exposure (Fig 6.8 L). The usage of nitrogenous carbon sources decreased again by 21 DAE in the Doon *V. anagallis-aquatica*-associated interstitial water communities, but elevated by 49 DAE in the West Montrose *V. anagallis-aquatica* associated treatment (Fig 6.8 L).

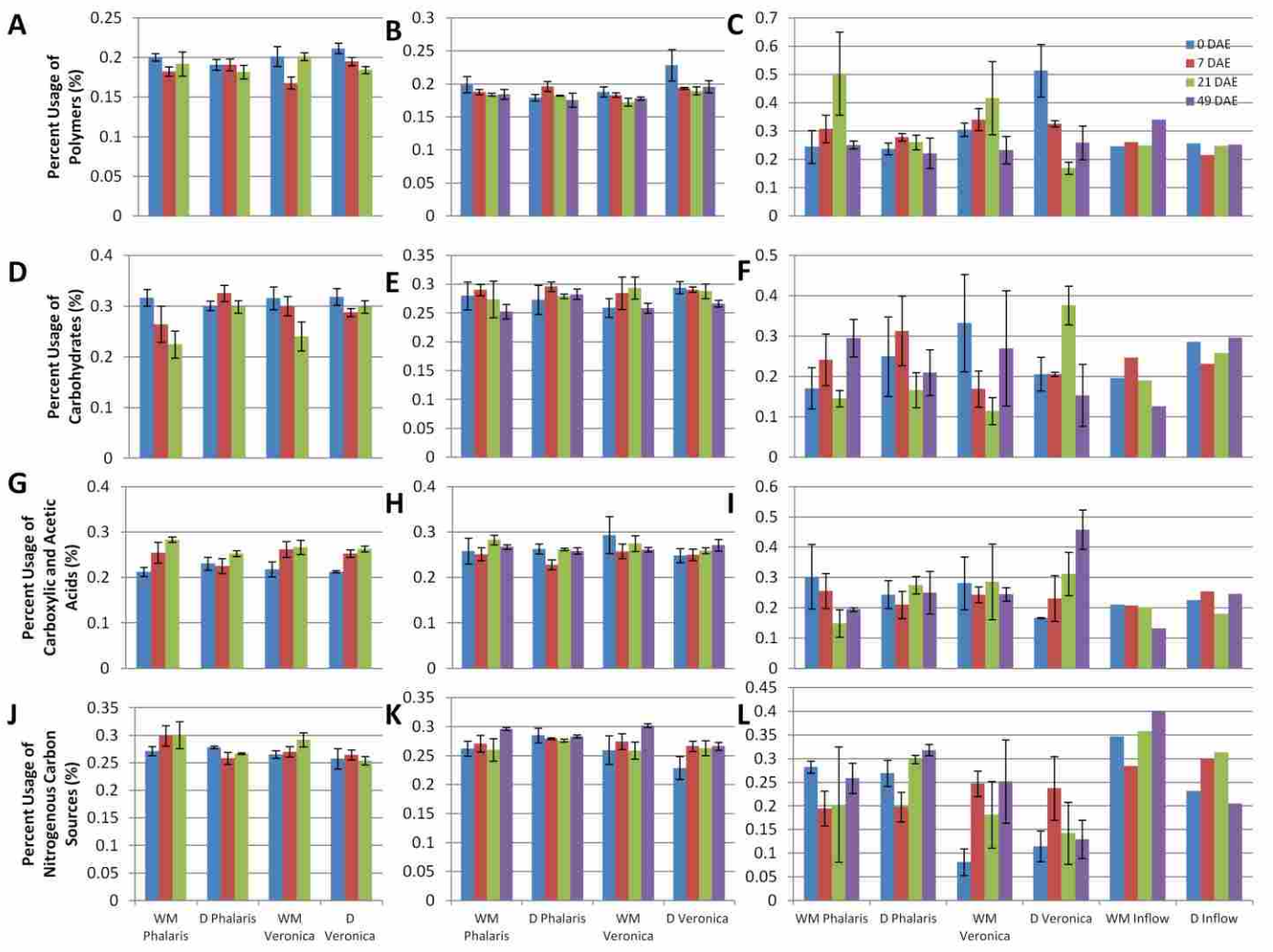

Figure 6.8 Percent usage of Biolog™ EcoPlate carbon sources belonging to the following groupings: polymers (A-C), carbohydrates (D-F), carboxylic and acetic acids (G-I) and nitrogenous carbon sources (J-L). Rhizoplane microbial communities are represented in graphs A, D, G and J, rhizosphere microbial communities are represented in graphs B, E, H and K, and inflow and interstitial water communities are represented in graphs C, F, I and L. Mesocosms receiving high water quality inflow are indicated with a WM (West Montrose) and mesocosms receiving low water quality inflow are indicated with a D (Doon). Bars represent averages of three experimental replicates and three technical replicates for rhizoplane, rhizosphere and interstitial water communities, inflow water communities are averages from technical replicates only. Error bars represent standard error from experimental replicates only.

6.3.2.3 Average Well Color Development and Metabolic Diversity

Average well color development, the average usage of all 31 different Biolog^{TM} carbon sources measured as an absorbance at 590 nm, differed significantly for some treatment types over the experimental timeline within rhizoplane communities. AWCD for *V. anagallis-aquatica* treatments did not change over the course of the experiment, and measurements for West Montrose and Doon treatments were not significantly different from each other (Fig 6.9 A). Alternatively, both West Montrose and Doon-treated *P. arundinacea* rhizoplane-associated microbial communities showed a decrease in AWCD at 21 DAE followed by an increase in AWCD close to values measured at 0 DAE, by 49 DAE. The effect of additional phosphorus on the metabolic diversity of carbon substrates used by rhizoplane microbial communities was similar among treatment groups (Fig 6.9 B). A decrease in metabolic diversity was observed in all communities at 21 DAE. In the *V. anagallis-aquatica* treatments metabolic diversity continued to decreased by 49 DAE. Both *P. arundinacea* treatments increased in metabolic diversity by 49 DAE compared to 21 DAE, although diversity values for both these microbial communities was still much lower than values observed at 0 DAE at this time.

AWCD in rhizosphere-associated communities did not change significantly from pre-treatment measurements after the addition of phosphorus (Fig 6.9C). Metabolic diversity was reduced in rhizosphere communities receiving the Doon (poor water quality) treatment by 7 DAE, while West Montrose-associated communities did not change significantly (Fig 6.9 D). The reduction in metabolic diversity observed post-treatment in the Doon mesocosms increased slightly by 21 DAE but decreased again by 35 DAE, and increased again by 49 DAE to values comparable with pre-exposure measurements.

AWCD for water-associated microbial communities did not change significantly after addition of 5 mg/L of P_2O_5 to the experimental mesocosms (Fig 6.9 E). However, metabolic diversity of the interstitial water communities associated with both *V. anagallis-aquatica* treatments exhibited an increase at 7 DAE, while the *P. arundinacea*-associated communities did not change significantly (Fig 6.9 F). While the West Montrose-treated *V. anagallis-aquatica*-associated mesocosms returned to metabolic diversity values similar to pre-treatment measurements by 21 DAE, the Doon-treated communities continued to exhibit elevated metabolic diversity until 49 DAE.

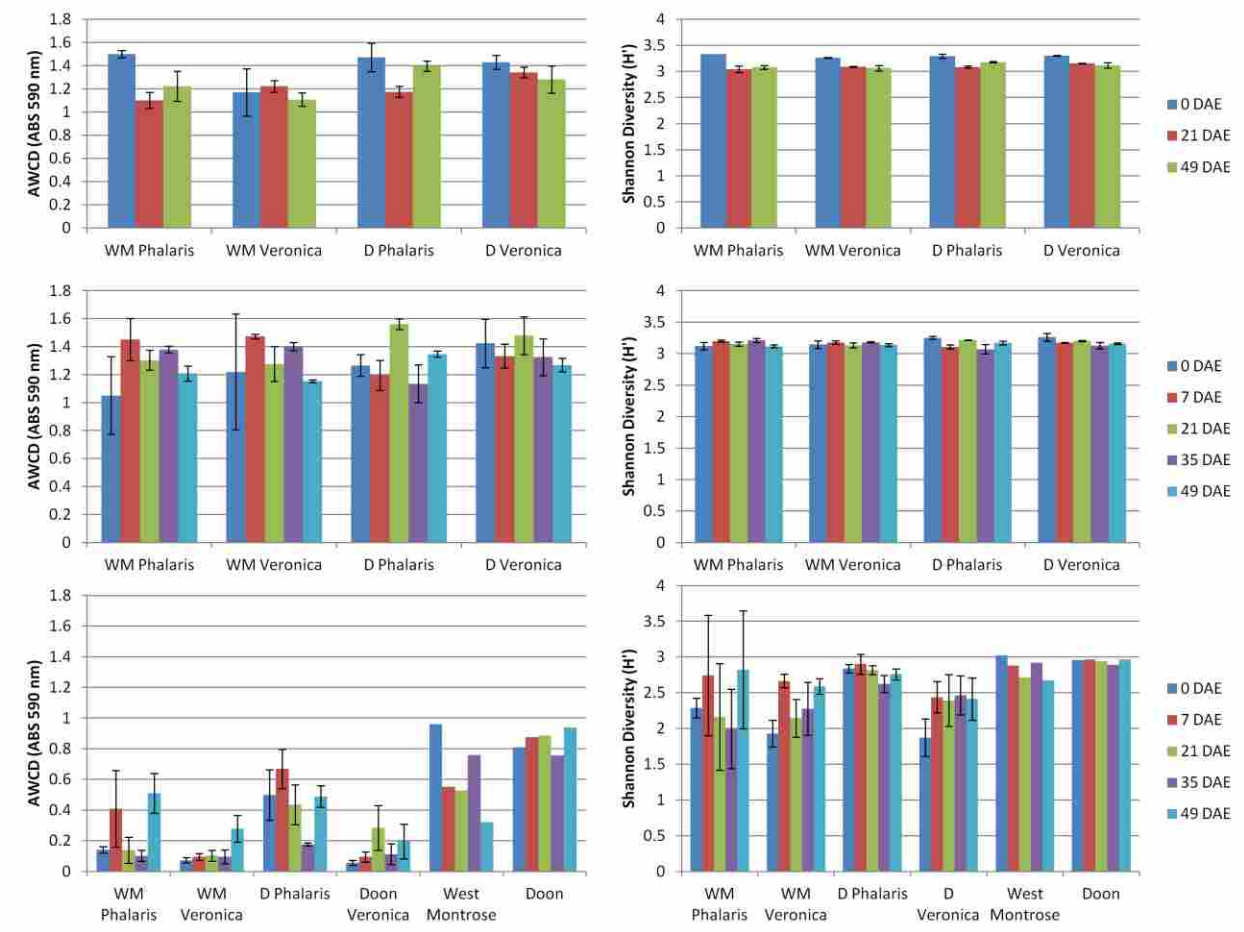

Figure 6.9 Average well color development (AWCD) (A,C,E) and metabolic diversity (B,D,F) of microbial communities from the rhizoplane (A-B), rhizosphere (C-D) and interstitial/inflow water communities (E-F) after exposure to 5 mg/L of P_2O_5. Mesocosms receiving high water quality inflow are indicated with a WM (West Montrose) and mesocosms receiving low water quality inflow are indicated with a D (Doon). Bars represent averages of 3 technical replicates and 3 experimental replicates. Error bars are representative of standard error for experimental replicates. Inflow water samples do not have experimental replicates (n=1).

6.3.3 Water chemistry and Water Quality Variables Associated with Mesocosms Receiving either High or Low Water Quality Treatments Post-Phosphorus Loading

6.3.3.1 Total Reactive Phosphorus

Prior to the addition of inorganic phosphorus into the mesocosms the concentrations of total reactive phosphorus in Doon and West Montrose inflow water were similar to each other and were both either equivalent to or less than the concentration of total reactive phosphorus present in outflow water across sampling events (Fig 6.10). Upon the addition of inorganic phosphorus to the mesocosm inflow water, measured levels of total reactive phosphorus increased significantly in both the Doon and West Montrose inflow water samples, although the increase in the West Montrose water was much higher than in the Doon water, despite the addition of the same amount of P_2O_2 to each water type. The amount of total reactive phosphorus measured in outflow water following phosphorus addition decreased considerably compared both to the previous time point and to inflow values across all treatment types, except the Doon *V. anagallis-aquatica* treatment. From 3 DAE to 49 DAE, total reactive phosphorus concentrations measured in West Montrose and Doon inflow water were similar. Outflow concentrations of total reactive phosphorus steadily increased over the experimental sampling time points until outflow levels resembled those prior to the addition of phosphorus at 49 DAE. At this time, once again, levels of total reactive phosphorus in outflow water were similar to that measured in the inflow water for all treatments. The percentage of total reactive phosphorus present in outflow water was similar among all four treatments.

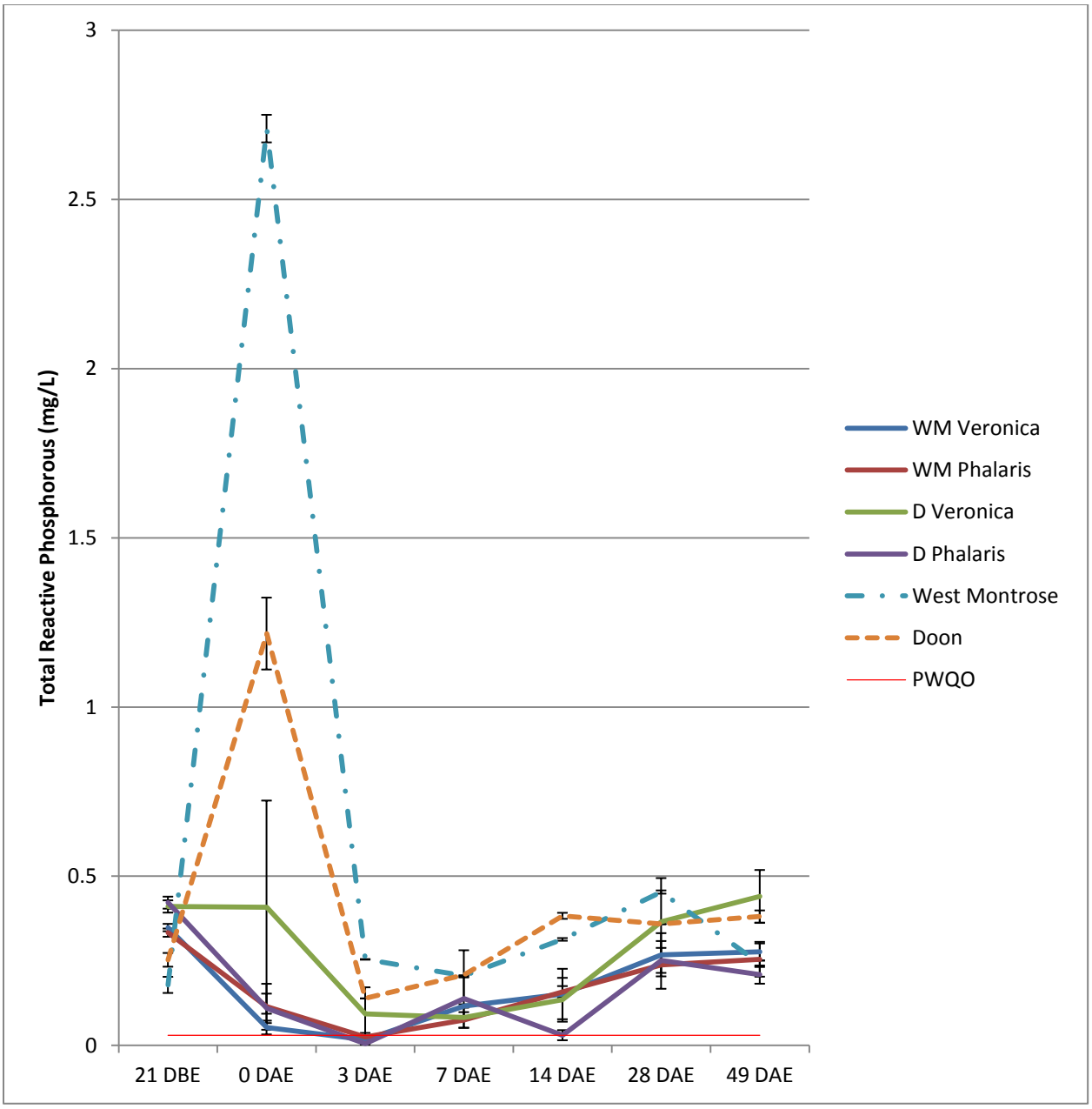

Figure 6.10 Total reactive phosphorus quantification (mg/L) in mesocosm outflow water and inflow water (West Montrose (WM) and Doon (D)) using HACH® water test kits at 21 days before exposure (DBE) to 5 mg/L of phosphorus as P_2O_5 up to 49 days after exposure (DAE). The Ontario provincial water quality objective (PWQO) for total reactive phosphorus in surface water sources is indicated in red. Error bars are representative of standard error (outflow water n=3, inflow water n=3).

