The Role of Urban Wetland Diversity and Function in Contaminant Fate

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Abstract

It is recognized that microbial transformations are the primary mechanism of organic contaminant removal in natural and constructed wetland systems. However, not much is known about urban wetland microbial communities or their functional capacity to process contaminants. The objective of this research was to first characterize the physiological and phylogenetic diversity of microbial communities of different urban wetland types using the BIOLOG™ method and through DGGE of 16S rRNA sequences. The capacity of urban wetlands to attenuate model chlorinated aromatic compounds (2,4-D and 3-CBA) was assessed by UPLC biodegradation and ^{14}C mineralization experiments. Toxicity tests were conducted to assess microbial tolerance to pollutant addition. In general, results indicate that urbanization has a homogenizing effect on microbial community structure and distribution within urban wetland systems, regardless of type. Urban wetlands also appear to have a limited capacity to remove chlorinated organic pollutants. Microbial community tolerance to chlorinated organic pollutants is relatively high, whereas heavy metal tolerance was found to coincide with history of contaminant exposure.

Keywords: Wetland, Biodegradation, Microbial Diversity, 2,4-D, UPLC, BIOLOG™, DGGE

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List of Abbreviations

AODC	Acridine Orange Direct Counts
AWCD	Average Well Colour Development
CFU	Colony Forming Unit
CLPP	Community Level Physiological Profiles
CW	Constructed Wetland
CRW	Contaminated Remnant Wetland Copper Sulfate
$CuSO_4$	Copper Sulfate
DAPI	4,6-diamidino-2-phe-nylindole
DGGE	Denaturing Gradient Gel Electrophoresis
dNTP	Deoxyribonucleotide Triphosphate
HPLC	High Pressure Liquid Chromatography
PCA	Principle Components Analysis
PC	Principle Component
PDA	Photo Diode Array
PCR	Polymerase Chain Reaction
ROL	Radial Oxygen Release
RW	Remnant Wetland
SF	Surface Flow

SSF	Sub Surface Flow
SIP	Stable Isotope Probing
SPME	Solid Phase Micro Extraction
UPGMA	Unweighted Pair Group Method with Arithmetic Mean
UPLC	Ultra-high Performance Liquid Chromatography
2,4-D	2,4-Dichlorophenoxyacetic acid
3-CBA	3-Chlorobenzoic acid

Chapter 1: General Introduction

1.1 Wetlands Defined

1.1.1 Natural Wetlands

Wetlands are transitional environments "ecotones" between terrestrial and aquatic environments and are found in every climate zone in the world except Antarctica (Mitra *et al*., 2005). They are defined as ecosystems that depend on permanent or periodic inundation and are characterized by reducing sediments and the presence of hydrophitic plant communities adapted to life in saturated environments (Spray and McGlothlin, 2004). There are a variety of wetland types and wetland delineation is an ongoing science. Common classification schemes used by scientists are either based on hydrology and landscape position (hydrogeomorphology) or by the wetland plant (macrophyte) community. The Canadian wetland classification system recognizes five wetland classes and they are swamps, marshes, bogs, fens and shallow open waters. They are defined on the basis of ecosystem processes such as hydrological regime; carbon budget, water chemistry, and characteristic vegetation cover (Zoltai and Vitt, 1995).

1.1.2 Constructed Wetlands

Constructed wetlands (CW) are artificial wetlands designed to mimic the structure and function of natural wetlands but do so in a more controlled environment. CWs are classified according to how water flows through the system. They are divided into surface flow (SF) and subsurface flow (SSF) systems. Surface flow wetlands are most similar to natural wetlands; however are not as widely used and consequently the least studied

(Truu *et al*., 2009). They generally have a soil bottom, a water depth of less than 0.4 m, are densely vegetated and may or may not include areas of open water (Haberl *et al*., 2009). In sub surface flow systems wastewater flows by gravity either horizontally or vertically through a bed of porous filter material (usually sand or gravel). The bed depth in SSF wetlands is typically between 0.6 and 1.0 m (Haberl *et al*., 2009).

1.1.3 Urban Wetlands

Urban wetlands include both natural and artificial wetlands that are found distributed within urban areas. These wetlands are structurally and functionally different because of the effects of urbanization. Most of the "natural" wetlands in urban areas are remnants of larger wetlands that have been modified and destroyed through anthropogenic activities such as being drained or filled in for agricultural and housing development activities (Lotze *et al*., 2006, Boyer and Polasky, 2004). The hydrology in these systems is altered through filling, ditching, diking draining and by physical geomorphological changes as a result of land movement associated with road and building construction (Ehrenfeld, 2000). Climate and air quality are also altered by urbanization and the water quality of urban wetlands is also typically characterized by elevated levels of nutrients, metals, and organic pollutants (Ehrenfeld, 2000). Artificial stormwater CWs are also included in this definition since they are commonly built within urban environments to collect excess storm water and are also subject to the effects of urbanization.

1.2 Wetlands and Ecosystem Services

Historically viewed as wastelands and breeding sites for malaria, wetlands have been under pressure to become economically beneficial. In urbanized areas of Canada and the United States agricultural and construction activities are responsible for wetland losses often exceeding 90% (Bedford *et al*., 1999, Woltemade, 2000). In Southern Ontario, loss of marshes in the Hamilton and Toronto area are estimated between 75 to 100% (Bedford, 1999). Recently however, attitudes about wetlands have changed in recognition of the multitude of ecosystem services they provide for humans and wildlife. Wetlands are integral parts of watersheds and play huge roles in terms of water storage, flood control, groundwater recharge and shoreline stabilization (Boyer and Polasky, 2004). On the global scale wetlands are major carbon sinks due to uptake and storage of CO_2 by wetland plants which decompose slowly under anaerobic conditions (Mitra *et al*., 2009). They also provide habitat that support a unique biodiversity of microbes, plants, insects and wildlife. The transition between terrestrial and aquatic habitats supports various amphibian, reptile and fish species. They also provide breeding and feeding areas for various migratory birds (Murray and Hamilton, 2010). Wetlands also have recreational value and provide a valuable food source for both humans and wildlife. Costanza *et al*. (1997) estimates the total value of ecosystem services provided by wetlands worldwide to be 15.5 trillion dollars per year, almost 46% of the total for all global ecosystems.

In addition to these services, the most valued ecosystem service is the capacity of wetlands to improve water quality and mitigate the transport of pollutants. Natural and constructed wetlands are used to treat a variety of wastewater sources such as secondary or tertiary effluent from municipal wastewater treatment facilities (Kadlec and Tilton,

1979), industrial effluent such as acid mine drainage (Kim *et al*., 2009), hydrocarbon effluent from the petroleum industry (Hadwin *et al*., 2006), agricultural and food wastewater from farm and animal productions (Haberl *et al*., 2002), as well as pesticide associated agricultural runoff (Moore *et al*., 2002). It is the unique combination of wetland components such as wetland hydrology, hydrophitic plant communities and hydric soils that work in concert with diverse microbial communities to create an environment that enhances the detoxification, transformation, and/or sequestration of virtually all forms of pollutants. These include pathogens, nutrients, sediments, simple dissolved organic matter, and also organic chemicals and heavy metals (Faulwetter *et al*., 2009, Gersberg *et al*., 1989, Imfeld *et al*. 2009). The mechanisms of contaminant removal in wetlands include both abiotic and biotic processes. Abiotic processes include sorption/sedimentation, humification, volatilisation, photodegradation. Biotic processes include both plant activity (i.e. phytoaccumulation, phytovolitilization and phytodegradation) and microbial activity (i.e bioaccumulation and biodegradation).