6.3.3.2 Nitrate

The amount of nitrate measured in mesocosm inflow water was not different between the West Montrose and Doon sites (Fig 6.11). Both source waters experienced a large increase in nitrate levels at 3 DAE which was likely attributable to an influx of inorganic nitrogen from runoff due to a rain event during water collection at that time. Aside from this sampling point, measurements of nitrate in the inflow water showed some variability across the experimental timeline and ranged between 2 and 9 mg/L. Increases in influent levels of nitrate typically coincided with a decrease in nitrate present in outflow water for all treatment types (e.g. 3 and 21 DAE) while comparably low levels of nitrate present in inflow water coincided with higher levels in outflow water. The concentration of nitrate in outflow water was similar among treatment groups, although the *P. arundinacea* and *V. anagallis-aquatica* treatments receiving Doon inflow water showed a trend towards higher nitrate removal rates compared to West Montrose treatments. The amount of nitrate measured in outflow mesocosm samples showed an overall decreasing trend over the sampling period, which also was observed in the inflow water nitrate concentrations.

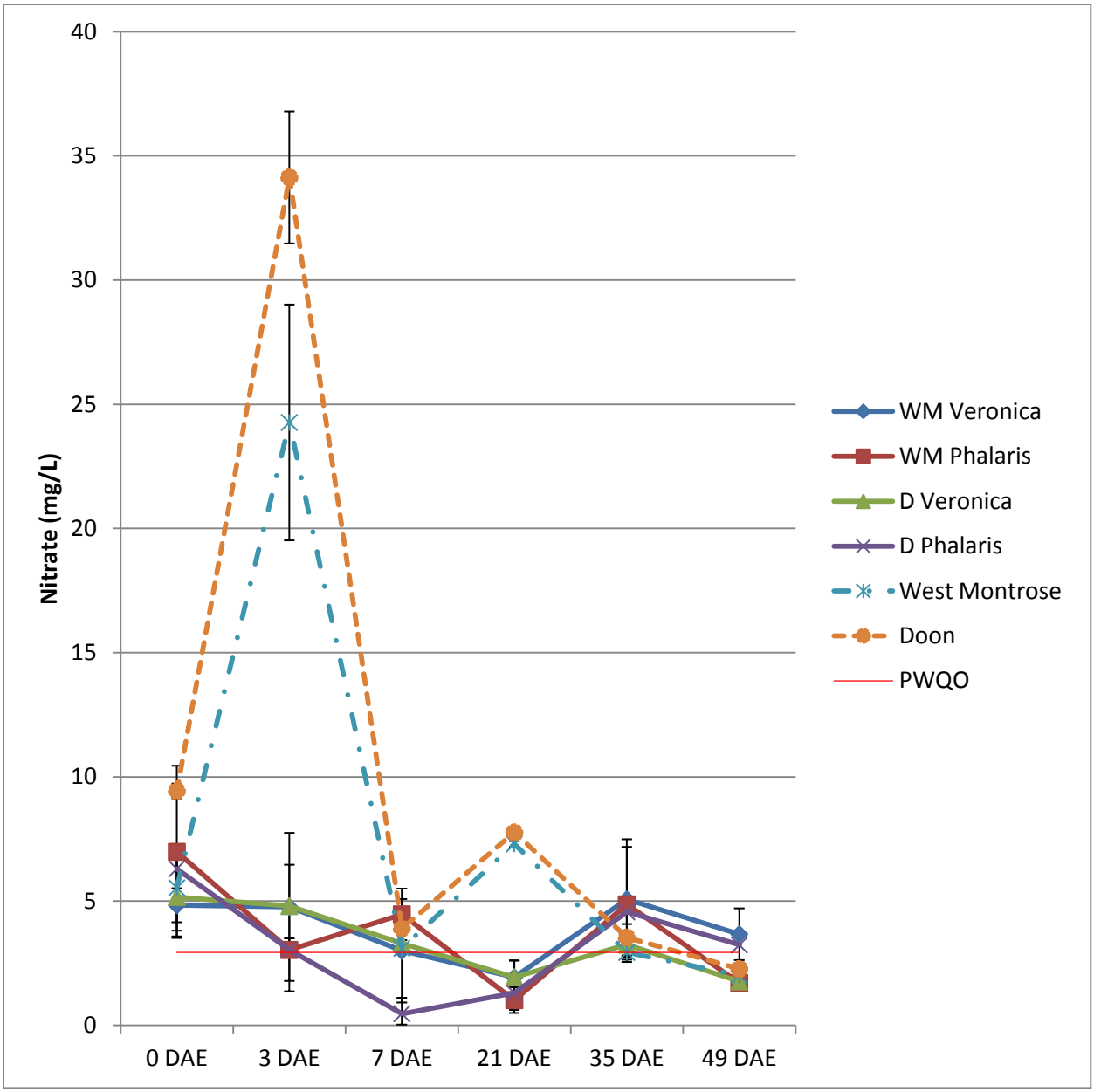

Figure 6.11 Nitrate quantification (mg/L) in mesocosm outflow water and inflow water (West Montrose (WM) and Doon (D)) using HACH® water test kits. The Ontario provincial water quality objective (PWQO) for nitrate in surface water sources is indicated in red. Error bars are representative of standard error (outflow water n=3, inflow water n=3).

6.3.3.3 Nitrite

Nitrite concentrations detected in West Montrose and Doon inflow water were different from each other across the experimental sampling events, however, neither site had consistently higher nitrite concentrations than the other and both sites had large variations in measured concentrations between sampling events (Fig 6.12). Levels of nitrite measured in inflow water were consistently below the provincial water quality objectives for source waters at all time-points other than 3 DAE for the Doon site (Loomer and Cooke, 2011). Levels of nitrite measured in outflow water were typically lower than inflow measurements for both of the Doon treatment groups (*P. arundinacea* and *V. anagallis-aquatica*). Alternatively, the West Montrose treatment groups showed more variability depending on the inflow levels. Higher inflow concentrations of nitrite were associated with higher removal rates and lower concentrations of nitrite in the inflow water correlated with poorer removal percentages. Differences among treatments for nitrite removal were present to some extent, with better removal being associated with the Doon treatments compared to the West Montrose treatments. This comparison was the strongest for the *V. anagallis-aquatica*-planted mesocosms which had much higher removal percentages for the Doon-treated mesocosms compared to the West Montrose-treated mesocosms.

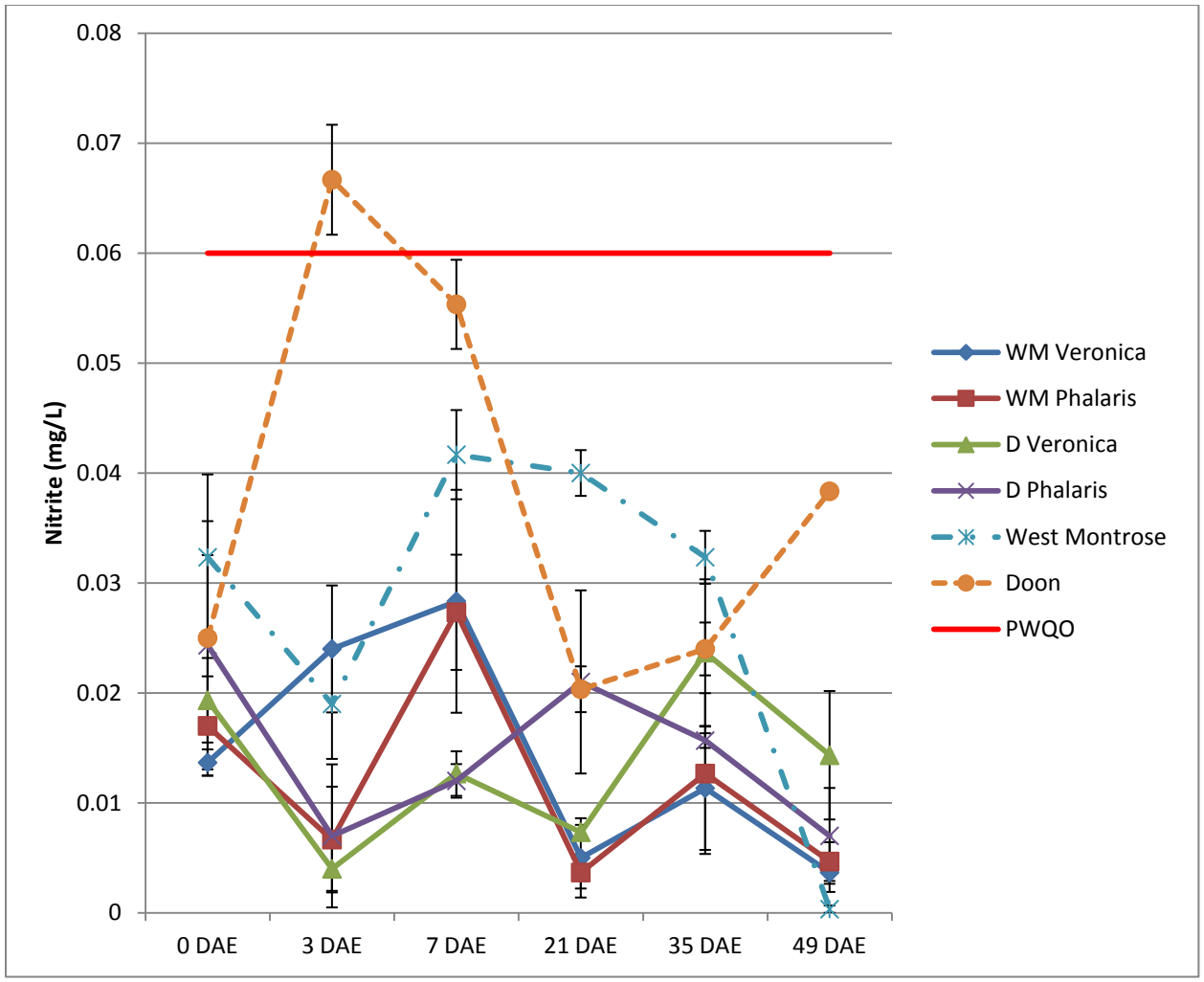

Figure 6.12 Nitrite quantification (mg/L) in mesocosm outflow water and inflow water (West Montrose (WM) and Doon (D)) using HACH® water test kits. The Ontario provincial water quality objective (PWQO) for nitrite in surface water sources is indicated in red. Error bars are representative of standard error (outflow water n=3, inflow water n=3).

6.3.3.4 Ammonia

Unionized ammonia concentrations measured in mesocosm inflow water were similar between West Montrose and Doon source waters during all sampling events (Fig 6.13). Similar to observations from nitrate and nitrite concentrations, there was a spike in unionized ammonia concentrations observed at 3 DAE associated with a rain event. Levels of unionized ammonia measured in inflow water were consistently higher than recommendations in the provincial water quality objectives for surface waters (Loomer and Cooke, 2011). Consistent differences in unionized ammonia concentrations from outflow water among the different treatments were not observed. Similarly, the removal efficiency for unionized ammonia in mesocosm outflow water did not differ significantly among experimental treatments, and on average concentrations in outflow water were higher than inflow water concentrations.

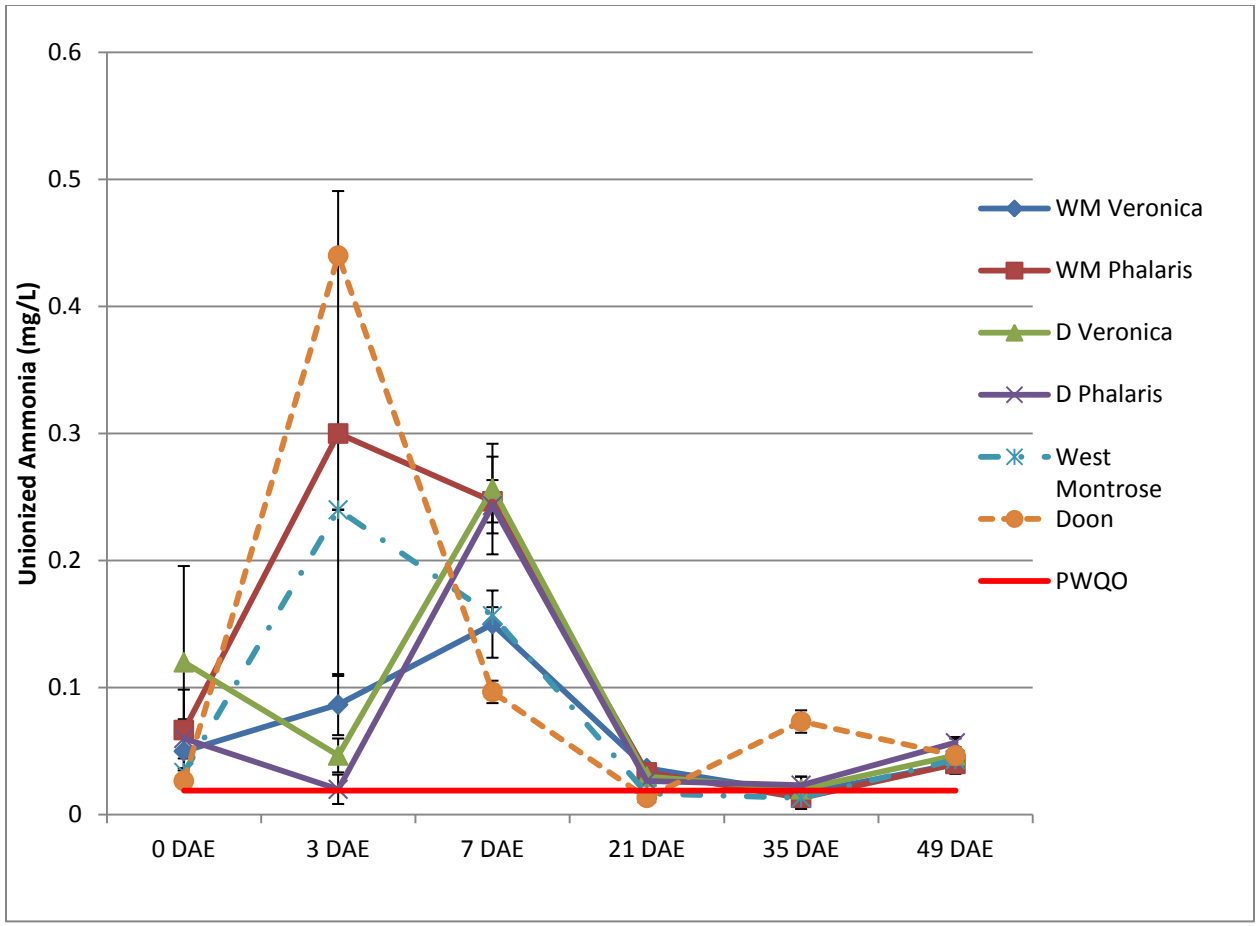

Figure 6.13 Unionized ammonia quantification (mg/L) in mesocosm outflow water and inflow water (West Montrose (WM) and Doon (D)) using HACH® water test kits. The Ontario provincial water quality objectives (PWQO) for ammonia in surface water sources is indicated in red. Error bars are representative of standard error (outflow water n=3, inflow water n=3).

6.3.3.5 Fecal Coliforms (Culture-Based)

Fecal coliforms measured in West Montrose and Doon inflow water both showed a decreasing trend over the sampling time period which was not reflected in the measured values for fecal coliforms present in outflow samples (Fig 6.14). Post-exposure to additional phosphorus, all treatments showed an increase in fecal coliform presence in mesocosm outflow water, however, this increase was greater in the West Montrose *P. arundinacea* and Doon *V. anagallis-aquatica* mesocosms. In the West Montrose *P. arundinacea* treatment fecal coliforms increased after phosphorus exposure and remained relatively constant across the rest of the sampling events. In the West Montrose *V. anagallis-aquatica* treatment fecal coliform numbers showed an increasing trend across the sampling events. The Doon *P. arundinacea* treatment showed the smallest increase in fecal coliforms directly after phosphorus exposure (7 DAE) however this increase became greater at 21 DAE and fluctuated at the remaining sampling points. The Doon *V. anagallis-aquatica* treatment had a large increase in fecal coliforms detected in outflow water, however, from 7 to 49 DAE these values decreased steadily to values close to those at 0 DAE by 49 DAE. There was a high degree of variability in the fecal coliform removal rate for mesocosm treatments among sampling events. However, removal rates tended to decrease over the course of the experiment in all treatment groups. The removal efficiency for fecal coliforms was similar among all treatments aside from the *V. anagallis-aquatica* treatments where removal efficiencies were much better for the Doon treatment than the West Montrose treatment.

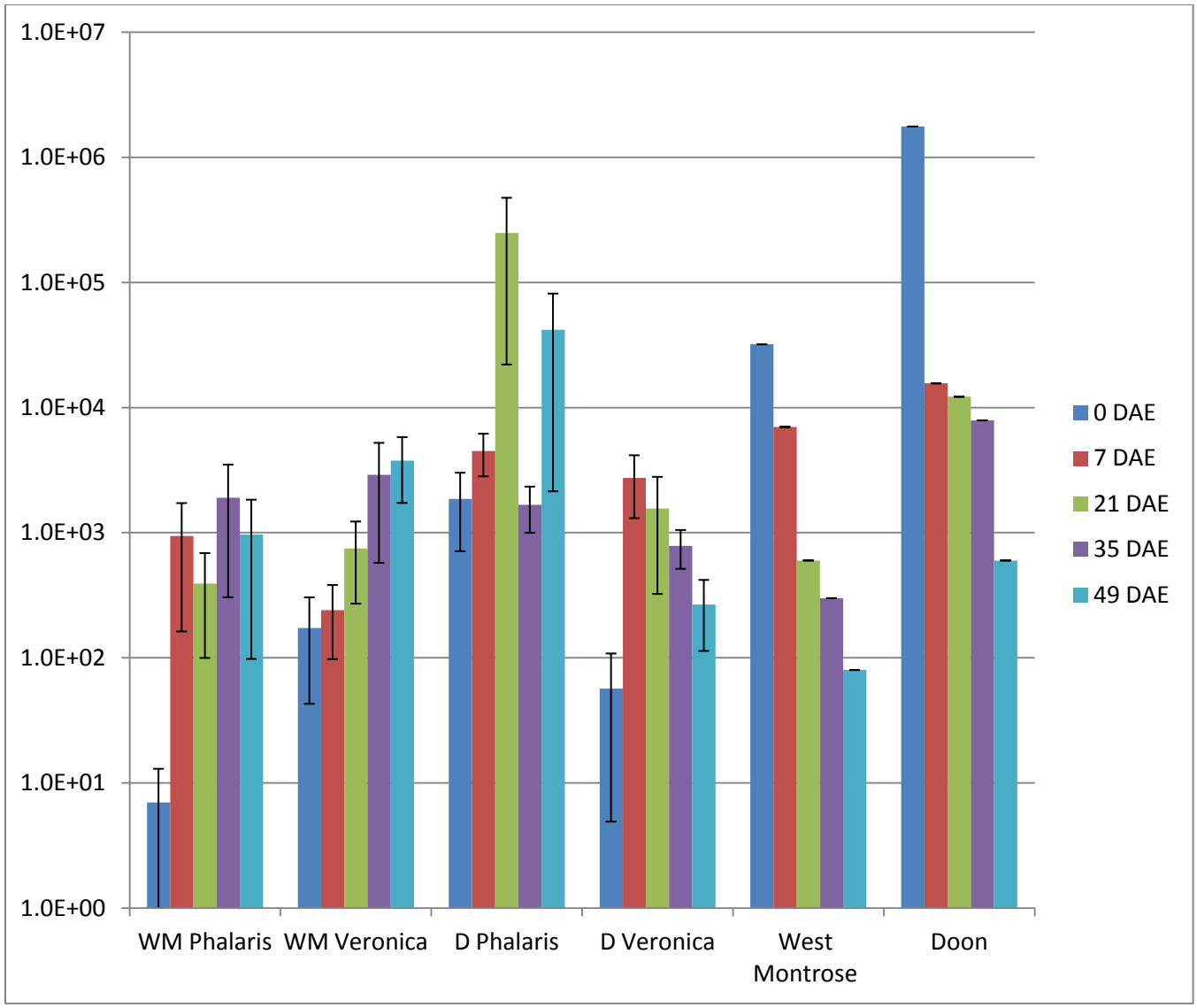

Figure 6.14 Fecal Coliforms (CFU/100 mL) present in mesocosm outflow water and inflow water (West Montrose (WM) and Doon (D)) as determined by the membrane fecal coliform method (m-FC). The Canadian recreational water quality guideline for fecal coliforms in surface water sources is 200 CFU/100 mL. Error bars are representative of standard error among experimental replicates (outflow water n=3, inflow water n=1).

6.4 Discussion

The hypothesis that microbial communities associated with wetland plants adapted to either high or low water quality conditions would respond to environmental perturbations (nutrient addition of 5 mg/L of phosphorus as P_2O_2) differently was examined with this experiment. Measurements of structural community change among the root-associated microbial communities in response to phosphorus addition did not support this hypothesis (Fig 6.1-6.5). Theoretically, the poor water quality effluent treatment would be more adapted to nutrient pulses and potentially respond differently than a higher water quality-adapted community under such conditions. However, similar structural community changes were observed in both of the water quality treatments. At 7 DAE to the phosphorus pulse, there was a large increase in structural similarity among the outflow water microbial communities, while the rhizosphere structural community profiles remained similar, and similar treatment-based groupings by water quality treatment were observed (Fig 6.2). At 21 DAE the increase in structural similarity that occurred directly after the phosphorus pulse had dissipated (Fig 6.3). There was an increase in structural similarity between the outflow and rhizoplane structural community profiles, where they became interchangeable and clustered in groupings containing both community types. There was also a large increase in clustering based on the associated plant species within the interstitial water/rhizoplane microbial community structural profiles. Some plant species-specific clustering was observed at 0 DAE within the rhizoplane (Fig 6.1), which correlated to one cluster of rhizoplane *P. arundinacea* associated communities (74% similarity, 4 samples) and one of *V. anagallis-aquatica* water/rhizoplane communities (67.3 % similarity, 5 samples). This plant species-specific correlation was expanded at 21 DAE to include almost all of the replicates within two larger clusters, one with *V. anagallis-aquatica* water/rhizoplane communities (49%

similarity, 9 samples) and one with *P. arundinacea* rhizoplane/water samples (45% similarity, 6 samples). Although the *P. arundinacea* cluster had a higher similarity, the *V. anagallis-aquatica* cluster contained more structural profiles, 9 of 12 versus 6 of 12 in the *P. arundinacea* cluster. Although we did observe clustering by water quality treatment, this seemed to be secondary to plant species-associated effects on community structure in this instance. By 35 and 49 DAE the plant species-specific effects appeared to diminish (although are retained to some extent exclusively in the rhizoplane) and water quality treatment and community type appear to drive structural similarity, as they did before the phosphorus pulse (Fig 6.4 and 6.5).

The clearest structural change to microbial communities observed in response to phosphorus addition is an increase in the influence of plant species on the structure of microbial wetland communities. Before the addition of the phosphorus, the influence of plant species on community structure was minimal and limited to the rhizoplane microbial community. The increased similarity between the rhizoplane and interstitial/outflow water communities following phosphorus treatment could be the result of an increase in sloughed off rhizoplane biofilm being shed in the outflow water. Other studies using lab-scale mesocosms to assess wetland-associated microbial communities have found that interstitial water communities are representative of the outermost attached biofilms retained within the mesocosm (Weber *et al.*, 2011, Helt *et al.*, 2012). Increased phosphorus availability has been shown to induce root elongation and root density in some plant species (e.g. Borch *et al.*, 1999, Ma *et al.*, 2003), which could result in an increase in biofilm sloughing from root surfaces. Plant species differ in their phosphorus uptake efficiency (e.g. Föhse *et al.*, 1988). They may also differ in their physiological responses to increased phosphorus availability. Hetrick *et al.* (1991) found that cool season grasses (*Bromus inermis, Elymus cinereus, Festuca arundinacea, Koeleria pyranidata* and *Lolium perenne*) responded

differently to phosphorus addition than warm season grasses (*Andropogon gerardii*, *Schizachyrium scopariumm Panicum virgatum, Bouteloua curtipendula, Sorghastrum nutans*), with warm season grasses exhibiting root architectural changes that were absent in the cool season grasses. Similarly, Chapin III and Shaver (1985) found that patterns of growth response to nutrient additions (including phosphorus) in Tundra plant species were highly variable by plant species. As such, differences in plant growth response between our experimental plant species could explain why there is an increased effect of plant species on microbial community structure in the rhizoplane resulting from phosphorus loading. Root-associated microbial communities are known to be affected by root elongation and changes in root architecture (e.g. DeAngelis *et al.,* 2009). Changes in root exudation profiles have also been shown to result from phosphorus addition in some plant species, which is an important driver of plant-associated microbial community structure (e.g. Lipton *et al.,* 1987). Alternatively, an increase in phosphorus availability could also result in changes to the mycorrhizal status of these plants, further resulting in changes to the plant-associated microbial communities which are affected by the presence of mycorrhizas (e.g. Marschner *et al.,* 2001, Hartman *et al.,* 2009). Both of the plant species studied were mycorrhizal and exhibited mycorrhizal colonization during the experiment (data not shown). Mycorrhizas play a substantial role in plant phosphorus acquisition (Smith *et al.,* 2011) and mycorrhizal colonization of plant roots can be dramatically altered by phosphorus availability, decreasing mycorrhizal presence and root colonization in some cases (e.g. Menge *et al.,* 1978, Asimi and Gianinazzi, 1980). Marshall (2013) found that when six species of wetland plants (*P. arundinacea, Echinochloa crus-galli, Solidago Canadensis, Eupatorium perforliatum* and *Verbena hastate*) were exposed to phosphorus concentrations ranging from 0.01 to 7 mg/L, there was a large degree of species-specific variability in changes to mycorrhizal status as a

result of phosphorus exposure. Marshall (2013) found that *P. arundinacea* exhibited inconsistent and minimal reductions in mycorrhizal colonization in response to phosphorus addition, which was not the common trend among the other plant species examined in this study. Thus, if *P. arundinacea* did not experienced a change in mycorrhizal colonization in response to phosphorus loading and *V. anagallis-aquatica* did, this could explain some of the changes to plant-species specific influence on community structure following the phosphorus addition.

Although cluster analyses did not identify water quality treatment-specific differences in community structural responses to the phosphorus pulse in most cases, we did observe a greater increase in the structural diversity of the outflow-based Doon water quality treatments at 7 and 21 DAE, compared to the West Montrose treatment (Fig 6.6). Within the rhizosphere and rhizoplane communities we did not see treatment specific differences in structural diversity, with all treatment types responding similarity to the phosphorus loading over the course of the experiment. It is interesting that the Doon treatment would experience a greater increase in structural diversity within the interstitial water communities, as although the same amount of P_2O_5 was added to the inflow water of both water quality treatments, the concentration of total reactive phosphorus present in Doon inflow water was lower than that of West Montrose water. As such, the resident microbial communities associated with the Doon treatment would have been exposed to less TRP but still exhibited a greater shift in structural community diversity. Beauregard *et al.* (2010) reported that microbial community structural diversity (measured for bacteria, fungi or arbuscular mycorrhizas) in soil associated with alfalfa receiving P_2O_5 fertilization was unaffected by the phosphorus addition over an 8 year long study. These findings agree with our observations for our *P. arundinacea* and *V. anagallis-aquatica*-associated microbial communities from the rhizosphere. However, Ahn *et al.* (2007) found that when

phosphorus was added in low (0.5 mg/L) and high (2.4 mg/L) quantities to lab-scale wetland mesocosms, bacterial community structural diversity in the mesocosm interstitial water was higher in the treatment with lower total reactive phosphorus. They speculated that the addition of phosphorus acted as a stressor reducing microbial diversity in the treatment with higher phosphorus availability. This finding would agree with our observations, in that the treatment with the higher measurable TRP load (West Montrose) exhibited the lower structural diversity value. However, it does not agree with our observation that upon phosphorus addition (which exhibited a significant increase in TRP from 0 to 7 DAE) species diversity actually increased compared to the previous sampling event in Doon-treated mesocosms for interstitial water communities. We suspect that the microbial communities associated with the Doon water treatments may be more adapted to nutrient pulses than the West Montrose-associated communities, as Doon is located immediately downstream from several WWTPs. Under the pre-phosphorus loading conditions, phosphorus would have likely been a limiting nutrient, only supporting abundant growth from organisms that were efficient at scavenging phosphorus. The nutrient pulse allowed other bacterial species, which were not as efficient at scavenging phosphorus (yet were still present in the community, below the detection limits of DGGE), to increase in abundance, resulting in enhanced structural community diversity. Souza *et al.* (2008) has suggested that low phosphorus availability reduces the intensity of horizontal gene transfer and reduces microbial diversity. Additionally, Allers *et al.* (2007) found that structural diversity within Rhodobacteriaceae was enhanced by phosphorus addition in marine water mesocosms. Additional research is required in order to understand how nutrient pulses may impact the structural of microbial communities associated with different types of environments. Our findings in conjunction with reports from the literature indicate that different community types

and bacterial groups respond differently to phosphorus loading, and their behavior is the result of multiple factors which are not currently well understood.

Of the changes that were observed to the structural community profiles in response to phosphorus addition, the least observable differences occurred within the rhizosphere. Rhizosphere microbial community structures appeared to remain relatively stable throughout the experiment. Rhizosphere community structures were largely influenced by water quality treatment throughout the experiment and clustering trends among experimental replicates remained relatively similar across sampling events (Fig 6.1 to 6.5). Edwards *et al.* (2014) reported that among microbial communities established in the bulk soil, rhizosphere and endosphere of rice (*Oryza* spp.), the structure of rhizosphere and bulk soil microbial communities were least impacted by changes in soil type. These results seem to indicate that the rhizosphere could be more structurally stable than other habitat/community types. Reports indicate that the rhizosphere has higher microbial diversity than other rhizo-compartments (Bulgarelli *et al.* 2012, Lundberg *et al.* 2012, Schlaeppi *et al.,* 2014, Edwards *et al.* 2014). This higher structural diversity could result in increased community stability under fluctuating environmental conditions. Increased diversity within the rhizosphere is assumed to be the result of distinctive environmental conditions which encompasses gradients of physicochemical conditions associated with distance from the plant root. These include gradients of oxygenation, carbon availability and pH, among others (Hartmann *et al.,* 2009).

The addition of phosphorus to the wetland mesocosms resulted in several different functional community shifts as measured by Biolog^{TM} EcoPlate carbon source usage. Similar to what we observed in the structural community changes, altered community function in response to the phosphorus pulse was different among community types (rhizosphere, rhizoplane,

interstitial water) (Fig 6.7). The rhizoplane-associated microbial communities exhibited the most dramatic shift in carbon source utilization profiles in response to phosphorus loading, followed closely by the interstitial water communities. Rhizosphere-associated microbial communities remained the most stable over the experimental time period. Interstitial water-associated microbial communities from mesocosms planted with *V. anagallis-aquatica* showed an increase in metabolic diversity 7 days following exposure (Fig 6.9). Pre-treatment carbon source utilization profiles were well separated by both water quality treatment and plant species treatment for principal component analyses of microbial rhizoplane communities (Fig 6.7). However, after the phosphorus exposure, the distinction between water quality treatments dramatically decreased, while plant species-specific differentiation was retained. Although *V. anagallis-aquatica*-associated communities regained some of the functional differentiation between water quality treatments, *P. arundinacea*-associated communities retained the changes observed at 7 DAE until the final sampling event at 49 DAE.