1.3 Mechanisms of Pollutant Removal in Wetland Systems

1.3.1 The Role of Microbes in Transformation and Biodegradation of Organic Contaminants

It is well recognized that the removal of organic pollutants in wetland systems is mainly driven by microbial mediated processes (Faulwetter *et al*., Haberl *et al*., 2003, Truu *et al*., 2009). Because many synthetic organic chemicals resemble naturally occurring organic compounds, they can be used by microorganisms as a source of carbon, energy, nutrients, and other elements required by the cell to sustain growth. Many

microbial enzymes can completely breakdown organic pollutants into inorganic components such as CO_2, N_2 and CH_4 in a process known as mineralization. Mineralization results in the complete breakdown of pollutants and returns essential elements back into the normal biogeochemical pool. It may be that some individual species of microbe may not completely mineralize a particular pollutant. However, the organic metabolites excreted by one species may be used by another species present in the community with the ability to convert the metabolite to CO_2. The net effect is still one of mineralization, although it is due to a consortium of microbial communities rather than just one individual species. It is important to also note that microbes can also break down organic pollutants without deriving any energy, carbon, or nutrients for growth. This type of transformation is known as co-metabolism and usually results in the accumulation of organic products that can be either more or less toxic than the parent compound (Alexander, 1995). However, the presence of diverse microbial communities usually results in one or more species' being able to utilise the accumulating organic products for growth, resulting in a net loss of organic contaminant.

1.3.2 Factors Affecting Biodegradation

1.3.2.1 Effects of Redox Processes

Effective mineralization depends on the type of microbial respiration occurring within the soil or sediment environment. Wetlands are usually inundated with water most of the time and since oxygen has a low diffusion rate though water, saturated sediments are usually depleted of oxygen. The depletion of oxygen creates a reducing environment with low redox potential, which promotes anaerobic microbial respiration. The type of

microbial respiration that occurs follows a predictable sequence within specific ranges of redox potential. As redox potential decreases, aerobic microbes are replaced by facultative aerobes and obligate anaerobes that use nitrate, manganese, iron, sulfate, and carbon compounds as alternate electron acceptors (Faulkner *et al*., 2004). The hydrology of a particular wetland system greatly affects the redox status of that system, and hence the type of microbial respiration that can occur. Redox conditions can be very important factors in determining the rate and extent of contaminant biodegradation. Certain organics such as petroleum based hydrocarbons are readily degraded under aerobic conditions but can persist under anaerobic conditions (Maier *et al*., 2009). However, the biotransformation of certain highly chlorinated pollutants (e.g pesticides such as aldrin) requires anaerobic conditions in order to remove chlorine atoms in a process known as reductive dehalogenation (Maier *et al*., 2009).

1.3.2.2 Genetic Potential

Microorganisms require the genetic potential to degrade certain types of xenobiotics such as chlorinated aromatics, for which the degradation pathways is assumed to be recent as a result of vast quantities of herbicide and pesticide production in the last 75 years (Fulthorpe *et al*., 1996). Although a variety of halogenated organics can also be formed naturally. The series of degradation steps resulting in mineralization are catalyzed by specific enzymes encoded within genes. These enzymes are synthesized within the cell, but can also be excreted to help initiate degradation reactions (Maier *et al*., 2009). Generally microbial biodegradation requires a period of adaptation to a particular xenobiotic compound where specific degradation enzymes are induced, followed by an increase in the population of the biodegrading organism. Repeat exposure

of a pollutant creates an environment in which a biodegradation pathway is maintained and biodegradation is enhanced (Spain *et al*., 1980). When naturally occurring analogues of a pollutant do not exist such as the case of chlorinated aromatics, a second type of adaptation is required involving a mutation or gene transfer (van der Meer *et al*., 2006).

1.3.2.3 Effect of Chemical Structure

Microbial degradation of organic chemicals is expected to strongly depend on the physicochemical properties of the contaminant. In general, high molecular weight compounds that are hydrophobic are more resistant to biodegradation than low molecular weight hydrophilic compounds. Hydrophilic compounds are dispersed in solution and are more accessible to microbial attack, whereas hydrophobic molecules are readily sorbed and biodegradation may depend on rates of desorption (Farenhorst *et al*., 2008). Hydrophobic molecules can also be present in non-aqueous phase liquids (NAPL) (Alexander, 1995). In these cases, microbial degradation is limited to the interfacial area between the non-aqueous and aqueous phases (Suthersan, 2002). Some species of microbes excrete surfactants and emulsifiers which increase the solubility of the hydrophobic molecules and subsequent biodegradation (Alexander, 1995). The actual chemical structure of a contaminant, such as the presence of secondary, tertiary or quaternary carbon atoms or functional groups such as chlorine, will also affect its microbial degradation (Imfeld *et al*., 2009).

1.3.2.4 Effect of Environmental Parameters and Sediment Physicochemical Properties

Important environmental and sediment characteristics that effect biodegradation include temperature, pH, salinity, organic matter content, soil moisture content and these

will be discussed separately. Temperature and pH are important factors as these parameters affect the rate at which the mineralization of organic contaminants occurs (Reddy and D'Angelo, 1997). For example the degradation of the herbicide 2,4-D has been shown to be most optimal between pH 7.6 and 8.0 and a temperature of 30^{o}C (Etinosa *et al*., 2007). Salinity is a common co-contaminant that can affect the biodegradation of pollutants. Salinity can have a variety of effects on microbial cells including disruption of tertiary protein structures, denaturing of enzymes and cell dehydration (Ulrich *et al*., 2009), with different species having different sensitivity to salinity stress. The amount of organic matter will influence microbial biomass as high amounts of organic matter generally support greater numbers of microorganisms than sediments with low organic matter, as organic matter provides electron and carbon sources required for microbial growth (Maier *et al*., 2009). Soil moisture content affects rates of aerobic biodegradation because oxygen diffusion through water is slow which limits oxygen replenishment. Optimal conditions for activity of aerobic soil microorganisms occur when the soil pore space is 38% to 81% filled with water because at this range water and oxygen availability is maximized (Maier *et al*., 2009).

1.3.3 Wetland Plants and Rhizosphere Effects

Wetland plants play several important roles in maintaining the diversity and functions of wetland systems. Next to microbial transformations, they are the other major biotic factors that influence pollutant fate. Plants lower the presence of pollutants through uptake, accumulation, degradation, and volatilization processes collectively referred to as phytoremediation (Singh and Jain, 2003). The plant root system also has numerous physical and chemical effects on the surrounding environment. Root growth

mechanically deforms the soil maintaining hydraulic properties and anchors the sediments protecting the surface from erosion (Shaw and Burns, 2007). The root zone (rhizosphere) also provides a suitable microenvironment that enhances microbial activity and density by providing favorable attachment sites as well as sources of carbon and energy through root exudates (Gagnon *et al*., 2007).

Plants exude organic compounds into the rhizosphere in a process known as rhizodeposition. Exudates such as enzymes and mucilage (i.e., exopolysaccharide) are actively secreted while certain compounds like sugars and amino and organic acids are passively leaked from intact cells (Shaw and Burns, 2007). The turnover of decaying or dead root cells contributes structural materials such as lignin and cellulose. An estimated 2% and 40% of photosynthetically fixed carbon is deposited in the rhizosphere as a result of exudation and root turnover, respectively (Shaw and Burns, 2007). Due to these processes, the rhizosphere typically has greater microbial numbers and activity compared to bulk soil, a phenomenon known as the rhizosphere effect. Compounds released from rhizodesposition may also act as effector molecules for transcriptional regulators of xenobiotic degradation pathways (Shaw and Burns, 2007). For example the degradation pathway for the herbicide 2,4-dichlorophenoxy acetic acid may be induced by root exudates such as 2,4 dichloromuconate (Shaw and Burns, 2007).

Wetland plants also have the ability to funnel oxygen into the rhizosphere creating a micro-aerobic environment through a process referred to as radial oxygen release (ROL) (Faulwetter *et al*., 2009). ROL promotes oxidation reactions, thus activating the breakdown of organics as well as the immobilization of oxidizable metals such as iron and sulfur (Neubaurer *et al*., 2007). Multiple studies have determined that removal of

organic pollutants is enhanced in planted soils versus non-planted soils (Gagnon *et al*., 2003, Imfeld *et al*., 2009). *Typha spp.* are common wetland plants that inhabit saturated soils and can be found growing at sites contaminated with organic and heavy metal pollution (Ye *et al*., 1997). According to Faulwetter *et al*. (2009) *Typha* has one of the highest rates of ROL at 1.41mg h^{-1} $plant^{-1}$ and has been used in constructed wetland systems designed to remove a variety of organic pollutants (Machate *et al*., 1997). Different plant species support different bacterial assemblages which results in varying degradative capacities for different types of pollutants. Therefore, choice of plant species can play a major role in enhancing the treatment efficiency of specific pollutants like heavy metals and organic contaminants.

1.3.4 Sorption to Sediment and Organic Matter

Sorption and sedimentation is a major abiotic mechanism of pollutant removal and often limits the biodegradation of contaminants (Suthersan, 2002). Most sorption occurs in the clay and organic matter fractions of soils and sediments. Clay minerals are composed of layers of silicon and aluminium that are tightly held together, and depending on the lattice structure of the clay can either absorb or adsorb organic compounds (Alexander, 1995). Large molecules are predominantly held to clays by hydrogen bonding and van der Waals forces while low molecular weight compounds involve ion exchange mechanisms. Clay minerals and colloidal organic matter have a net negative charge and attract cations such as Mg^{2+} Ca^{2+}, and K^{+}. A positively charged organic molecule may also displace a cation on the surface of clay and thus adsorb to the clay (Alexander, 1995). The potential for a charged molecule to displace a cation on the surface of clay is known as the cation exchange capacity (CEC). Pollutants may also be

adsorbed by organic matter in the same manner, as humic substances also bear negative charges (Suthersan, 2002).

Soil organic matter is generally divided into two groups; non-humic and humic substances. Non-humic substances are not unique to soils and are comprised of organic compounds that belong to recognizable classes such as polysaacharides, simple sugars, amino acids, proteins, lignins, nucleic acids, and a variety of organic acids (Suthersan, 2002). Most of these substances are easily degradable and can be utilized as substrates by soil or sediment microorganisms. In contrast, humic substances comprise a heterogeneous mixture of chemically unidentifiable macromolecules that are synthesized in soil and are resistant to both chemical degradation and microbial attack (Alexander, 1995).

Humic substances are formed during the decay process via transformation of organic matter by microbial activity. The humus fraction can be divided into three complex heterogeneous mixtures known as humin, fulvic acid, and humic acid (Alexander, 1995). These can form complexes with high molecular weight organic pollutants by attachment of the pollutant to a reactive site on the surface of the organic colloid or by incorporation of the compound into the structures of humic and fulvic acids which are formed microbiologically. Such complexing has been reported for a variety of organic pollutants (e.g. PAHs, PCB's, and BTEX aromatics) (Suthersan, 2002)

The physical structure of the soil also influences the bioavailability of a contaminant to microbial degradation. The small pore sizes characteristic of clays (0.5-0.8 μm) are too small for microbes (average size 2 μm) to fit into, and therefore act as a barrier to degradation (Mihelcic *et al*., 1993). In addition, the surface properties of the

soils effect the location of microbial attachment. For example, it was reported that 60% of total bacteria in soil were located on organic matter-coated particles which only made up 15% of total surface area, while only 0.02% of microorganisms were found on sand grain surface (Mihelcic *et al.*, 1993).

1.4 Microbial Community Structure and Function in Disturbed Wetland Systems

Wetlands contain many groups of microorganisms including bacteria, fungi, algae, protozoa and viruses. These microorganisms do not live in isolation from each other, but rather live together and interact within a microbial community. In this thesis the term "microbial community" mainly refers to the bacterial community although algae, fungi, viruses and protozoa could not be excluded from the biochemical based studies that were performed. It is also important to note that the vast majority of microbial biomass in wetland sediments consists mainly of heterotrophic bacteria, fungi and protozoan. The diversity, density and distribution of microbial communities are shaped by numerous environmental factors with hydrology being one of the most important. Because of intermittent flooding and draining, wetlands support both aerobic and anaerobic microbial communities. These communities can be found floating in the water column, growing on plants, litter, and within microhabitats created in the soil environment that support a range of conditions for microbial growth.

Microorganisms carry out fundamental processes in wetland systems. As primary decomposers they degrade detritus, maintain soil structure and play integral roles in the cycling of carbon, nutrient and energy towards upper trophic levels. Microbial species also carry out specialized functions that drive the biogeochemical cycling of carbon,

nitrogen, phosphorus, sulfur and iron (Spray and McGlothlin, 2004). They are also responsible for the majority of organic contaminant degradation because of their unique ability to use organic contaminants as energy rich substrates for growth through complex enzyme driven processes (Faulwetter *et al*., 2009). These microbial driven processes are largely influenced by the extent and composition of microbial diversity at a given wetland site.

In general the distribution of microbial communities in wetland sediments follows either competitive or non-competitive diversity patterns. In competitive diversity patterns, overall microbial diversity is low diversity with a few dominant species better adapted to environmental conditions eliminating other species by competitive exclusion. In non-competitive diversity patterns the overall diversity is higher and no particular species dominates as a result of superabundant resources, resource heterogeneity (as to avoid competition by specialization), spatial isolation, and non equilibrium conditions (where more populations are maintained under fluctuating environmental conditions) (Zhou *et al*., 2002).