As previously stated, rhizoplane-associated microbial communities exhibited the most dramatic functional community shift as a result of the phosphorus pulse (Fig 6.7 and 6.9). The metabolic diversity in microbial communities associated with all treatments decreased dramatically at 21 DAE. It appeared that by 49 DAE, metabolic diversity started to increase again in each of the *P. arundinacea* treatments, however *V. anagallis-aquatica* diversity measurements continued to decline. These trends were mirrored in measurements for average well color development, a calculation of total community consumption of the Biolog^{TM} EcoPlate carbon sources (Fig 6.9). As in the interstitial water-associated microbial community functional profiles, pre-exposure communities exhibited clear groupings by water quality and plant species treatments when analyzed with PCA (Fig 6.7). However, post-exposure these distinctions were

lost, although mostly recovered by 49 DAE. Furthermore, post-exposure there was a reduction in variability among the mesocosm replicates receiving Doon water which did not occur in West Montrose treated mesocosms (Fig 6.7).

Unlike with the other two community types the rhizosphere did not exhibit plant species-specific or water quality-specific functional differentiation pre-exposure. Some shifts in functional community profiles were observed at 7 DAE, however this only applied to the microbial communities associated with the Doon *P. arundinacea* treatment. The grouping patterns for the other treatments remained the same throughout the experiment. However, differentiation of the Doon *P. arundinacea*-associated microbial community was visible again at 49 DAE. The metabolic diversity of both *P. arundinacea* and *V. anagallis-aquatica* Doon-treated rhizosphere communities decreased 7 days following the phosphorus pulse, while West Montrose treated communities did not exhibit any significant change (but showed a trend towards increasing metabolic diversity). While diversity measurements remained relatively constant in the West Montrose-treatment microbial communities, Doon-treatment communities fluctuated between sampling events, showing an initial decrease in metabolic diversity 7 DAE followed by an increase at 21 DAE to pre-treatment levels, then another decrease and increase at 49 DAE. Due to the fact that changes in the Doon treatments seemed to fluctuate over multiple time points, not just immediately after the addition of inorganic phosphorus. As such, the functional shifts observed at 7 and 49 DAE are likely the result of something else specific to the Doon treatment water besides the inorganic nutrient loads measured in the experiment. Unfortunately, this is one of the drawbacks associated with using experimental conditions that more closely mirror those of natural environments. By using water taken from natural sources instead of composing our own synthetic freshwater, we introduced more variables into the

experiment. As we were not able to complete a full analysis of biotic (e.g. quantification of viruses and grazers) and abiotic (e.g. heavy metals, dissolved organic carbon, inorganic micropollutants) parameters for all water we collected, we cannot comment on additional factors that may have contributed to the trends we observed in our dataset.

With regards to microbial community stability by rhizo-compartment, our functional community data closely resembled observations made from structural community profiles. The functional community characteristic of the rhizoplane changed the most following phosphorus addition. As well, the rhizosphere exhibited the least measureable functional shift following phosphorus exposure among the community types sampled. There are few studies available in the literature that assess the functional characteristics of more than one type of microbial community in association with the plant root. As such, it is difficult to contextualize these findings in a broader sense. As previously stated, the structural stability that has been observed for rhizosphere microbial communities may be a result of increased structural diversity within this rhizo-compartment (Bulgarelli *et al.*, 2012; Lundberg *et al.*, 2012; Schlaeppi *et al.*, 2014; Edwards *et al.*, 2014). It is possible that this may also explain why the rhizosphere appears to be more functionally stable as well. Ecosystem diversity has been shown to be correlated positively with ecosystem stability (MacArthur, 1955; May, 1973). One possibility as to why we observe this correlation is due to functional redundancy with increased structural diversity (Briones and Raskin, 2003). Degens *et al.* (2001) found that functionally diverse soil microbial communities were more resistant (functionally) to perturbation from disturbance (dropping pH, increasing salinity, heavy metal contamination) than microbial communities which had less inherent functional diversity. Furthermore, Griffiths *et al.* (2000) found a positive correlation between high microbial diversity and community functional resilience. Community diversity in soil

samples were reduced artificially by fumigation with chloroform so that communities with varying levels of structural diversity were obtained. Communities with higher structural diversity were associated with increased denitrification, nitrification and methane oxidation. Furthermore, the more structurally diverse microbial communities were more resistant to perturbations by transient temperature increases (40°C) and $CuSO_4$ addition, and demonstrated greater resiliency. Stability was determined by measuring community decomposition rates for grass residues before and several times after imposed stress.

Despite receiving the same water treatment and residing within the same mesocosm, the microbial community subtypes within this experiment responded very differently to the phosphorus addition (Fig 6.9). Metabolic diversity in water-associated microbial communities from *V. anagallis-aquatica* treatments increased, while those communities from *P. arundinacea* planted mesocosms exhibited no change. Rhizosphere and rhizoplane associated microbial communities exhibited a decrease in metabolic diversity following the phosphorus treatment. Although, in the rhizosphere this decrease was only associated with the Doon treatments and was smaller than the decrease observed within the rhizoplane microbial communities. We expected to see a similar change in functional measurements across all community types in response to phosphorus loading, or simply a lack of change in those communities which were more resistant to perturbation. The presence of opposite tendencies in different communities is perplexing. Due to the different physical, chemical and biological characteristics of each community type and the nature of the disturbance the phosphorus addition may have affected each community type differently. For example, the interstitial water communities exhibited significantly lower pre-treatment total carbon source usage and metabolic diversity than the other community types (rhizosphere and rhizoplane). Microbial communities in water also typically experience lower

total microbial abundance and structural diversity than plant-associated communities (although we did not find this to be true in our measurements) (Wetzel, 1975; Wassel and Mills, 1983). Low metabolic activity and diversity associated with relatively high structural diversity in water associated communities could indicate metabolic suppression due to limited nutrient availability in water habitats. Aquatic environments are typically nutrient-poor and favour the growth of fastidious organisms that can grow slowly under nutrient-limiting conditions, such as oligotrophs (Roszak and Colwell, 1987). This is in contrast to organisms that can only grow at higher nutrient concentrations but can survive nutrient limitation by various physiological adaptations, which are termed eutrophs (Roszak and Colwell, 1987). The addition of phosphorus may have stimulated activity in the eutrophic portion of the water-associated microbial community resulting in an increase in metabolic diversity that we did not observe for the other community types. In contrast, the plant-associated microbial communities, which are not as nutrient-limited, would experience the phosphorus addition differently and thus respond accordingly. Furthermore, each community sub-types was structurally unique and there is some evidence that different groups of microorganisms may respond to changes in nutrient availability differently (Horner-Devine *et al.,* 2003). The most important lesson from this data is that we cannot make generalizations about how a microbial community will react to something based on the response of other community types. Many studies observe microbial communities from strictly a functional or structural perspective and focus on a single community type. This only provides a small amount of insight into the complete system, as we have shown here that water quality, plant species and phosphorus loading impacts microbial communities differently depending on whether they are in the rhizosphere, rhizoplane or water phase of the wetland. Furthermore,

structural changes may not always reflect functional changes and a multifaceted approach to studying microbial communities increases our understanding of the complete system.

Adding phosphorus to the inflow water of the mesocosms reduced the concentration of reactive phosphorus present in the outflow water (Fig 6.10). Outflow TRP concentrations and removal percentages did not differ significantly among treatments. Pre-exposure outflow concentrations of TRP exceeded the inflow concentrations. This seems counterintuitive as we would expect that by increasing the amount of phosphorus entering the system we would also increase in the amount of phosphorus exiting the system. The most likely explanation for this is that the mesocosms were phosphorus-limited before the loading phase so microorganisms were actively trying to release phosphorus from organic molecules via mineralization or through solubilisation of bound inorganic phosphorus in soil (Harder and Dijkhuizen 1983, Mohammadi, 2012). These processes increased the inorganic phosphorus (TRP) in the system so that it would be available for the microbial community, but as a result, also increased the concentration of reactive phosphorus in outflow water. Once phosphorus was present in a readily acquirable form through the addition of inorganic phosphate in the inflow water, the microorganism and plants removed it from the system and the microorganisms stopped releasing additional phosphorus from the soil and organic phosphorus stores. This pattern of response to phosphorus loading was also observed in an experiment by Ahn *et al.* (2007) where either 2.4 mg/L or 0.5 mg/L of phosphorus was added to mesocosms planted with *Schoenoplectus tabernaemontani.* The phosphorus removal percentage was significantly higher in high phosphorus treatments (79% vs. -14%) while the amount of free phosphorus in the systems was comparable between treatments (0.89 mg/L of phosphorus in the high phosphorus treatment and 0.39 mg/L of phosphorus in the low phosphorus treatment). This experiment also used unplanted controls, and phosphorus

removal efficiencies were comparable between planted and unplanted systems. This indicates that the majority of the phosphorus removal was being carried out by the microorganisms in the system (combined with equivalent amounts of removal from soil binding in both planted and unplanted controls). Similarly, the contribution of total phosphorus retention in natural stream systems attributed to microbial uptake has been reported to be as high as 80 to 91%, with the remainder being attributed to abiotic processes (Elwood *et al.*, 1981; Newbold *et al.*, 1983).

As stated previously, despite equivalent additions of P_2O_5 to both source waters, lower TRP concentrations were recorded in the Doon water treatment (Fig 6.10). The addition of 5 mg/L of phosphorus as P_2O_5 resulted in lower measurements than the 5 mg/L in both source waters. However, the TRP concentration in West Montrose water was more than double that of what was measured in Doon water. Phosphate has a high affinity for binding to iron and aluminum under acidic conditions and calcium under basic conditions. The pH of both water treatments were slightly basic (pH 8.2-8.4) which indicates that chemical processes removing phosphorus as precipitate would be largely attributed to Ca^{2+} ions (Reddy *et al.*, 1999). It is likely that the Doon site had higher water column concentrations of these dissolved ions, resulting in more inorganic phosphorus being removed from the system and lower resulting TRP concentrations being measured in this water quality treatment. This is an interesting observation as the poor water quality site actually exhibited a higher capacity to buffer against perturbations in water quality compared to the more pristine site.

Although we did not intentionally add a pulse of inorganic nitrogen, we observed a spike in nitrate and unionized ammonia in water from both water quality sites three days after the phosphorus spike (Fig 6.11-6.13). We attributed this spike to rain events that occurred during that time which would have increased the amount of runoff into the Grand River. Despite not

having planned this spike, it provided valuable information that we were able to learn from. Unlike with the phosphorus addition, concentrations of nitrate and ammonia were higher in Doon water compared to West Montrose water, likely due to higher inputs at Doon compared to West Montrose (more remote, less populated, lower land use). Despite the difference in inorganic nitrogen as nitrate entering the mesocosms between water quality treatments, no differences were observed in mesocosm outflow levels of nitrate between the West Montrose and Doon-treated mesocosms. This indicates that both treatment systems were equally capable of handing the increased nitrate load. When the systems received inorganic nitrogen as nitrate, outflow levels dropped compared to previous time points where inflow nitrate levels were fairly low (7-10 mg/L compared to 30-40 mg/L) (Fig 6.11). Similarly, removal percentages improved dramatically with nitrate loading in all mesocosms. This is a similar pattern to that observed with the phosphorus loading, indicating that when nitrate levels in the incoming water were low, more phosphorus was being released from the system, but when availability increased the nitrate was instead retained. Microbial populations are the primary drivers of nitrogen transformations in soil and are well equipped to release previously unavailable forms of nitrogen under limiting conditions (e.g. Kuypers *et al.*, 2018). A similar response was also observed with the increased unionized ammonia concentrations in mesocosm inflow water (Fig 6.13). However, the decrease in outflow water unionized ammonia concentrations following the increase in inflow concentrations was observed to a lesser extent in the West Montrose mesocosms compared to the Doon-treated mesocosms (especially the West Montrose *P. arundinacea* treatment). This could be the result of differences in nitrogen-cycling microbial populations between the two different treatments with regard to ammonia transformations (nitrification), although more advanced methods of microbial community structural analysis would be required to confirm this

hypothesis. Furthermore, nitrification requires oxygen input, while nitrate removal does not (Jaimeson *et al.,* 2003). Any differences in dissolved oxygen levels, or plant root-associated oxygenation between Doon and West Montrose could also lead to differences in ammonia removal efficiencies between the two treatments (Jaimeson *et al.*, 2003). Plants also have different affinities for inorganic nitrogen assimilation as either nitrate or ammonia (Haynes and Goh, 1978). Plant uptake and preference for nitrate and ammonia is species-specific and can be influenced by environmental factors such as temperature and pH, as well as by plant age (Haynes and Goh, 1978). Thus, differences in how the systems responded to the inorganic nitrogen influx as either nitrate or ammonia could also be influenced by assimilation of these compounds by plants.

Unlike observations of nitrate and ammonia concentrations, at 3 DAE we only measured a spike in nitrite concentration in Doon-associated inflow water (Fig 6.12). Nitrite concentrations at West Montrose remained similar to previously recorded values. Historical measurements from the Doon site over our 2-year study period have consistently measured relatively high nitrite concentrations at Doon compared to other sites along the Grand River. This has been confirmed in provincial water quality monitoring network data (data not shown, provincial water quality network, 2012). The differences between these two sites is likely a result of the nature of the inputs received by each site, with Doon receiving significant amounts of WWTP effluent which has been shown to contain elevated levels of nitrite (Allerman, 1985). The Doon-treated mesocosms tended to have better removal percentages for nitrite than the West Montrose-treated mesocosms during most sampling events. We suspect that this is the result of microbial communities associated with the Doon treatment being accustomed to the higher nitrite levels characteristic of that sampling location.

Fecal coliform concentrations in outflow water generally increased in all mesocosms regardless of treatment type following phosphorus loading (Fig 6.14). Fecal bacteria, like any other microorganism, require nutrients for growth. It appears that their proliferation within the mesocosms was stimulated by inorganic phosphorus addition. Similar findings were reported by Chudoba *et al.* (2013) who found that phosphorus addition as either inorganic or organic compounds stimulated fecal coliform growth circumstantially in constructed treatment wetlands of costal North Carolina. Interestingly, nitrogen addition did not result in the same effects. Similarly, Toothman *et al.* (2009) found that phosphorus loading in aquatic sediments, where phosphorus was previously limiting, was positively correlated with fecal coliform and fecal *Enterococcus* abundance within tidal creeks. While the mesocosms were capable of efficiently removing inorganic nutrient loads from inflow waters, the opposite response was observed for fecal coliform presence in outflow water. In constructed wetland systems receiving influent loads where high phosphorus levels are a concern, additional considerations may need to be made regarding the control of potential human pathogenic bacteria which may have previously not been a concern in the influent load.

6.5 Conclusions

We hypothesized that microbial communities associated with mesocosms adapted to either high or low water quality treatments from sites along the Grand River, ON would respond differently to phosphorous loading. Specifically, we predicted that the microbial communities associated with the water quality site experiencing greater anthropogenic impacts (Doon) would be more resistant to the effects of the phosphorus loading and potentially also have greater phosphorus removal capacities. However, evidence to support this hypothesis was not found. Structural and functional community shifts were observed in wetland plant-associated microbial

communities in response to phosphorus loading. However, the quantity and quality of these changes were dependent on the type of microbial community (not the water quality treatment), with rhizoplane and water- associated communities responding more dramatically than rhizosphere-associated communities. Furthermore, previous exposure to either high or low water quality or the presence of either *P. arundinacea* or *V. anagallis-aquatica* did not influence the ability of the wetland mesocosms to remove phosphorus from the system. All treatments performed equally well. Differences in removal capacities for nitrate and ammonia also did not differ between treatments. Nitrite removal efficiency was higher in the low water quality-treated mesocosms which experienced historically high nitrite levels. Phosphorus loading had a negative influence on fecal coliform abundance in wetland mesocosm outflow water, indicating the potential for phosphate influxes into naturally phosphorus-limited systems to stimulate coliform abundance in natural wetlands.