Contaminated wetlands are disturbed by contaminant stress and typically exhibit a competitive diversity pattern where the microbial community has a low diversity and is dominated by few species adapted to pollutant stress (Hadwin *et al*., 2006, Kim *et al*., 2009, Nicromat *et al*., 2006, Ravit *et al*., 2003, Trevors, 1998). A microbial community with low diversity dominated by a few resistant species may have serious implications for wetland processes. Reductions in microbial diversity have been shown to correspond to decreases in specialized niche functions such as methanogenesis, nitrification, denitrification and mineralization of organic pollutants (Baldwin *et al*., 2006, Girvan *et*

al. 2005, Griffiths *et al*., 2000). For example Girvan *et al*. (2005) showed that microbial communities disturbed by toxicant stress had a reduced capacity to mineralize 2,4 dichlorophenol.

1.5 Fate of Chlorinated Aromatic Xenobiotics in Urban Wetlands

Urban wetlands receive anthropogenic chlorinated aromatic compounds originating from a variety of sources such as agriculture, domestic or landfill runoff (Boyer and Polasky, 2004, Groffman and Crawford, 2004). Chlorinated aromatic compounds include all pollutants that contain at least one chlorine atom covalently bonded to an aromatic ring. Common types of these pollutants include organochlorine pesticides and herbicides used for residential and commercial golf course lawn care. Polychlorinated biphenyls (PCB's) are another type of chlorinated aromatic compound used in a variety of applications such as lubricants, heat transfer fluids, paints, adhesives, pesticide extenders, and these can be found in municipal landfill leachate runoff (Wyndham and Straus, 1998).

The environmental fate of chlorinated aromatics in wetlands systems depends largely on the physicochemical properties of the contaminant as this will influence its persistence, solubility, sorption, volatility, photo-degradability and amenability for microbial transformations. Take, for instance, the phenoxy herbicide 2,4-dichloropehnoxy acetic acid (2,4-D). Introduced in the 1940's 2,4-D has been the most widely used herbicide over the last 60 years for control of broadleaf weeds in agriculture, golf and residential turf, and forestry operations as well as aquatic weeds (Gaultier *et al*., 2009). 2,4-D has the potential to contaminate urban wetlands because once the herbicide

is applied, wind erosion and rainfall events can transport the contaminant into nearby wetlands (Gaultier *et al*., 2009). 2,4-D has a high aqueous solubility (900 mg/L at 25^{o}C, pH 7, Gaultier *et al*., 2009) and is readily dissolved and transported as runoff after a rainfall event. 2, 4-D is usually applied as an ester formulation and when in water is hydrolyzed to its anionic form within a day, therefore persistence of the 2,4-D anion is of primary concern (Walters, 1999). The 2,4-D anion has a lower sorption potential due to repulsion of the negative charges of sediments. 2,4-D also has a low vapor pressure ($1.4x10^{-7}$) and a low Henry's Law constant ($1.76x10^{-12}$) therefore little movement of 2,4-D is expected between the air/water barrier and volatilization and loss through volatilization is negligible. 2,4-D has the potential to photodegrade with an aqueous photolysis half life of 13 days, however microbial biodegradation is considered the primary route of degradation (Walters, 1999).

Common model compounds used for studying the microbial degradation of chlorinated aromatic pollutants include 2,4-dichlorophenoxy acetic (2,4-D) and 3-Chlorobenzoic acid (3-CBA) because of their simple structure (Morimoto *et al*., 2005). 3-CBA is one of the major intermediates in the degradation of PCBs and was found to be a major contaminant in landfill leachate (Wyndham and Straus, 1998). Bacteria capable of degrading 2,4-D and 3-CBA have been isolated from soils previously contaminated with these pollutants, and also, but to a lesser extent, from pristine soils (Fulthorpe *et al*., 1996, Morimoto *et al*., 2005). They can be divided into three groups based on experimental measured growth characteristics and evolutionary relationships (Kamagata *et al*., 1997). Isolates from pristine soils are generally slow growing and have catabolic genes that differ in terms of sequence similarity. The best studied representative of 2,4-D

degraders is *Wautersia eutropha* JMP134, a group I isolate harbouring the catabolic plasmid pJP4 (Shaw and Burns, 2007). This plasmid contains the *tdf* genes required for the degradation of 2,4-D and 3-CBA.

However both plasmid and chromosomal-encoded enzymatic steps are involved in the degradation pathway (Perkins *et al*., 1990). The first step of 2,4-D degradation is cleavage of the acetate side chain by an α-ketoglutarate-dependent dioxygenase encoded by the *tfd*A gene to produce 2,4-dichlorophenol (Fukumori and Hassinger, 1993). 2,4-dichlorophenol is catabolised to 3,5 dichlorocatechol by enzymes encoded in the *tfd*B and *tfd*CDEF operons and 3,5 dichlorocatechol is then degraded by ortho ring cleavage to produce tricarboxylic acid (TCA) cycle intermediates (Perkins *et al*., 1990, Shaw and Burns, 2007). 2,4-D can be completely degraded under aerobic and anaerobic conditions. Under reducing conditions 2,4-D undergoes reductive dechlorination and is further degraded to methane and carbon dioxide (Chang *et al*., 1998). Reductive dechlorination can be either a co- metabolic process or linked to respiration in a process known as dehalorespiration were the reduction of the halogenated compound is coupled to ATP production (Holliger and Shumacher, 1994)

1.6 The Role of Urban Wetland Community Structure and Function in Contaminant Fate

Anthropogenic contaminants are ubiquitous in today's urban environment. Contaminants such as heavy metals and synthetic organic compounds are received by urban ecosystems either through local point source pollution (e.g., industrial landfill leachate) or diffuse non point source pollution from multiple inputs over large areas (e.g.,

paved surface runoff). As a consequence, the air we breathe, the source water we drink and the soil in which we grow our food are contaminated with these chemicals. Fortunately, ecosystems that receive pollutants can, through natural processes, sequester and transform pollutants. These transformations ultimately influence the potential exposure and resulting toxicity to humans and wildlife.

Wetlands are unique ecosystems that can be found within urban areas and they are recognized for their intrinsic ability to naturally attenuate virtually all forms of aquatic pollution. The ability of wetlands to remediate pollutants has been known for years and natural wetlands have been and are still used to treat wastewater today (Kadlec and Tilton, 1979), although in recent times the use of constructed wetlands has become a much more effective and popular option because they can be engineered to optimize the removal processes that occur in natural wetlands (Haberl *et al*., 2003). Most wetlands are used to treat domestic wastewater and target pollutants such as pathogens, nutrients and simple forms of dissolved organic matter. However wetlands can also remove more complex organic chemical pollutants. In fact the natural attenuation of different petroleum based and volatile organic pollutants has been observed in freshwater wetlands (Lorah and Olsen, 1999, Mills *et al*., 2003). The attenuation of organic pollutants in wetland systems is achieved through various complex biological, chemical and physical interactions that take place within the association of sediments, wetland plants and microorganisms. The removal mechanisms in constructed wetland systems are well known and have been studied in detail, and it is recognized that microbial biotransformation is the primary factor in organic pollutant removal in wetland systems (Faulwetter *et al*., 2009, Haberl *et al*., 2003, Imfeld *et al*., 2009).