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Chapter 7

Summary and Reccomendations

7.1 Research Hypotheses and Objectives

The relevant hypotheses and objectives that were under investigation throughout this thesis research are as follows:

3) Natural and constructed wetland systems highly impacted by anthropogenic activities will differ from less affected systems in community composition, function, remediation capabilities (ability to remove contaminants and pollutants from water) and response to environmental changes.
    a. Use an *in situ* field-based approach to examine similarities and differences among the microbial communities associated with a wetland plant (*P. arundinacea*) at sampling locations with contrasting water quality characteristics from a structural and functional perspective (Chapter 3).
    b. Use an *ex situ* mesocosm-based approach to look at structural and functional differences between microbial communities associated with wetland plants treated with water from a low water quality sampling location and from a high water quality sampling location using lab-scale constructed wetland mesocosms (Chapter 5). The mesocosm-based approach will reduce some of the variability associated with field-based research and allow us to test the ability of the different wetland communities to remove contaminants.
    c. Use an *ex situ* mesocosm-based approach to examine how wetland-associated communities adapted to either high or low water quality conditions will respond to environmental perturbations by simulating a rain event with associated run-off .

This will be achieved by loading inorganic phosphorus into the mesocosms. Changes to community structure, function and remedial capabilities will be monitored after the phosphorus loading event (Chapter 6).

4) The microbial community composition of wetland plants will differ among species. Different plant species will harbour unique microbial communities that vary in their community compositions, functionality, remediation capabilities and in their response to perturbations in water quality.
    a. Use an *in situ* field-based approach to compare community structural and functional characteristics between different plant species (*I. versicolor*, *P. natans*, *V. spicata*) at field locations with contrasting water quality characteristics (Chapter 4).
    b. Use an *ex situ* lab-based approach to compare the community structure, function and remediation capabilities of wetland-associated microbial communities in mesocosms planted with different plant species (*P. arundinacea* and *V. anagallis-aquatica*) receiving contrasting water quality treatments (Chapter 5).
    c. Compare the ability of high and low water quality-treated microbial communities associated with either *P. arundinacea* or *V. anagallis-aquatica* to resist perturbations in water quality by loading mesocosms with phosphorus (Chapter 6).

7.2 Summary of Major Findings

Summarized below are the major conclusions and trends which have resulted from this research. I have presented them as they correspond to the major research hypotheses and objectives stated above.

1a. Research objective 1a was addressed in Chapter 3, where rhizoplane and rhizosphere communities associated with *P. arundinacea* and river water-associated microbial communities from the Grand River were examined at 6 different sampling locations. These sampling locations had been given water quality designations of high, moderate or poor by the Grand River Conservation Authority based on their inorganic nutrient pollution levels from historical data. Community type (e.g. rhizoplane, rhizosphere or water) was the primary determinant of microbial community structural and functional characteristics. Structural community profiles and functional carbon source utilization profiles clustered together with other samples from the same community type during all sampling events. Microbial community structural profiles from all sample types exhibited some sampling location-specific secondary clustering patterns, however these did not relate to site water quality designations. The only exception to this trend was during the sampling event in July 2015, where rhizosphere samples exhibited secondary sub-groupings related to site water quality designations. However, further investigation into the data revealed that the relationship among the rhizosphere microbial community structural profiles in July was probably the result of physicochemical gradients along the Grand River (e.g. pH, dissolved oxygen and conductivity) and not due to the inorganic nutrient loads at each site. Inorganic nitrogen and phosphorus concentrations did not follow expected trends among sites with regard to water quality designations. Sample location and water quality designations did not clearly

influence carbon source utilization profiles from any sample type during any of the sampling events.

1b. Research objective 1b was met in Chapter 5 whereby lab-scale wetland mesocosms were planted with *P. arundinacea* and *V. anagallis-aquatica* and exposed to water from a high water quality site or a low water quality site for 91 days. The results from this phase of the research revealed a clear effect of water quality treatment on the structural characteristics of rhizoplane, rhizosphere and interstitial water-associated microbial communities. The influence of water quality treatment on the structure of these microbial communities was apparent as early as 14 DAE. The influence of water quality treatment on community functional characteristics was observed to the greatest extent in the rhizoplane and to a lesser extent in the rhizosphere and interstitial water microbial communities. The effects of water quality treatments on functional carbon source utilization profiles associated with microbial communities were greater in *P. arundinacea*-associated communities compared to *V. anagallis-aquatica*-associated communities. Different water quality treated mesocosms did not differ in their abilities to remove inorganic nutrients or total fecal coliforms from incoming water. However, the amount of *E. coli*-specific genetic material detected in the rhizoplane, rhizosphere and interstitial water of wetland mesocosms differed by water quality treatment. Higher loads of *E. coli* were measured in the high water quality treatment-associated samples for interstitial water and rhizosphere soil (only associated with *P. arundinacea*). However, concentrations of *E. coli* detected in the rhizoplane of *V. anagallis-aquatica* were higher in the poor water quality treatment.

1c. Objective 1c was addressed in Chapter 6 where lab-scale wetland mesocosms adapted to either high or low water quality conditions were exposed to 5mg/L of inorganic phosphorus and

the associated microbial communities were monitored for 49 DAE. The removal of phosphorus from incoming water did not differ between the water quality treatments, both of which responded to the increased phosphorus concentration in the incoming water by increasing the efficiency of phosphorus removal for the system. Structural and functional community shifts occurred in response to phosphorus loading within both water quality treatments. Structural and metabolic diversity within the rhizoplane was reduced following phosphorus exposure. Within the rhizosphere, structural diversity was unaffected. However, metabolic diversity was reduced, but only in those communities receiving the poor water quality treatment. There was no difference between the water quality treatments in the ability of mesocosms to remove fecal coliforms. However, all mesocosms experienced an increase in these microorganisms in response to phosphorus loading.

2a. Objective 2a was addressed in Chapter 4 where three species of wetland plants (*I. versicolor, P. natans* and *V. spicata*) were selected at two sampling locations with contrasting water quality characteristics. Structural community data revealed a strong influence of sampling location on the structural community profiles from all sample types and all plant species. However, the degree of structural similarity between microbial communities from the two different sampling locations differed by plant species. Structural similarity between sampling locations was highest for *V. spicata* and lowest for *I. versicolor*, while *P. natans* was intermediate. Similarily, the influence of sampling location on functional community characteristics was plant species-specific. *V. spicata* did not exhibit differences in carbon source utilization by sampling location. *I. versicolor* exhibited distinct carbon source utilization profiles for rhizosphere and rhizoplane communities by sampling location. Alternatively, *P. natans* only exhibit sampling location-specific effects on carbon source utilization profiles within the rhizoplane. The presence of

potentially pathogenic microorganisms associated with fecal contamination differed between the plant species. Specifically, *Enterococcus* species genetic material was completely absent from the rhizosphere of *P. natans* at both sampling locations.

2b. Research objective 2b was addressed in Chapter 5 where *P. arundinacea* and *V. anagallis-aquatica* were exposed to water from a high and low water quality site in lab-scale wetland mesocosms. Microbial community structural profiles were primarily determined by community type and secondarily by water quality treatment. Evidence for plant species-specific influences on microbial community structure were not apparent until 91 DAE and at that time, only in rhizoplane and interstitial water microbial communities. Functional community profiles were distinct between the two plant species for rhizoplane-associated microbial communities. *P. arundinacea* exhibited distinct carbon source utilization profiles by water quality treatment in rhizosphere and interstitial water-associated microbial communities as well, while *V. anagallis-aquatica* did not.

2c. Research objective 2c was addressed in Chapter 6 where lab-scale wetland mesocosms planted with *P. arundinacea* and *V. anagallis-aquatica* adapted to either high or low water quality conditions were exposed to 5 mg/L of inorganic phosphorus. Structural community changes as a result of the short term phosphorus loading were similar for both plant species-associated microbial communities. However, functional community shifts in carbon source utilization following phosphorus exposure differed by plant species. Specifically, the microbial communities associated with the *P. arundinacea* treatment receiving poor water quality inflow water seemed to resist changes especially within the rhizosphere and rhizoplane. Furthermore, *V. anagallis-aquatica*-associated microbial communities in interstitial water exhibited an increase in the usage of nitrogenous carbon sources, while *P. arundinacea*-associated communities

exhibited a decrease, following phosphorus loading. Furthermore, metabolic diversity in *P. arundinacea*-associated rhizoplane microbial communities showed an increase at 49 DAE following the initial decreased seen in all rhizoplane microbial communities at 21 DAE. This rebound observed in the *P. arundinacea*-associated rhizoplane microbial communities was absent from those associated with *V. anagallis-aquatica.* Furthermore, metabolic diversity in *V. anagallis-aquatica*-associated mesocosm interstitial water increased following phosphorus loading, while *P. arundinacea*-associated communities did not exhibit any change.

7.3 Significance of the Research

The research presented in this thesis represents the first studies to assess the effects of water quality associated with anthropogenic impact, from a combined structural and functional microbial community perspective, on multiple microbial community types within wetland-associated habitats. The limited research currently available in the literature addressing this topic only considers a single microbial community type, typically rhizosphere or interstitial water communities, in isolation. We have shown that it is important to monitor the impact of an environmental stressor on all applicable microbial community types as they respond in an individualistic manner to the same condition.

Our findings have shown that the microbial communities in association with different species of wetland plants respond to environmental stressors (in the form of changes to water quality or sampling location with different anthropogenic impacts) in a plant species-dependent manner. More specifically, the degree of structural and functional changes experienced by the associated microbial communities in response to the external stimulus will differ depending on the associated plant species. The research presented in this thesis is the first time that this has

been demonstrated in the literature for plants and microbial communities within a wetland environment.

Significant quantities of DNA from *Salmonella* spp., *E. coli* and *Enterococcus* spp. were associated with the rhizosphere (except for *P. natans* and *Enterococcus*) and rhizoplane microbial communities of our study species. This included evidence from mesocosm studies and/or field studies for the following wetland plant species: *I. versicolor*, *P. arundinacea, P. natans, V. anagallis-aquatica* and *V. spicata.* There have been only a few reports in the literature indicating that *Salmonella* and *Enterococcus* occur naturally (i.e. without experimental introduction) within the rhizosphere and/or rhizoplane of some species of plants (Germida and Sicilano, 2001; Berg *et al.*, 2002; Maougal *et al.*, 2014; Mukhtar *et al.*, 2016). We have provided the first evidence for this among wetland plant species, and for *Salmonella* and *E. coli,* within the rhizoplane biofilm specifically. Furthermore, to the best of our knowledge, this is the first time the exclusion of an entire genus of bacteria (*Enterococcus*) from the rhizosphere of a specific plant species (*P. natans*) has been reported.

7.4 Recommendations for Future Research

1. One of the major themes throughout the different experiments conducted was that structural and functional characteristics of microbial communities from wetlands are determined by the community type. Water quality, sampling location or plant species may also influence structural and functional community characteristics under different circumstances. As such, we recommend that when investigations are being made into the effects of a particular set of environmental circumstances (e.g. contaminant loading, temperature, physical disturbance etc.) on the structure and function of wetland-

associated microbial communities (natural or constructed), the investigators should examine all associated community types separately, as they seem to respond differently to the same environmental changes. Furthermore, if resources do not permit this then the rhizoplane microbial community seems to be the most sensitive to environmental change from our data. If investigators are only interested in seeing whether or not a specific set of conditions causes an effect in microbial community structure and function the rhizoplane may be most applicable as a conservative indicator.

2. Plant species were shown to be differentially affected by the water quality treatments and by short term phosphorus loading. As such, when investigating the effects of environmental change on natural or constructed wetland-associated microbial communities, it is important to sample communities from more than one plant species as we have shown that rhizoplane and rhizosphere microbial community structure and function will respond to environmental changes differently depending on the associated plant species.
3. Our experiment with short term phosphorus loading in wetland mesocosms showed that the addition of inorganic phosphorus to wetland systems can result in an increase in the concentration of fecal coliforms exiting the system. Special attention should be paid to natural or constructed wetland systems typically limited in reactive phosphorus, when experiencing an event temporarily increasing the amount of phosphorus available in that system. Although the wetland may be capable of removing the additional phosphorus load, a corresponding increase in fecal coliforms entering receiving water bodies may present a public health concern.

7.5 Future Directions

1. Differences in the removal of inorganic nutrients among wetland mesocosms containing different plant species or receiving different water quality treatments were not observed. This could be due to the relatively small differences in inorganic nutrient concentrations occurring between the two water quality treatments used in this study. It is possible that with a more significant difference between the inorganic nutrient concentrations of the different water quality treatments, a difference in removal capabilities between the water quality treatments may be observed. Future research should use wetland mesocosms planted with *P. arundinacea* and *V. anagallis-aquatica* receiving either synthetic high and low water quality treatments with greater differences in inorganic nutrient concentrations or use secondary treated WWTP effluent of different strengths (e.g. full strength vs. 1/10) to better observe potential differences among treatments.
2. One of the most interesting findings that came out of this research was that the rhizosphere of *P. natans* does not contain *Enterococcus.* Future research should investigate the cause of this absence. The most likely causes are competitive exclusion or the production of an antimicrobial compound by an organism specific to this rhizosphere community. Future research should focus on determining why *Enterococcus* is absent from this rhizosphere community and whether or not this mechanism may have an application in constructed wetland technology or public health.

7.6 References

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Appendix A: Supplementary Tables

Table A1. Factor Loadings and Eigenvalues for the first two principal components of PCAs of Biolog™ Carbon Source Utilization Profiles of Microbial Communities in the Rhizosphere of *P. arundinacea.*

	May		July		August		October		November	
	PC1	PC2	PC1	PC2	PC1	PC2	PC1	PC2	PC1	PC2
Pyruvic acid methyl ester	0.037	-0.523	0.339	-0.714	0.471	0.473	0.471	0.473	0.432	0.197
Tween 40	-0.178	0.349	-0.342	**0.838**	0.443	**0.618**	0.443	**0.618**	0.141	-0.593
Tween 80	-0.273	-0.082	-0.338	**0.861**	**0.752**	0.125	**0.752**	0.125	-0.143	-0.523
α-Cyclodextrin	-0.369	**0.697**	-0.700	-0.277	0.033	0.030	0.033	0.030	-0.514	0.311
Glycogen	-0.423	-0.144	**0.582**	**0.659**	0.348	-0.176	0.348	-0.176	0.369	-0.465
D-Cellobiose	0.014	-0.460	**0.778**	0.312	-0.371	-0.060	-0.371	-0.060	0.125	**0.581**
α-D-Lactose	-0.107	-0.613	0.086	-0.081	0.060	-0.735	0.060	-0.735	-0.529	0.261
β-Methyl-D-Glucoside	**0.745**	0.132	0.770	0.108	-0.110	**0.612**	-0.110	**0.612**	-0.200	**0.851**
D-Xylose	-0.223	-0.700	-0.686	0.222	-0.197	-0.360	-0.197	-0.360	-0.722	-0.036
i-Erythritol	-0.237	-0.153	-0.172	-0.299	**0.579**	-0.260	**0.579**	-0.260	-0.793	-0.430
D-Mannitol	**0.771**	0.180	**0.742**	-0.100	-0.509	-0.130	-0.509	-0.130	-0.014	**0.645**
N-Acetyl-D-Glucosamine	**0.805**	0.112	**0.713**	**0.567**	-0.685	0.017	-0.685	0.017	0.038	**0.804**
D-Glucosaminic acid	**0.562**	0.458	-0.291	-0.100	0.258	-0.538	0.258	-0.538	**0.578**	-0.244
Glucose-1-Phosphate	-0.433	-0.463	-0.575	0.385	0.373	-0.510	0.373	-0.510	-0.515	0.463
D,L-α-Glycerol Phosphate	-0.051	-0.002	0.311	-0.632	0.755	-0.010	0.755	-0.010	-0.277	-0.494
D-Galactonic acid γ-Lactone	0.486	-0.382	**0.691**	-0.453	-0.079	**0.840**	-0.079	**0.840**	-0.516	0.316
D-Galacturonic acid	**0.797**	0.197	0.345	**0.640**	-0.021	**0.666**	-0.021	**0.666**	**0.596**	-0.191
2-Hydroxy benzoic acid	-0.508	0.024	-0.002	-0.580	-0.434	-0.336	-0.434	-0.336	-0.374	0.439
4-Hydroxy benzoic acid	**0.713**	0.223	-0.413	-0.023	-0.516	-0.095	-0.516	-0.095	**0.751**	0.074
γ-Hydroxybutyric acid	-0.226	**0.524**	-0.290	-0.100	**0.564**	-0.070	**0.564**	-0.070	-0.435	-0.520
Itaconic acid	0.334	0.177	0.200	0.227	-0.627	0.202	-0.627	0.202	-0.679	-0.393
α-Ketobutyric acid	-0.666	0.361	-0.455	-0.442	**0.513**	-0.294	**0.513**	-0.294	-0.521	-0.340
D-Malic acid	0.354	0.472	**0.567**	-0.297	-0.006	-0.019	-0.006	-0.019	**0.875**	-0.253
L-Arginine	-0.633	-0.103	-0.026	-0.743	0.002	-0.397	0.002	-0.397	0.244	-0.163
L-Asparagine	**0.652**	-0.454	**0.900**	-0.078	-0.104	**0.817**	-0.104	**0.817**	**0.901**	0.016
L-Phenylalanine	-0.416	-0.363	-0.699	0.235	0.239	0.236	0.239	0.236	-0.483	0.189
L-Serine	**0.664**	-0.093	0.445	**0.558**	-0.750	-0.194	-0.750	-0.194	0.457	-0.165
L-Threonine	-0.572	0.377	-0.527	0.083	**0.729**	-0.133	0.729	-0.133	-0.645	-0.298
Glycyl-L-Glutamic acid	-0.560	0.216	-0.726	-0.363	0.620	0.132	0.620	0.132	-0.653	-0.601
Phenylethyl-amine	-0.435	**0.815**	-0.239	-0.323	-0.114	0.056	-0.114	0.056	0.001	-0.632
Putrescine	0.269	0.239	**0.551**	-0.494	0.361	0.077	0.361	0.077	0.399	-0.274
Eigenvalues	7.555	4.659	8.533	6.316	6.261	4.702	6.261	4.702	8.117	5.828

* Carbon sources contributing significantly to positive principal component scores are bolded while carbon sources contributing significantly to negative principal component scores are underlined

Table A2. Factor Loadings and Eigenvalues for the first two principal components of PCAs of Biolog™ Carbon Source Utilization Profiles of Microbial Communities in the Rhizosphere of *P. arundinacea.*

	May		July		August		October		November	
	PC1	PC2	PC1	PC2	PC1	PC2	PC1	PC2	PC1	PC2
Pyruvic acid methyl ester	**0.875**	-0.143	-0.218	0.347	**0.736**	-0.235	**0.736**	-0.235	**0.627**	-0.228
Tween 40	**0.729**	-0.306	0.005	**0.627**	**0.912**	-0.044	**0.912**	-0.044	0.327	0.408
Tween 80	0.094	-0.518	-0.228	0.440	**0.733**	0.372	**0.733**	0.372	**0.705**	0.170
α-Cyclodextrin	0.051	**0.513**	**0.869**	-0.093	-0.002	**0.521**	-0.002	**0.521**	-0.589	0.321
Glycogen	**0.934**	-0.086	-0.076	**0.523**	-0.707	-0.017	-0.707	-0.017	-0.553	0.210
D-Cellobiose	0.063	0.165	0.062	**0.721**	-0.509	-0.613	-0.509	-0.613	-0.827	0.059
α-D-Lactose	-0.050	**0.814**	**0.673**	-0.288	-0.516	-0.212	-0.516	-0.212	-0.625	**0.517**
β-Methyl-D-Glucoside	**0.596**	-0.228	-0.553	0.456	-0.522	-0.602	-0.522	-0.602	-0.602	-0.398
D-Xylose	-0.328	**0.859**	**0.786**	-0.039	0.048	**0.652**	0.048	**0.652**	-0.270	**0.720**
i-Erythritol	**0.530**	0.444	0.156	-0.462	**0.516**	0.495	**0.516**	0.495	0.340	**0.715**
D-Mannitol	0.379	-0.309	-0.810	-0.327	**0.706**	-0.099	**0.706**	-0.099	-0.554	-0.381
N-Acetyl-D-Glucosamine	**0.730**	-0.272	-0.626	0.469	-0.653	0.257	-0.653	0.257	-0.766	-0.186
D-Glucosaminic acid	-0.229	**0.676**	-0.091	-0.695	0.179	0.372	0.179	0.372	0.240	-0.160
Glucose-1-Phosphate	0.033	**0.855**	**0.671**	-0.253	-0.357	0.010	-0.357	0.010	-0.372	**0.591**
D,L-α-Glycerol Phosphate	**0.816**	-0.003	-0.001	-0.306	0.324	-0.557	0.324	-0.557	0.400	**0.507**
D-Galactonic acid γ-Lactone	-0.431	-0.528	-0.323	0.256	**0.911**	-0.153	**0.911**	-0.153	0.073	-0.473
D-Galacturonic acid	-0.822	-0.405	-0.772	-0.411	**0.663**	-0.057	**0.663**	-0.057	0.321	-0.221
2-Hydroxy benzoic acid	-0.801	0.340	**0.818**	0.250	-0.549	0.305	-0.549	0.305	0.366	0.352
4-Hydroxy benzoic acid	-0.796	-0.103	0.149	-0.351	-0.221	**0.756**	-0.221	**0.756**	**0.860**	-0.109
γ-Hydroxybutyric acid	-0.224	-0.233	0.171	0.468	0.017	**0.660**	0.017	**0.660**	**0.693**	0.360
Itaconic acid	-0.556	0.346	0.055	-0.382	-0.470	-0.103	-0.470	-0.103	0.454	0.251
α-Ketobutyric acid	0.078	**0.713**	**0.652**	-0.379	-0.365	0.125	-0.365	0.125	**0.502**	0.005
D-Malic acid	-0.411	-0.454	0.154	0.246	-0.679	0.014	-0.679	0.014	0.270	-0.198
L-Arginine	-0.920	0.007	-0.326	-0.689	**0.602**	0.176	**0.602**	0.176	**0.571**	-0.251
L-Asparagine	-0.880	-0.274	-0.834	-0.270	**0.966**	-0.023	**0.966**	-0.023	0.315	-0.653
L-Phenylalanine	-0.324	**0.659**	0.461	-0.594	-0.365	0.005	-0.365	0.005	0.403	**0.706**
L-Serine	-0.789	-0.106	-0.584	-0.606	-0.571	**0.523**	-0.571	**0.523**	-0.121	-0.636
L-Threonine	-0.212	0.072	**0.526**	-0.014	-0.062	**0.526**	-0.062	**0.526**	-0.176	**0.550**
Glycyl-L-Glutamic acid	0.208	**0.673**	**0.647**	-0.150	-0.489	0.243	-0.489	0.243	0.497	0.424
Phenylethyl-amine	-0.759	-0.305	**0.714**	0.419	-0.595	-0.393	-0.595	-0.393	**0.753**	-0.232
Putrescine	-0.878	-0.200	-0.247	0.113	**0.569**	-0.169	**0.569**	-0.169	**0.660**	-0.323
Eigenvalues	10.783	6.255	8.270	5.450	9.778	4.424	9.778	4.424	8.399	5.340

* Carbon sources contributing significantly to positive principal component scores are bolded while carbon sources contributing significantly to negative principal component scores are underlined

Table A3. Factor Loadings and Eigenvalues for the first two principal components of PCAs of Biolog™ Carbon Source Utilization Profiles of Microbial Communities in Grand River Water.

	May		July		August		October		November	
	PC1	PC2	PC1	PC2	PC1	PC2	PC1	PC2	PC1	PC2
Pyruvic acid methyl ester	-0.709	0.420	**0.700**	-0.217	**0.855**	-0.310	0.432	0.197	**0.752**	-0.507
Tween 40	**0.546**	0.463	0.058	**0.906**	0.409	0.213	0.141	-0.593	0.198	-0.145
Tween 80	-0.119	0.155	-0.329	0.439	**0.608**	**0.674**	-0.143	-0.523	-0.727	-0.607
α-Cyclodextrin	**0.798**	-0.242	0.041	0.025	0.430	0.194	-0.514	0.311	-0.089	-0.032
Glycogen	**0.540**	0.236	**0.895**	-0.160	-0.388	-0.263	0.369	-0.465	**0.731**	-0.136
D-Cellobiose	0.241	-0.241	-0.381	**0.847**	-0.650	0.320	0.125	**0.581**	0.253	-0.049
α-D-Lactose	0.357	-0.586	-0.524	0.297	**0.952**	-0.029	-0.529	0.261	0.431	0.493
β-Methyl-D-Glucoside	-0.621	-0.579	-0.743	0.007	-0.238	-0.312	-0.200	**0.851**	-0.503	**0.827**
D-Xylose	**0.821**	-0.072	-0.310	-0.476	0.057	**0.729**	-0.722	-0.036	0.034	**0.834**
i-Erythritol	-0.111	-0.357	0.470	-0.770	**0.688**	-0.302	-0.793	-0.430	**0.963**	0.012
D-Mannitol	-0.537	0.344	**0.608**	-0.035	**0.578**	-0.605	-0.014	**0.645**	-0.399	**0.533**
N-Acetyl-D-Glucosamine	-0.568	-0.469	0.466	-0.807	**0.717**	0.172	0.038	**0.804**	-0.979	-0.074
D-Glucosaminic acid	-0.404	-0.047	-0.674	-0.635	**0.798**	0.330	**0.578**	-0.244	**0.916**	-0.248
Glucose-1-Phosphate	**0.753**	-0.304	-0.695	-0.322	-0.229	0.417	-0.515	0.463	-0.096	**0.841**
D,L-α-Glycerol Phosphate	0.013	0.007	**0.607**	-0.615	0.290	0.256	-0.277	-0.494	0.288	-0.776
D-Galactonic acid γ-Lactone	-0.718	0.037	-0.720	0.465	-0.671	-0.440	-0.516	0.316	-0.679	0.431
D-Galacturonic acid	-0.707	-0.095	-0.385	**0.672**	-0.687	-0.173	**0.596**	-0.191	-0.366	0.018
2-Hydroxy benzoic acid	0.317	-0.378	-0.833	-0.460	**0.714**	-0.480	-0.374	0.439	**0.701**	0.188
4-Hydroxy benzoic acid	-0.362	0.200	0.158	-0.326	-0.318	**0.532**	**0.751**	0.074	-0.772	-0.246
γ-Hydroxybutyric acid	0.096	-0.546	**0.719**	-0.263	0.437	-0.323	-0.435	-0.520	**0.524**	**0.830**
Itaconic acid	0.125	**0.513**	**0.778**	-0.492	**0.801**	0.140	-0.679	-0.393	-0.306	-0.185
α-Ketobutyric acid	0.269	-0.552	-0.815	-0.493	-0.079	**0.570**	-0.521	-0.340	0.190	-0.335
D-Malic acid	0.093	**0.892**	**0.590**	-0.109	-0.155	-0.707	**0.875**	-0.253	-0.494	0.360
L-Arginine	-0.135	0.262	0.035	-0.366	-0.746	0.112	0.244	-0.163	0.148	-0.800
L-Asparagine	-0.661	-0.247	-0.167	-0.827	-0.840	-0.092	**0.901**	0.016	-0.466	-0.673
L-Phenylalanine	0.113	**0.626**	-0.745	-0.496	-0.078	**0.597**	-0.483	0.189	-0.176	**0.916**
L-Serine	0.100	0.451	-0.665	-0.602	-0.905	-0.148	0.457	-0.165	-0.207	-0.568
L-Threonine	**0.688**	0.081	-0.570	-0.668	-0.609	-0.191	-0.645	-0.298	**0.924**	-0.053
Glycyl-L-Glutamic acid	**0.661**	0.182	-0.163	-0.077	**0.679**	**0.608**	-0.653	-0.601	**0.980**	0.024
Phenylethyl-amine	**0.653**	0.238	-0.649	-0.639	**0.636**	-0.560	0.001	-0.632	**0.866**	0.206
Putrescine	-0.268	0.681	0.038	-0.162	-0.329	**0.906**	0.399	-0.274	-0.446	-0.753
Eigenvalues	7.581	4.969	9.906	8.114	10.913	5.895	8.117	5.828	10.562	8.069

* Carbon sources contributing significantly to positive principal component scores are bolded while carbon sources contributing significantly to negative principal component scores are underlined

Table A4. Factor Loadings and Eigenvalues for the first two principal components of PCAs of Biolog™ Carbon Source Utilization Profiles of Microbial Communities in all Sample Types obtained from the Grand River Water.

	May		July		August		October		November	
	PC1	PC2	PC1	PC2	PC1	PC2	PC1	PC2	PC1	PC2
Pyruvic acid methyl ester	**0.520**	0.053	**0.931**	0.064	-0.649	**0.593**	**0.855**	-0.310	**0.875**	-0.143
Tween 40	-0.357	**0.583**	**0.861**	0.095	-0.742	**0.578**	0.409	0.213	**0.729**	-0.306
Tween 80	-0.416	-0.788	**0.506**	0.015	0.123	**0.694**	**0.608**	**0.674**	0.094	-0.518
α-Cyclodextrin	0.187	**0.576**	-0.408	**0.573**	-0.258	-0.207	0.430	0.194	0.051	**0.513**
Glycogen	-0.241	**0.655**	**0.858**	0.025	-0.865	-0.186	-0.388	-0.263	**0.934**	-0.086
D-Cellobiose	-0.449	0.119	0.244	0.091	-0.300	-0.446	-0.650	0.320	0.063	0.165
α-D-Lactose	0.277	-0.008	-0.275	**0.604**	-0.183	-0.470	**0.952**	-0.029	-0.050	**0.814**
β-Methyl-D-Glucoside	0.060	-0.136	**0.830**	0.079	-0.502	-0.238	-0.238	-0.312	**0.596**	-0.228
D-Xylose	-0.782	-0.579	-0.374	**0.747**	-0.150	-0.363	0.057	**0.729**	-0.328	**0.859**
i-Erythritol	**0.880**	-0.319	0.475	-0.154	0.071	**0.517**	**0.688**	-0.302	**0.530**	0.444
D-Mannitol	0.406	0.494	**0.715**	-0.336	-0.362	0.409	**0.578**	-0.605	0.379	-0.309
N-Acetyl-D-Glucosamine	-0.296	**0.933**	**0.889**	-0.231	-0.718	-0.217	**0.717**	0.172	**0.730**	-0.272
D-Glucosaminic acid	**0.971**	-0.168	-0.464	0.281	0.083	-0.420	**0.798**	0.330	-0.229	**0.676**
Glucose-1-Phosphate	0.301	-0.195	-0.174	0.465	-0.419	-0.262	-0.229	0.417	0.033	**0.855**
D,L-α-Glycerol Phosphate	**0.807**	0.219	**0.728**	-0.200	-0.798	0.265	0.290	0.256	**0.816**	-0.003
D-Galactonic acid γ-Lactone	-0.113	-0.745	**0.809**	-0.116	0.308	0.757	-0.671	-0.440	-0.431	-0.528
D-Galacturonic acid	0.470	-0.376	-0.780	-0.466	**0.832**	0.302	-0.687	-0.173	-0.822	-0.405
2-Hydroxy benzoic acid	-0.331	-0.395	-0.597	**0.520**	0.143	-0.569	**0.714**	-0.480	-0.801	0.340
4-Hydroxy benzoic acid	-0.393	**0.660**	-0.857	-0.234	**0.659**	-0.426	-0.318	**0.532**	-0.796	-0.103
γ-Hydroxybutyric acid	**0.788**	-0.559	-0.251	-0.511	0.323	0.152	0.437	-0.323	-0.224	-0.233
Itaconic acid	**0.943**	-0.271	-0.709	0.097	0.436	-0.410	**0.801**	0.140	-0.556	0.346
α-Ketobutyric acid	0.483	-0.135	-0.034	0.194	-0.145	-0.212	-0.079	**0.570**	0.078	**0.713**
D-Malic acid	-0.883	-0.013	-0.118	-0.222	-0.238	-0.401	-0.155	-0.707	-0.411	-0.454
L-Arginine	**0.681**	-0.382	-0.859	-0.287	**0.877**	0.148	-0.746	0.112	-0.920	0.007
L-Asparagine	-0.527	-0.491	-0.722	-0.629	**0.850**	0.465	-0.840	-0.092	-0.880	-0.274
L-Phenylalanine	-0.771	-0.391	-0.191	0.465	0.100	-0.218	-0.078	**0.597**	-0.324	**0.659**
L-Serine	0.363	-0.208	-0.800	-0.402	**0.768**	-0.506	-0.905	-0.148	-0.789	-0.106
L-Threonine	**0.600**	**0.747**	-0.372	0.225	-0.177	0.029	-0.609	-0.191	-0.212	0.072
Glycyl-L-Glutamic acid	**0.941**	-0.300	0.030	0.058	-0.343	-0.330	**0.679**	**0.608**	0.208	**0.673**
Phenylethyl-amine	0.398	**0.911**	-0.738	0.246	**0.715**	-0.425	**0.636**	-0.560	-0.759	-0.305
Putrescine	-0.599	-0.224	-0.718	-0.541	**0.812**	0.217	-0.329	**0.906**	-0.878	-0.200
Eigenvalues	10.520	7.228	12.103	3.979	8.695	5.097	10.913	5.895	10.783	6.255

* Carbon sources contributing significantly to positive principal component scores are bolded while carbon sources contributing significantly to negative principal component scores are underlined

Table A5. Physicochemical Characteristics of the Water Column During Sampling Events at Six Study Sites in the Grand River Watershed.