Urban wetlands in particular receive substantial inputs of pollutants such as excess nutrients, heavy metals and organic chemicals through various non point sources of urban runoff. (Boyer and Polasky, 2004, Groffman and Crawford, 2004). However few studies have investigated the removal of organic chemicals from urban wetlands (Kohler *et al*., 2004, Maillard *et al*., 2011, Thurston, 1999), and even less is known about microbial contributions. In this thesis three types of urban wetlands commonly found in cities and suburban areas were identified and investigated. These include constructed wetlands (CW) for stormwater control, contaminated remnant wetlands (CRW) adjacent to abandoned landfills, and natural remnant wetlands (RW). Our aim is to better understand the ecological structure of these urban wetland communities and their ability to tolerate and biodegrade contaminants in runoff pollution.

1.7 Research Hypothesis, Goals and Objectives

Although much is known about the capacity of wetlands to process contaminants, little is known about the potential for different urban wetland types to attenuate surface runoff pollution. In particular, it is not known if differing urban wetlands have unique microbial communities, and if their capacity to biodegrade organic contaminants also differs. To date, urban wetlands have been largely ignored with respect to their ability to remediate pollution. The conventional wisdom is that urban wetlands are degraded systems with low biodiversity, and hence low functional capacity.

In this thesis I investigated three types of cattail dominated urban wetlands commonly found in cities and suburban areas. These include a mature (+20 years) constructed wetland (CW) designed to collect stormwater runoff from a residential area, a

contaminated remnant wetland (CRW) that has historically been receiving point source inputs of heavy metal and organic pollutants from a landfill site, and a natural remnant wetland (RW) that receives both urban and mixed-forest runoff. I hypothesize that each urban wetland type would have different structural and functional characteristics based on differences in physicochemical properties, biological community structure and historical contaminant exposures. For example, microbial communities exposed to point source pollution such as landfill leachate are expected to have biological communities that are adapted to tolerate and process elevated contaminant concentrations found in leachate. Thus the aim of this research was to better understand the sediment and microbial community structure of different urban wetlands types and how they compare in terms of their capacity to attenuate organic pollutants found in runoff. To this end the following research objectives were met:

1- Characterize the abiotic features of each wetland by evaluating the physical structure, organic content, salinity, pH and moisture content of wetland sediments
2- Characterize the microbial communities at each site by measuring microbial biomass, activity and diversity, including physiological and phylogenetic diversity.
3- Assess the biodegradation potential of each wetland system by comparing rates of biodegradation of two model chlorinated aromatic compounds (2,4-Dichlorophenoxyacetic acid (2,4-D) and 3-Chlorobenzoate (3-CBA)
4- Assess the tolerance of each microbial community to organic and heavy metal pollutants via toxicity assays.

Chapter 2. Characterization of Sediment Physicochemical and Microbiological Properties in Urban Wetland Communities

2.1 Introduction

Wetlands sediments have unique physicochemical conditions as a result of their unique hydrology and plant communities. For example, oxidation reduction conditions are maintained by intermittent flooding and draining in combination with oxygen diffusion through the water column and active oxygen transport to the rhizosphere by wetland plants. These features create different oxic-anoxic interfaces that influence: (1) The type of microbial respiration that occurs and (2) the concomitant shifts in terminal electron acceptors that influence rates of organic chemical degradation (Imfeld *et al*., 2009). Also, wetland sediments typically have high organic matter because more carbon is produced by plants than can be decomposed under anaerobic conditions. When evaluating the pollutant removal efficiency of wetland systems another important parameter is pH, which will influence the range at which particular biochemical reactions occur (Imfeld *et al*., 2009). In addition, both pH and organic matter influence the sorption and bioavailability of organic pollutants.

Because the mineralization of organics is largely microbial-mediated, measurements of microbial biomass and activity are valuable indicators when assessing potential organic pollutant removal. Physicochemical properties such as pH, organic matter, moisture content, salinity and particle size distribution determine the activity, occurrence and spatial distribution of specific microbial biomass in different wetland types (Truu *et al*., 2009, Van der Valk, 2006). Sediments with high microbial biomass are

expected to correspond to relatively high levels of microbial activity that support a variety of degradation pathways (Voos and Graufman, 1997). In fact the size of microbial biomass has been shown to relate to herbicide degradation capacity (Voos and Graufman, 1997). The most direct method for quantifying microbial biomass is through staining and microscopic observations, and this method has been used as a reference for more indirect methods such as dilution plating (Gaunt *et al*., 1995). Direct bacterial counts have been used to estimate biomass in different freshwater wetland ecosystems (Gaunt *et al*., Gsell *et al*., 1997, Gsell *et al*., 2004, Maurice and Leff, 2002). Microbial activity is used to describe the vast range of activities carried out by microorganisms and various methods are used to determine activity. Enzymatic assays are commonly used to assess microbial activity, and because organics are deactivated by enzymatic conversions, they could serve as potential indicators of the ability of wetlands to transform these pollutants (Reddy and D'angelo, 1997). The hydrolysis of fluorecein diacetate (FDA) is an established enzymatic assay used to assess total microbial activity. FDA is hydrolyzed by enzymes involved in the degradation of organic matter and may act as a broad spectrum indicator for the potential degradation of organic chemicals.

No previous studies have characterized the sediment or microbial properties in the urban wetlands investigated in this study, and little is known about sediment and microbial characteristics of urban wetlands in general. These wetlands may have inherent sediment and microbial features that would ultimately influence their functional capacity to biodegrade pollutants. The objective of this study was to compare and characterize three different urban wetland types through measurements of sediment physicochemical properties and microbial biomass and activity. Microbial biomass was assessed through

direct counts using acridine orange staining and epifluorescent microscopy, and activity was assessed by rates of FDA hydrolysis. These measured properties will then be compared to those of natural wetland systems to see how the different urban wetland types compare.

2.2 Materials and Methods

Description of Study Sites

The study sites are located are Oshawa, ON within 15 kilometres of each other and are subject to the same local climate regime (Figure 1). The three urban wetland types include: Constructed Wetland (CW), Contaminated Remnant Wetland (CRW), and Remnant Wetland (RW). Each site is dominated by cattails (*Typha spp.*) therefore rhizosphere effects are expected to be similar. According to the Canadian Wetland Classification System all three wetland types fit the description of a Riparian Meltwater Channel Marsh (The National Wetland Working Group, 1997).

Constructed Wetland (CW)

The CW was located in a residential subdivision and was built to collect storm water runoff. This wetland was constructed at least 20 years ago and follows the design of a typical surface flow wetland. This wetland receives the highest inputs of non point source pollution originating from runoff of paved surfaces and residential lawns. Therefore the types of pollutants expected to enter this system might include various fertilizers, herbicides/pesticides, hydrocarbons, heavy metals, and excess levels of salinity (Thurston, 1999).