Site	Date	Water Temp (°C)	pH	DO (mg/L)	TDS (mg/L)	ORP (mV)	Conductivity (Cus/cm)	TSS (mg/L)
Bridge & Lancaster	01/06/2015	12.1	8.18	12.04	378.95	23.4	439.6	9
	13/07/2015	23.1	8.19	14.16	321.1	23.8	473.4	19
	12/08/2015	19.9	8.37	9.7	339.06	122.7	471.7	3
	06/10/2015	14.5	8.29	8.66	289.9	7.5	355.7	5
Conestogo River	28/05/2015	19.4	8.13	8.39	313.3	80	341.4	7
	17/08/2015	25.3	8.47	6.5	317.85	152.4	491.18	5
	21/07/2015	23.3	8.41	10.62	344.5	23.9	509	6
	01/10/2015	13.6	8.59	9.49	265.85	26.92	322.1	4
Doon	28/05/2015	23.2	8.96	19.77	500.5	35.4	744	16
	13/07/2015	25.7	8.56	14.13	396.5	24.7	615	2
	12/08/2015	21.6	8.55	9.2	429	163.12	619	8
	01/10/2015	14.6	8.31	12.68	362.85	31.05	472.1	7
Glen Morris	01/06/2015	14.7	8.19	12.07	468	10.5	582	15
	21/07/2015	25.3	8.58	12.7	481	37.5	747	5
	17/08/2015	26.3	8.77	8.82	487.5	179.2	768	13
	06/10/2015	14.2	8.55	11.38	494	21.5	607	10
Shand Dam	20/05/2015	9.5	8.03	12.33	299.65	45.1	323.7	29
	13/07/2015	18	7.86	10.23	289.55	47.1	386.2	20
	17/08/2015	21.3	8.24	8.9	302.25	229	432.2	6
	01/10/2015	16.1	8.46	7.93	229.45	0.7	291.2	5
West Montrose	20/05/2015	16.3	8.66	15.02	365.3	32.3	485.8	17
	21/07/2015	20.5	8.2	10.12	312	25.8	439.2	7
	12/08/2015	18.7	8.25	10.2	330.2	175.2	444.7	3
	06/10/2015	13.9	8.03	7.62	263.25	70.2	319	6

Table A6. Physicochemical Characteristics of Pore-Water during Sampling Events at Six Study Sites in the Grand River Watershed.

Site	Date	Temperature Water (°C)	pH	DO (mg/L)	TDS (mg/L)	ORP (mV)	Conductivity (Cus/cm)
Shand Dam	20/05/2015	9.4	7.55	2.2	352.3	44.9	385
	13/07/2015	18.1	7.63	2.2	297.7	-51.9	398.1
	17/08/2015	21.8	7.99	4.6	168.35	3.3	244.6
	01/10/2015	14.525	7.6875	2.905	247	15.1	306.1
West Montrose	20/05/2015	15.025	7.63	4.98	486.2625	29.775	611.775
	21/07/2015	17.75	7.345	3.5075	371.8	-32.125	492.3
	12/08/2015	16.1	7.2075	3.35	620.75	42.4	790.25
	06/10/2015	13.6	7.2375	3.5775	569.4	-58	679.175
Conestogo River	28/05/2015	19.425	7.83	5.73	348.3125	39.325	425.45
	17/08/2015	22.9	7.9075	3.7025	232	22.525	338.95
	21/07/2015	24.275	8.0075	3.0525	247.9075	152.125	378.25
	01/10/2015	13.575	7.9925	5.9775	262.9	27.3675	324.75
Bridge & Lancaster	01/06/2015	12.7	7.9925	9.4325	379.5125	21.1	447.175
	13/07/2015	23.525	8.125	5.525	283.2375	15.85	421.35
	12/08/2015	19.725	8.1525	2.525	265.1625	115.4825	373.775
	06/10/2015	14.375	8.2	7.7	291.6875	12.875	357.75
Doon	28/05/2015	20.7	7.79	11.16667	654.2167	30.36667	592.1333
	13/07/2015	23.4	7.445	3.4825	342.575	-34.575	437.9
	12/08/2015	22.25	8.4125	3.005	476.125	149.35	690.25
	01/10/2015	13.075	7.5725	4.925	748.8	26.475	877.925
Glen Morris	01/06/2015	12.55	7.5475	4.345	527.6	142.175	784.75
	21/07/2015	19.45	7.655	3.04	367.15	24	498.9
	17/08/2015	20.375	7.57	3.5	375.1375	156.3675	525.125
	06/10/2015	13.5	7.7475	4.84	533	21.725	640.5

Table A7. Factor Loadings and Eigenvalues for the First Two Principal Components of the PCA for Biolog™ Carbon Source Utilization by Microorganisms Present in all Sample Types and those Associated with *P. natans, V. spicata* and *I. veriscolor* Individually from Sampling in October and November 2015.

	All Samples		*Iris Versicolor*		*Potamogeton natans*		*Veronica spicata*	
	PC1	PC2	PC1	PC2	PC1	PC2	PC1	PC2
Pyruvic acid methyl ester	0.601	0.094	0.623	0.196	0.829	-0.041	0.644	**0.430**
Tween 40	-0.118	**0.617**	-0.104	**0.563**	-0.115	-0.149	0.012	**0.559**
Tween 80	0.187	**0.709**	0.579	**0.387**	0.535	0.354	0.726	0.314
α-Cyclodextrin	0.638	0.188	0.754	-0.094	0.759	0.287	0.576	-0.383
Glycogen	0.514	0.360	0.766	0.109	0.656	-0.423	0.594	0.205
D-Cellobiose	**0.847**	-0.034	**0.876**	-0.003	**0.959**	-0.027	0.793	-0.180
α-D-Lactose	0.493	0.347	0.610	-0.201	0.595	0.223	0.662	0.341
β-Methyl-D-Glucoside	0.709	0.355	0.767	**0.451**	**0.923**	-0.079	0.849	0.159
D-Xylose	0.509	0.123	0.741	-0.076	0.751	**0.506**	0.525	0.262
i-Erythritol	0.423	-0.501	0.161	-0.379	0.460	0.311	0.503	-0.358
D-Mannitol	0.711	**0.504**	0.863	0.370	**0.902**	-0.253	0.864	0.303
N-Acetyl-D-Glucosamine	0.696	0.220	0.793	0.209	0.891	-0.146	0.659	-0.087
D-Glucosaminic acid	0.395	0.036	0.133	**0.760**	0.528	0.237	0.363	0.299
Glucose-1-Phosphate	0.198	**0.459**	0.158	**0.441**	0.528	0.462	0.483	**0.513**
D,L-α-Glycerol Phosphate	0.353	0.363	0.459	-0.491	0.143	**0.696**	0.423	**0.800**
D-Galactonic acid γ-Lactone	0.586	-0.210	0.670	-0.057	0.790	-0.237	0.604	-0.147
D-Galacturonic acid	0.782	0.270	**0.881**	0.240	**0.909**	-0.257	**0.894**	0.077
2-Hydroxy benzoic acid	0.540	-0.639	0.535	-0.379	0.707	0.328	0.509	-0.769
4-Hydroxy benzoic acid	**0.855**	-0.253	**0.910**	-0.003	**0.918**	-0.270	0.896	-0.231
γ-Hydroxybutyric acid	0.303	-0.109	0.411	0.052	0.296	0.117	0.674	-0.081
Itaconic acid	0.627	-0.610	0.664	-0.383	0.875	0.115	0.547	-0.714
α-Ketobutyric acid	0.440	0.113	0.497	-0.296	0.425	**0.778**	0.524	0.260
D-Malic acid	0.740	-0.195	0.781	-0.490	0.874	-0.226	0.765	-0.191
L-Arginine	0.760	-0.236	0.815	0.372	0.825	-0.351	**0.906**	-0.243
L-Asparagine	**0.844**	-0.077	0.849	0.286	0.893	-0.359	**0.925**	-0.075
L-Phenylalanine	0.234	0.115	0.483	-0.485	0.266	**0.678**	0.290	-0.022
L-Serine	**0.824**	0.109	**0.910**	0.241	0.893	-0.381	**0.914**	-0.073
L-Threonine	0.298	**0.570**	0.693	-0.367	0.203	0.379	0.343	**0.738**
Glycyl-L-Glutamic acid	0.516	0.147	0.511	-0.427	0.465	**0.654**	0.766	0.281
Phenylethyl-amine	0.650	-0.564	0.661	-0.369	0.894	-0.072	0.677	-0.530
Putrescine	**0.826**	-0.216	**0.876**	0.168	0.851	-0.187	**0.901**	-0.180
Eigenvalues	10.979	4.039	14.015	3.788	15.798	4.115	14.074	4.548

* Carbon sources contributing significantly to positive principal component scores are bolded while carbon sources contributing significantly to negative principal component scores are underlined

Table A8. Factor Loadings and Eigenvalues for the First Two Principal Components of the PCA for Biolog^{TM} Carbon Source Utilization by Microorganisms Present in Mesocosm Rhizoplane Samples at 0, 49 and 91 DAE to Different Water Quality Sources.

		0 DAE		49 DAE		91 DAE	
Class of Carbon Source	**Carbon source**	**PC1**	**PC2**	**PC1**	**PC2**	**PC1**	**PC2**
Polymers	Tween 40	-0.103	0.78	0.798	-0.366	0.747	0.076
	Tween 80	0.181	0.704	0.357	**-0.654**	0.707	-0.132
	α-Cyclodextrin	0.517	0.043	**-0.567**	-0.305	**-0.505**	0.155
	Glycogen	0.754	0.07	0.253	0.008	0.776	-0.332
Carbohydrates	Pyruvic acid methyl ester	-0.068	0.315	-0.012	-0.425	**-0.712**	-0.438
	D-Cellobiose	0.674	-0.472	0.216	0.863	0.789	-0.422
	α-D-Lactose	-0.19	**-0.544**	**-0.578**	0.306	**-0.716**	-0.226
	β-Methyl-D-Glucoside	0.645	-0.483	**-0.602**	0.338	0.065	0.369
	D-Xylose	-0.469	**-0.555**	-0.368	0.57	-0.215	**-0.532**
	i-Erythritol	-0.039	**-0.559**	-0.467	0.092	**-0.574**	**-0.525**
	D-Mannitol	**-0.506**	0.317	0.804	0.402	-0.07	**-0.62**
	N-Acetyl-D-Glucosamine	0.865	-0.284	-0.29	0.451	0.534	0.478
	Glucose-1-Phosphate	0.762	0.051	**-0.783**	0.001	0.293	0.147
	D,L-α-Glycerol Phosphate	0.816	-0.302	0.037	-0.032	0.396	0.47
Carboxylic and acetic acids	D-Glucosaminic acid	**-0.785**	0.215	**-0.637**	-0.422	**-0.751**	-0.104
	D-Galactonic acid γ-Lactone	**-0.639**	-0.37	**-0.641**	-0.01	-0.41	-0.081
	D-Galacturonic acid	-0.062	0.65	0.807	-0.014	0.219	0.616
	2-Hydroxy benzoic acid	**-0.636**	-0.461	**-0.577**	0.144	-0.362	0.02
	4-Hydroxy benzoic acid	0.363	0.588	0.834	0.133	0.809	-0.46
	γ-Hydroxybutyric acid	**-0.538**	0.317	0.311	0.707	-0.021	0.638
	Itaconic acid	**-0.577**	-0.019	0.125	0.694	-0.478	0.521
	α-Ketobutyric acid	0.428	-0.094	-0.116	**-0.764**	0.156	0.216
	D-Malic acid	-0.046	0.539	0.428	0.137	0.52	0.395
Amino acids	L-Arginine	-0.086	-0.057	0.488	**-0.594**	0.883	-0.06
	L-Asparagine	-0.303	0.79	0.917	-0.163	0.789	-0.429
	L-Phenylalanine	-0.2	**-0.638**	-0.348	-0.533	-0.408	0.576
	L-Serine	0.299	0.791	0.612	0.206	-0.189	0.605
	L-Threonine	0.718	0.011	-0.289	**-0.592**	**-0.727**	-0.156
	Glycyl-L-Glutamic acid	0.643	-0.065	-0.298	0.431	-0.157	-0.268
Amides/amines	Phenylethyl-amine	-0.139	**-0.537**	-0.029	**-0.594**	0.201	-0.169
	Putrescine	0.496	0.557	0.815	-0.092	0.092	0.441
	Eigenvalue	8.06	6.688	8.812	5.891	8.764	4.822

* Carbon sources contributing significantly to positive PC scores along an axis are underlined. Carbon sources contributing significantly to negative PC scores are in bold.

Table A9. Factor Loadings and Eigenvalues for the First Two Principal Components of the PCA for Biolog^{TM} Carbon Source Utilization by Microorganisms Present in Mesocosm Rhizosphere Samples at 0, 49 and 91 DAE to Different Water Quality Sources.

		0 DAE		49 DAE		91 DAE	
Class of Carbon Source	**Carbon source**	**PC1**	**PC2**	**PC1**	**PC2**	**PC1**	**PC2**
Polymers	Tween 40	**-0.873**	0.2	0.91	0.161	**-0.956**	0.08
	Tween 80	**-0.711**	0.196	0.893	-0.314	0.966	0.134
	α-Cyclodextrin	0.756	-0.144	**-0.727**	0	**-0.639**	-0.255
	Glycogen	0.872	-0.158	-0.459	-0.352	-0.107	**-0.594**
Carbohydrates	Pyruvic acid methyl ester	-0.233	0.069	0.098	0.42	-0.155	0.386
	D-Cellobiose	0.936	-0.104	**-0.588**	**-0.7**	**-0.921**	0.023
	α-D-Lactose	0.42	**-0.501**	**-0.798**	0.168	-0.126	-0.433
	β-Methyl-D-Glucoside	0.943	0.018	**-0.59**	-0.176	**-0.893**	-0.107
	D-Xylose	-0.18	**-0.492**	-0.117	0.819	**-0.502**	0.731
	i-Erythritol	0.421	**-0.383**	-0.382	**-0.579**	**-0.714**	-0.253
	D-Mannitol	**-0.734**	-0.161	0.617	-0.001	0.966	0.062
	N-Acetyl-D-Glucosamine	0.794	0.177	-0.484	-0.442	**-0.748**	-0.441
	Glucose-1-Phosphate	0.587	0.299	**-0.675**	-0.267	**-0.768**	0.014
	D,L-α-Glycerol Phosphate	0.645	0.553	**-0.656**	-0.373	0.992	0.024
Carboxylic and acetic acids	D-Glucosaminic acid	**-0.27**	0.326	-0.352	0.651	0.783	-0.411
	D-Galactonic acid γ-Lactone	-0.387	**-0.229**	**-0.662**	0.391	**-0.742**	-0.198
	D-Galacturonic acid	**-0.712**	0.28	0.887	-0.169	**-0.524**	0.807
	2-Hydroxy benzoic acid	-0.153	**-0.469**	-0.388	0.118	-0.451	-0.24
	4-Hydroxy benzoic acid	**-0.552**	0.436	0.829	-0.206	0.959	0.234
	γ-Hydroxybutyric acid	**-0.808**	**-0.346**	0.519	0.766	**-0.853**	0.155
	Itaconic acid	**-0.78**	-0.173	-0.255	0.729	**-0.899**	0.146
	α-Ketobutyric acid	0.46	0.491	0.095	0.196	-0.03	-0.437
	D-Malic acid	0.927	0.131	0.149	-0.457	0.909	0.242
Amino acids	L-Arginine	**-0.795**	-0.004	0.787	-0.367	**-0.902**	-0.078
	L-Asparagine	**-0.813**	0.28	0.938	-0.216	**-0.818**	0.424
	L-Phenylalanine	0.065	**-0.346**	-0.444	-0.213	**-0.816**	-0.171
	L-Serine	**-0.802**	0.468	0.685	-0.083	0.961	0.216
	L-Threonine	**0.538**	0.151	**-0.606**	0.05	0.003	**-0.794**
	Glycyl-L-Glutamic acid	0.414	0.76	-0.362	0.478	0.866	-0.426
Amides/amines	Phenylethyl-amine	0.809	**-0.388**	-0.113	**-0.737**	**-0.728**	-0.015
	Putrescine	0.422	0.698	0.163	0.324	0.929	0.024
	Eigenvalue	13.364	3.942	10.62	5.527	17.919	3.93

* Carbon sources contributing significantly to positive PC scores along an axis are underlined. Carbon sources contributing significantly to negative PC scores are in bold.

Table A10. Factor Loadings and Eigenvalues for the First Two Principal Components of the PCA for Biolog^{TM} Carbon Source Utilization by Microorganisms Present in Mesocosm Water Samples Including Interstitial and Inflow Water at 0, 49 and 91 DAE to Different Water Quality Sources.

		0 DAE		49 DAE		91 DAE	
Class of Carbon Source	**Carbon source**	**PC1**	**PC2**	**PC1**	**PC2**	**PC1**	**PC2**
Polymers	Tween 40	-0.336	-0.142	-0.436	0.069	-0.056	**-0.596**
	Tween 80	-0.119	-0.372	0.028	-0.319	-0.174	**-0.581**
	α-Cyclodextrin	-0.17	0.28	**-0.544**	**-0.539**	0.179	-0.493
	Glycogen	**-0.707**	-0.241	-0.456	-0.125	0.14	**-0.693**
Carbohydrates	Pyruvic acid methyl ester	0.538	-0.036	-0.41	-0.286	-0.285	**-0.501**
	D-Cellobiose	-0.322	**-0.687**	0.246	0.782	**-0.559**	0.602
	α-D-Lactose	-0.094	-0.286	-0.173	0.16	-0.432	-0.251
	β-Methyl-D-Glucoside	0.698	-0.058	0.693	-0.364	-0.364	0.014
	D-Xylose	-0.164	**-0.527**	-0.469	0.125	-0.353	0.112
	i-Erythritol	-0.238	**-0.68**	-0.219	0.725	-0.052	-0.167
	D-Mannitol	-0.014	-0.345	0.408	**-0.504**	0.071	-0.267
	N-Acetyl-D-Glucosamine	0.534	-0.249	0.763	0.011	0.175	0.205
	Glucose-1-Phosphate	0.084	-0.391	**-0.672**	0.041	-0.103	-0.341
	D,L-α-Glycerol Phosphate	0.109	0.06	0.003	0.44	-0.315	-0.259
Carboxylic and Acetic Acids	D-Glucosaminic acid	-0.338	0.254	**-0.58**	0.472	0.337	-0.379
	D-Galactonic acid γ-Lactone	-0.169	-0.039	0.635	-0.009	0.402	0.416
	D-Galacturonic acid	0.04	0.226	0.542	-0.189	0.59	0.7
	2-Hydroxy benzoic acid	-0.105	**-0.76**	-0.235	0.331	-0.419	-0.13
	4-Hydroxy benzoic acid	-0.364	0.42	**-0.683**	**-0.589**	-0.384	-0.263
	γ-Hydroxybutyric acid	-0.495	0.52	-0.394	0.451	0.445	**-0.522**
	Itaconic acid	-0.324	0.372	-0.129	0.6	0.83	0.13
	α-Ketobutyric acid	**-0.606**	**-0.63**	-0.341	0.419	0.825	0.036
	D-Malic acid	**-0.636**	-0.035	-0.369	**-0.561**	-0.239	0.245
Amino Acids	L-Arginine	0.072	0.635	-0.299	-0.448	0.284	-0.336
	L-Asparagine	0.736	-0.013	-0.17	**-0.62**	0.037	**-0.526**
	L-Phenylalanine	**-0.675**	0.433	-0.404	-0.054	0.866	0.213
	L-Serine	0.159	0.808	**-0.599**	**-0.58**	0.629	**-0.66**
	L-Threonine	**-0.563**	0.422	-0.12	0.66	0.746	-0.012
	Glycyl-L-Glutamic acid	**-0.532**	-0.265	-0.43	0.423	0.45	**-0.636**
Amides/Amines	Phenylethyl-amine	-0.474	0.647	**-0.621**	-0.198	0.044	**-0.672**
	Putrescine	0.834	0.18	0.549	-0.223	0.575	0.44
	Eigenvalue	5.891	5.552	6.406	5.653	5.962	5.591

* Carbon sources contributing significantly to positive PC scores along an axis are underlined. Carbon sources contributing significantly to negative PC scores are in bold.

Table A11. Factor Loadings and Eigenvalues for the First Two Principal Components of the PCA for Biolog^{TM} Carbon Source Utilization by Rhizoplane Microorganisms Associated with *P. arundinaceae* and *V. anagallis-aquatica* at 0, 49 and 91 DAE to 5 mg/L of Phosphorus.

	0 DAE		21 DAE		49 DAE	
	F1	F2	F1	F2	F1	F2
Pyruvic acid methyl ester	**-0.712**	-0.438	**-0.569**	0.111	0.163	-0.363
Tween 40	0.747	0.076	0.698	-0.372	0.933	-0.028
Tween 80	0.707	-0.132	0.654	0.010	0.948	0.035
α-Cyclodextrin	**-0.505**	0.155	**-0.509**	-0.238	**-0.579**	-0.326
Glycogen	0.776	-0.332	**-0.548**	-0.137	**-0.819**	0.003
D-Cellobiose	0.789	-0.422	**-0.702**	-0.282	**-0.635**	-0.377
α-D-Lactose	**-0.716**	-0.226	**-0.857**	0.261	-0.085	-0.450
β-Methyl-D-Glucoside	0.065	0.369	**-0.774**	-0.403	**-0.868**	-0.210
D-Xylose	-0.215	**-0.532**	-0.487	0.414	-0.053	**-0.541**
i-Erythritol	**-0.574**	**-0.525**	-0.225	**-0.695**	0.508	-0.094
D-Mannitol	-0.070	**-0.620**	0.716	-0.225	**-0.562**	0.217
N-Acetyl-D-Glucosamine	0.534	0.478	**-0.657**	-0.494	**-0.788**	-0.069
D-Glucosaminic acid	**-0.751**	-0.104	0.033	0.654	-0.394	0.683
Glucose-1-Phosphate	0.293	0.147	**-0.683**	**-0.565**	**-0.614**	-0.108
D,L-α-Glycerol Phosphate	0.396	0.470	**-0.733**	-0.183	-0.486	-0.214
D-Galactonic acid γ-Lactone	-0.410	-0.081	-0.093	0.703	-0.465	**-0.522**
D-Galacturonic acid	0.219	0.616	0.669	-0.460	0.805	-0.408
2-Hydroxy benzoic acid	-0.362	0.020	0.108	0.460	-0.068	0.492
4-Hydroxy benzoic acid	0.809	-0.460	0.658	0.157	0.576	0.047
γ-Hydroxybutyric acid	-0.021	0.638	0.593	-0.228	0.725	0.004
Itaconic acid	-0.478	0.521	0.271	-0.455	0.083	**-0.680**
α-Ketobutyric acid	0.156	0.216	0.664	-0.463	0.107	0.538
D-Malic acid	0.520	0.395	-0.100	0.372	0.058	0.952
L-Arginine	0.883	-0.060	0.667	0.333	0.888	0.237
L-Asparagine	0.789	-0.429	0.613	-0.349	0.816	-0.368
L-Phenylalanine	-0.408	0.576	-0.057	**-0.588**	-0.260	-0.030
L-Serine	-0.189	0.605	0.818	-0.332	0.839	-0.319
L-Threonine	**-0.727**	-0.156	-0.372	-0.262	**-0.554**	-0.056
Glycyl-L-Glutamic acid	-0.157	-0.268	-0.416	-0.475	**-0.802**	0.091
Phenylethyl-amine	0.201	-0.169	-0.094	0.717	0.008	0.864
Putrescine	0.092	0.441	0.888	-0.002	0.708	0.013
Eigen Value	8.764	4.822	10.238	5.218	11.357	4.900

* Carbon sources contributing significantly to positive principal component scores are bolded while carbon sources contributing significantly to negative principal component scores are underlined

Table A12. Factor Loadings and Eigenvalues for the First Two Principal Components of the PCA for Biolog™ Carbon Source Utilization by Rhizosphere Microorganisms Associated with *P. arundinaceae* and *V. anagallis-aquatica* at 0, 49 and 91 DAE to 5 mg/L of Phosphorus.

	0 DAE		21 DAE		49 DAE	
	F1	F2	F1	F2	F1	F2
Pyruvic acid methyl ester	-0.155	0.386	0.396	0.142	**-0.665**	0.565
Tween 40	**-0.956**	0.080	**-0.523**	**-0.614**	0.657	0.232
Tween 80	0.966	0.134	**-0.640**	0.464	0.190	0.161
α-Cyclodextrin	**-0.639**	-0.255	0.453	**-0.521**	**-0.501**	-0.022
Glycogen	-0.107	**-0.594**	0.586	0.160	0.663	-0.362
D-Cellobiose	**-0.921**	0.023	0.622	**-0.614**	0.018	**-0.613**
α-D-Lactose	-0.126	-0.433	0.378	**-0.647**	-0.439	0.733
β-Methyl-D-Glucoside	**-0.893**	-0.107	0.700	-0.251	-0.428	**-0.561**
D-Xylose	**-0.502**	0.731	-0.189	-0.388	**-0.570**	0.350
i-Erythritol	**-0.714**	-0.253	-0.331	-0.115	**-0.607**	0.024
D-Mannitol	0.966	0.062	0.003	-0.465	0.710	-0.249
N-Acetyl-D-Glucosamine	**-0.748**	-0.441	0.522	-0.290	-0.168	**-0.628**
D-Glucosaminic acid	0.783	-0.411	-0.100	0.607	0.436	-0.403
Glucose-1-Phosphate	**-0.768**	0.014	0.504	**-0.544**	-0.320	-0.130
D,L-α-Glycerol Phosphate	0.992	0.024	0.662	0.227	-0.002	**-0.619**
D-Galactonic acid γ-Lactone	**-0.742**	-0.198	-0.109	0.681	**-0.594**	0.587
D-Galacturonic acid	**-0.524**	0.807	**-0.655**	**-0.640**	0.518	-0.104
2-Hydroxy benzoic acid	-0.451	-0.240	0.023	0.817	-0.497	**-0.676**
4-Hydroxy benzoic acid	0.959	0.234	**-0.686**	**-0.515**	0.787	-0.057
γ-Hydroxybutyric acid	**-0.853**	0.155	-0.080	**-0.595**	0.736	-0.087
Itaconic acid	**-0.899**	0.146	0.246	-0.216	0.641	0.032
α-Ketobutyric acid	-0.030	-0.437	-0.102	-0.136	**-0.526**	-0.336
D-Malic acid	0.909	0.242	**-0.515**	0.174	-0.101	0.408
L-Arginine	**-0.902**	-0.078	**-0.687**	0.008	0.490	0.729
L-Asparagine	**-0.818**	0.424	**-0.708**	-0.492	0.707	0.397
L-Phenylalanine	**-0.816**	-0.171	0.235	0.436	-0.172	-0.369
L-Serine	0.961	0.216	-0.175	-0.341	-0.163	0.358
L-Threonine	0.003	**-0.794**	0.585	0.584	0.408	-0.154
Glycyl-L-Glutamic acid	0.866	-0.426	0.794	0.056	-0.428	-0.178
Phenylethyl-amine	**-0.728**	-0.015	**-0.734**	0.523	-0.300	-0.497
Putrescine	0.929	0.024	**-0.545**	0.098	0.327	0.450
Eigen Value	17.919	3.930	7.652	6.738	14.365	3.551

* Carbon sources contributing significantly to positive principal component scores are bolded while carbon sources contributing significantly to negative principal component scores are underlined

Table A13. Factor Loadings and Eigenvalues for the First Two Principal Components of the PCA for Biolog^{TM} Carbon Source Utilization by Interstitial and Inflow Water Microorganisms Associated with *P. arundinaceae* and *V. anagallis-aquatica* at 0, 49 and 91 DAE to 5 mg/L of Phosphorus.

	0 DAE		21 DAE		49 DAE	
	F1	F2	F1	F2	F1	F2
Pyruvic acid methyl ester	**-0.559**	0.602	-0.408	-0.361	0.744	-0.359
Tween 40	-0.056	**-0.596**	-0.328	-0.339	-0.138	0.738
Tween 80	-0.174	**-0.581**	0.653	-0.407	-0.014	0.454
α-Cyclodextrin	0.179	-0.493	0.450	0.046	**-0.578**	0.555
Glycogen	0.140	**-0.693**	-0.056	0.298	**-0.712**	0.003
D-Cellobiose	-0.285	**-0.501**	-0.382	0.706	-0.372	**-0.617**
α-D-Lactose	-0.432	-0.251	0.266	-0.133	0.042	0.231
β-Methyl-D-Glucoside	-0.364	0.014	0.146	0.622	-0.420	**-0.734**
D-Xylose	-0.353	0.112	**-0.566**	-0.277	-0.421	0.128
i-Erythritol	-0.052	-0.167	0.418	-0.019	0.484	0.454
D-Mannitol	0.071	-0.267	0.715	0.345	**-0.547**	**-0.541**
N-Acetyl-D-Glucosamine	0.175	0.205	-0.450	0.435	**-0.522**	**-0.699**
D-Glucosaminic acid	0.337	-0.379	0.466	-0.298	0.363	0.418
Glucose-1-Phosphate	-0.103	-0.341	-0.194	-0.089	**-0.680**	-0.111
D,L-α-Glycerol Phosphate	-0.315	-0.259	-0.006	-0.116	**-0.505**	0.123
D-Galactonic acid γ-Lactone	0.402	0.416	0.033	**-0.518**	0.480	0.214
D-Galacturonic acid	0.590	0.700	**-0.634**	0.376	0.432	-0.396
2-Hydroxy benzoic acid	-0.419	-0.130	-0.215	-0.386	0.823	-0.049
4-Hydroxy benzoic acid	-0.384	-0.263	0.584	-0.122	0.307	0.350
γ-Hydroxybutyric acid	0.445	**-0.522**	-0.167	-0.008	0.433	0.141
Itaconic acid	0.830	0.130	0.667	0.076	0.587	0.204
α-Ketobutyric acid	0.825	0.036	0.522	-0.264	-0.058	0.110
D-Malic acid	-0.239	0.245	0.650	-0.239	**-0.513**	0.681
L-Arginine	0.284	-0.336	0.731	0.024	-0.374	0.378
L-Asparagine	0.037	**-0.526**	0.412	-0.269	-0.136	0.635
L-Phenylalanine	0.866	0.213	0.654	0.448	**-0.511**	0.117
L-Serine	0.629	**-0.660**	0.888	0.281	-0.299	-0.136
L-Threonine	0.746	-0.012	0.214	0.767	**-0.552**	0.355
Glycyl-L-Glutamic acid	0.450	**-0.636**	0.554	0.491	-0.375	0.515
Phenylethyl-amine	0.044	**-0.672**	0.652	-0.104	-0.088	-0.107
Putrescine	0.575	0.440	0.640	-0.302	-0.076	0.499
Eigen Value	5.962	5.591	7.672	3.892	6.517	5.462

* Carbon sources contributing significantly to positive principal component scores are bolded while carbon sources contributing significantly to negative principal component scores are underlined