Contaminated Remnant Wetland (CRW)

The CRW was located in the riparian zone of an urban creek, and has been receiving point source pollution (i.e. industrial landfill leachate) containing a variety of heavy metals and organics for at least 30 years. Approximately 1 million tonnes of industrial waste were land-filled at this site. The majority of the waste originated from General Motors automotive manufacturing plant and contained a variety of industrial and operational materials including metals and paint sludge, oils and industrial solvents (Crutcher and Mosher, 1991).

Remnant Wetland (RW)

This RW is likely the remnant of a once larger natural wetland that has been modified by urbanization. The RW is part of the riparian zone of an urban creek and is situated between a busy street, agricultural fields and residential subdivisions. This site does not directly receive any known point sources of pollutants but potentially receives runoff from the various sources surrounding the wetland and this could include a variety of organic and heavy metal pollutants.

Figure 1. Aerial Map showing location of urban wetlands investigated in this study. Coordinates CW: (43°56'48.15"N, 78°52'36.08"W) RW: (43°55'24.49"N, 78°55'22.30"W) CRW: (3°55'22.36"N, 78°50'7.37"W)

2.2.4 Wetland Sediment Sample Collection

Sediment samples were collected from the rhizosphere of each site. They were collected from 3 sample locations in parallel to each other in a west to east transect across each wetland. A 4 meter buffer zone from the edge of the wetlands was left to eliminate edge effects. Four sediment cores were collected from a 1-m^2 quadrant and mixed together in sterile whirlpack© bags. Samples were immediately placed in a cooler at 4 ^oC and transported to the laboratory and remained stored at 4 ^oC until analysis. Samples used for analysis of sediment physical properties and bacterial enumeration were collected on

April 10/2010 and analyzed within 2 weeks of collection. Samples used for FDA analysis were collected November 26/2011 and analyzed in January.

2.2.5 Wetland Physicochemical Properties

2.2.5.1 Soil Organic Matter Analysis

Total Organic Carbon (TOC) was assessed using an ashing procedure (Carter and Gregorich, 2008). Air dried soil samples (2 g) were transferred into ceramic crucibles and placed into a muffle furnace at 550 ^{o}C for 24 hours. Samples were removed, allowed to cool in a desiccator cabinet, and weighed at hourly intervals until no change in weight was observed. Total organic carbon is measured as % weight lost during combustion and calculated as follows:

Ash = [(A-C)/(B-C)]*100

Where: A = weight of crucible + ash (g)

B = weight of empty crucible (g)

C = weight of empty crucible + sample (g)

2.2.5.2 Soil Moisture Content

The moisture content of sediments was determined by measuring the amount of water lost after drying samples at 105-110 ^{o}C for 24 hours. Samples (25 g) were transferred to a stoppered Erlenmeyer flask. The flask was dried with the lid removed, allowed to cool in a desiccator, and re-weighed with the lid. Moisture content is determined by the following calculations:

H_2O lost = Weight Moist Soil (g) – Weight Oven Dry Soil (g)

Dry mass% = H_2O lost / mass oven dried soil (g) x 100%.

Wet mass% = H_2O lost / mass wet soil (g) x 100%

2.2.5.3 Soil pH determination

Soil pH was determined as described by Watson and Brown (1998). Samples (10 g) of wet sediment were mixed with 15 mL of deionized H_2O and allowed to settle for 10 min. Measurements were taken with a calibrated Oakton pH/Ion 510 meter that was inserted into the sediment slurry and swirled gently.

2.2.5.4 Sediment Salinity

Sediment salinity was determined using a Cole-Palmer handheld salt refractometer. Pore water was pressed from sediment samples pressed in a 5 mL syringe. One to two drops of pore water were placed on the refractometer faceplate, held to the light and % salinity (TDS g/L) measurement was made.

2.2.5.5 Sediment Particle Size Distribution

Sediment texture was determined using the hydrometer method (Tan, 2005). Sediments (50g) were dispersed in 800 ml water adjusted to pH 11 with NaOH with a magnetic stir bar in a beaker. The soil suspension was transferred to a 1 L graduate cylinder and hydrometer readings were taken twice at 40s and after 2 hr to allow the different fractions of (silt+ clay) and (clay) to settle. Hydrometer readings were corrected for temperature:

% Sand + % Silt + % Clay = 100%

40 s reading : %(silt + clay) = (40 s reading /50) *100

2 hr reading: % clay = (2 hr reading /50)*100

2.2.6 Determination of Colony Forming Units (CFU) by Dilution Plating

Dilution plating is used to estimate the number of viable heterotrophic bacteria in an environmental sample**.** Bacteria are cultured on specific carbon sources and therefore only a subpopulation of the whole community is detected**.** However dilution plating is a quick, inexpensive and reliable technique that generally yields good estimates of microbial numbers in a given community. Cells were extracted by adding 5 g of sediment to 35 mL of sterile 10mM phosphate buffer (pH 7) and vortexing at high speed for 1 min. The cell extract was serial diluted down to a 10^{-6} dilution and each dilution plated on R2A agar plates in triplicate. R2A is commonly used media for plate counts and containing low amounts of a large variety of nutrients and growth factors required for bacterial growth (Reasoner and Geldreich, 1985). The dilution that resulted in growth of between 30-300 colonies was chosen for counts. The number of microorganisms in the original sample is calculated using the average number of colonies (Avg), the volume of inoculant (Vol1), volume of suspension (Vol2), the dilution factor (Dil) and the mass of the original sediment sample as follows:

Colonies mL^{-1} of suspended sample = Avg x Dil / Vol 1

Colonies g^{-1} of suspended sample = Num x Vol 2 /Mass soil added (g)

2.2.7 Acridine Orange (AO) Staining and Epifluorescence Microscopy

In this method cells are fixed with a preservative, stained with a fluorescent dye, filtered onto black polycarbonate filters and counted under a fluorescent microscope. This

method is used for estimating of total microbial numbers and is superior to culture based methods as all viable and non viable cells are stained and counted, including the microorganisms that cannot be cultured in laboratory. The two most commonly used fluorescent dyes are acridine orange (AO) and 4,6-diamidino-2-phe-nylindole (DAPI). AO was chosen over DAPI, since DAPI has been shown to yield underestimates when working with sediment samples (Kepner and Pratt, 1994). Acridine orange binds to adjacent phosphate groups in both DNA and RNA. When bound to double stranded nucleic acids the dye shows bright green fluorescence, when bound to single stranded nucleic acid it emits a red orange fluorescence (Kepner and Pratt, 1994).

The acridine method used in this study was adapted from (Hobbie *et al*., 1977 and Bolter *et al*., 2006). Cells were preserved by mixing 5 g of sediment with 35 mL 10 mM phosphate buffer containing 1% formalin. The cells were then dispersed by ultrasonication at 40-50 KHz for 2.5 min at 20 ^{o}C. Ultrasonication was chosen as it was shown to be more effective than homogenization when working with sandy sediments (Ellery and Schleyer, 1984). The volume of cell suspension used for staining must be adjusted to achieve appropriate bacterial density, and this is determined by a process of trial and error. The final amount of cell suspension used for staining was 250 μL, 500 μL and 700 μL of a 10^{-2} dilution for RW, CW and CRW sediment samples respectively. The cells were stained with a final AO concentration of 90.9 μg/mL for 3 minutes and filtered onto black polycarbonate filters (25 mm, 0.2 μm pore size). A cellulose acetate backings filter was used to promote better dispersion onto the filter (Kepner and Pratt, 1994). The polycarbonate filter was placed between two slides and two drops of immersion oil. All slides were prepared in triplicate and counted the day of preparation. Slides were counted

using a Leica DMIL epifluorescence microscope with blue light filters. Pictures of the field of view were taken with a Nikon DSL 1 camera. Between 20 and 50 cells were counted in 15 randomly located fields. The total bacteria count (TBC) per g of soil was determined by the following calculation:

$$TBC = (DxBxM)/W$$

Where: TBC = Total bacteria count per g of soil

D = dilution caused by suspension and subsequent sampling of soil

B = mean count of bacteria per counting area

M = microscope factor (filtration area/area of counting field)

W = weight of oven dry sample

2.2.8 Measurement of Total Microbial Activity by Fluorescein Diacetate (FDA) Hydrolysis

In this method microbial activity is assessed by measuring enzymatic rates of fluorescein diacetate (FDA) hydrolysis to fluorecein, a coloured end product that is measured by UV-vis spectrophotometry. FDA is hydrolysed by non specific esterases, lipases and proteases found within cellular membranes or as free extracellular enzymes released by active microbial cells (Adam and Duncan, 2001). These enzymes are involved in the decomposition of organic matter and are produced by the major decomposers, bacteria and fungi, which process more than 90% of the energy flow in a typical soil system. Therefore FDA hydrolysis is thought to reflect total microbial activity. FDA hydrolysis has also been shown to correlate well with measurements of

microbial biomass of pure and mixed cultures including ATP content and cell density (Stubberfield and Shaw, 1990).

The FDA method used in this study was adapted from (Shaw and Burns, 2006, Adam and Duncan, 2001). Sediments were first normalized for water content by centrifugation at 4000 rpm for 5 min and portions were transferred to a sterile 6 well plate and incubated for 2 days before starting of the FDA experiments. After incubation 1 g of sediment was added to a 50 mL falcon tube with 7.5 mL of sterile water. Aliquots (0.1 mL) from a 1000 µg/mL FDA stock solution were added to each treatment, mixed by vortexing, and incubated at 30 ^{o}C for 30 min in on a rotary incubator. Immediately after 30 minutes the reaction was terminated by addition of 7.5 mL of a 2:1 chloroform:MeOH solution. The tubes were centrifuged at 300 g for 2 min and 1.5 mL of the upper phase supernatant transferred to an eppendorf tube and microcentrifuged at 16,500 rpm for 5 min to remove fine particulates. The supernatant was analyzed for fluorescein by measurement of absorbance at 490 nm using a Genesys 20 Thermo Spectronic UV-vis spectrophotometer. Sediment blanks without the addition of FDA were prepared to correct for background absorbance. Abiotic controls were prepared by autloclaving the sediments for 40 minutes at 121 ^{o}C. All treatments were done in triplicate. The mass of fluorescein produced in each treatment was determined using a calibration curve constructed with standards made with fluorescein salt. The amount of FDA hydrolysis activity is determined by dividing the dry weight of soil and is expressed as µg fluorescein g soil $^{-1}$ 0.5 h $^{-1}$.

2.2.9 Statistical Analysis

Data was first tested for normal distribution by the Shapiro-Wilk's test and for equal variance using the Brown and Forsythe's test for homogeneity of variances ($p \leq 0.05$). Statistically significant differences among all treatment means was analyzed by one way Analysis of Variance (ANOVA). If there was a significant difference ($p \leq 0.05$) pairwise comparison of means was performed using Tukey's Range Test. All Data was analyzed with SigmaPlot 11.0 software package.

2.3 Results

2.3.1 Physicochemical Properties of Urban Wetland Sediments

The sediment physicochemical properties for each urban wetland are presented in Table 1. Each site had a similar neutral pH ranging from 7.05-7.21. Salinity at the CW site was significantly higher than the RW and CRW sites which had comparable salinity levels. The CRW was completely saturated with moisture content over 100% while the other sites had moisture contents above 50%. Organic matter content was highest for the CRW at 18.43% compared to 7.49% and 8.08% for the CW and RW sites respectively. The soils at each site were mainly composed of sand.

Table 1. Sediment physicochemical properties collected April, 2010 from each Wetland site CW: Constructed Wetland CRW: Contaminated Remnant Wetland RW: Remnant Wetland

	Study Sites		
Factor	**CW**	**CRW**	**RW**
pH	7.05 ± 0.01	7.21 ± 0.02	7.06 ± 0.02
Salinity (ppt)	19 ± 0.88 *	3 ± 0.52	2
Moisture (% dry wt.)	69.5 ± 21.8	182 ± 67.8	59.7 ± 12.5
Organic Matter (%)	7.49 ± 1.19	18.4 ± 5.92	8.08 ± 2.49
Physical Structure			
Sand (%)	87.3	68.3	78.8
Silt (%)	9	23	17
Clay	3.7	8.9	4
Soil Type	Sand	Sandy Loam	Loamy Sand

ANOVA, Tukey P≥0.05, +/- standard error (SE), * Significant Difference

2.3.2 Microbial Biomass and Activity

Microbial biomass could not be determined by plate counts on R2A agar because of indeterminant growth patterns. In general, dilutions < 10^{-3} had too many colony forming units to count and dilution > 10^{-3} had very little to no growth. Therefore direct counts using acridine orange staining was used to estimate biomass because this technique is culture independent and estimates the total number of bacteria in the sample,

rather than just those species than can grow on R2A. The microbial biomass at each site was comparable ranging from 1.09 to 1.32 x 10^8 cells $g^{-1}$and were not statistically different between sites (Figure 2). Microbial activity was highest at the CRW with rates of FDA hydrolysis that were double that of the other two sites (Figure 3). The CW and RW had comparable rates of FDA hydrolysis.

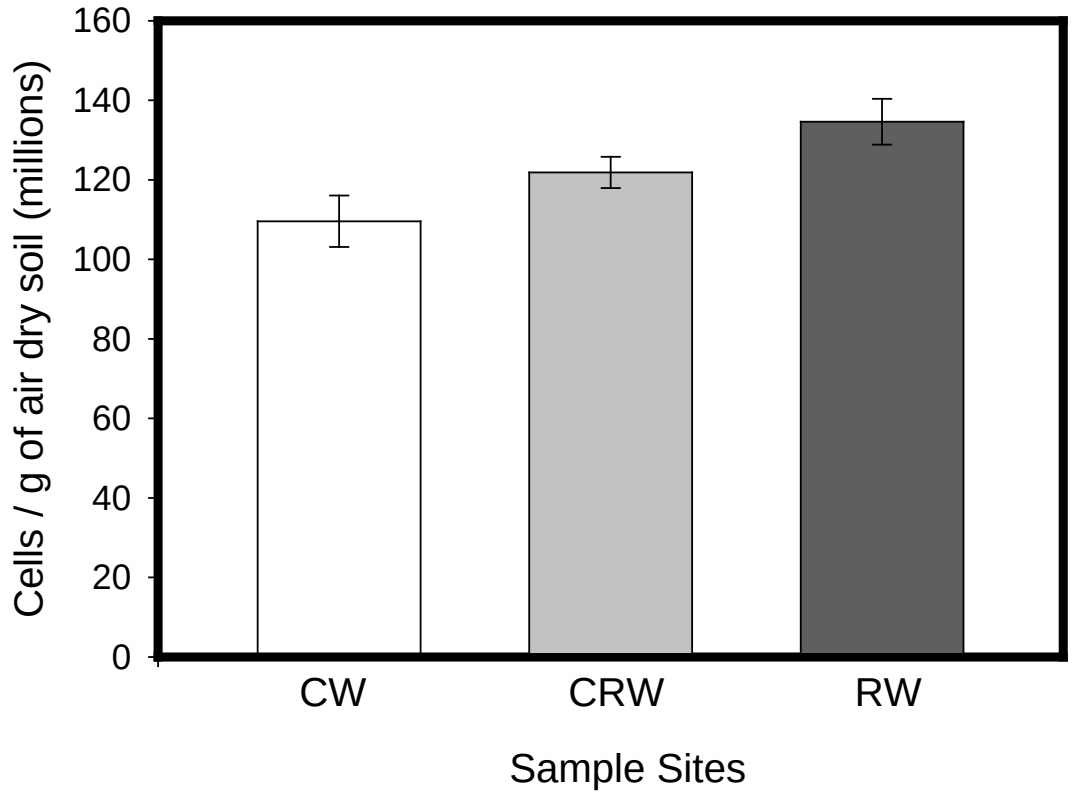


Figure 2. Total bacterial enumeration of sediment samples using acridine orange (n=3), +/- standard error (SE), ANOVA of ln transformed data, Tukey, p>0.05

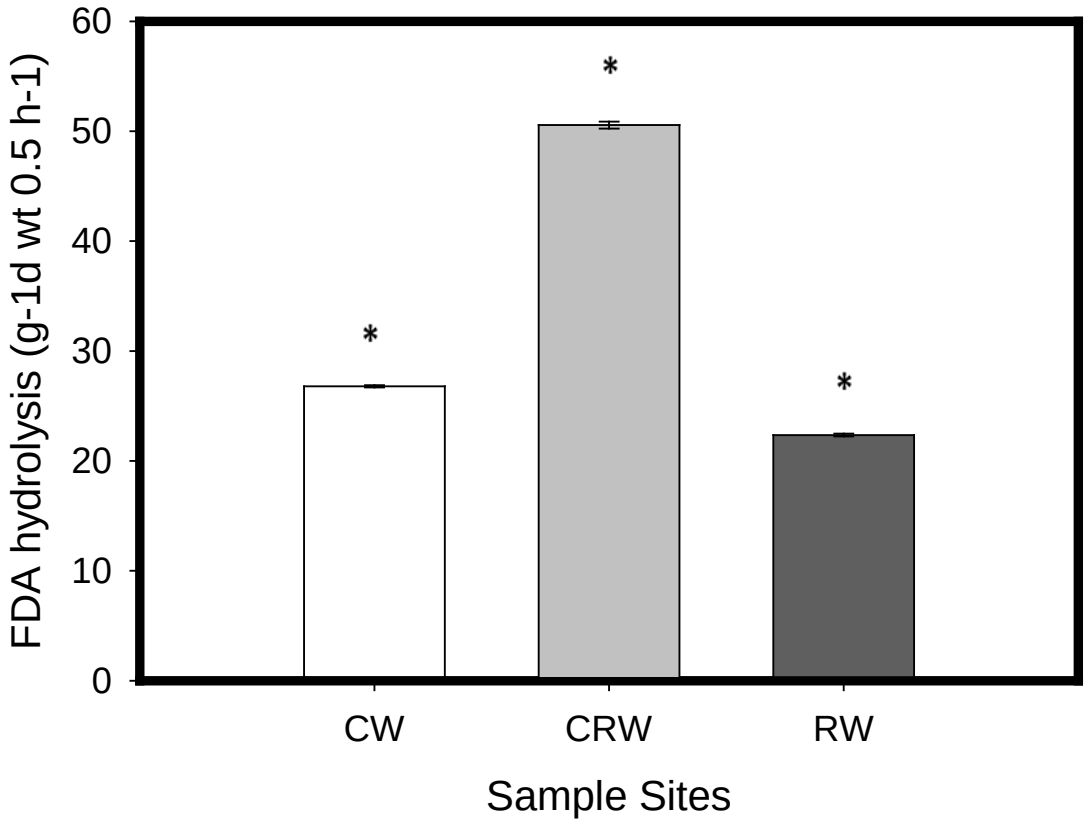


Figure 3. FDA hydrolysis from sediment samples collected at each wetland site +/- standard error (SE), ANOVA, Tukey, $p>0.05$, *significantly different

Discussion

The measured sediment physicochemical properties did not show great variation between sites except for salinity at the CW which was significantly higher. This may be explained by the fact this CW is located within a residential area and directly receives stormwater runoff from roadways. We collected our samples in the spring and this wetland would have been receiving residual road salt runoff from winter applications and spring thaw snowmelt (Novotny *et al*., 2008).

The biomass at each of the urban wetlands determined by acridine orange direct counts (AODC) was comparable in the range of 10^{8} cells $^{-1}$ g^{-1} which is an order of magnitude lower than what has been reported for other wetland sediments (Gaunt *et al*., 1995, Gsell *et al*., 1997, Gsell *et al*., 2004, Ipsilantis and Sylvia, 2007). AODC from sediments of an alkaline fen wetland, rice field wetlands and nutrient impacted wetlands were in the range of 10^{-9} to 10^{-10} g^{-1} dry mass soil. Seasonal effects may explain our lower than expected bacterial counts. Bacterial counts are driven by temperature and the presence of easily degradable organic matter (Ipsilanti and Sylvia, 2007). Our samples were collected in the spring while cell counts are typically higher in the summer when temperatures are elevated, or in the fall when plant litter provides a source of easily degradable organic matter that sustains greater microbial growth (Groffman *et al*., 1996, Ipsilantis and Sylvia, 2007). Urban wetlands also typically receive increased inputs of surface runoff containing pollutants such as heavy metals and road salt and may cause lower bacterial densities. Both heavy metal and high salinity impacted environments have shown to have reduced microbial biomass compared to non-impacted environments (Kuperman and Carreiro, 1997, Tripathi *et al*., 2006).

The low bacterial counts in our study may also be explained by the high sand content at all of our sites (>67%). AODC counts of aquifer sediments have been found to correlate with soil texture where counts decrease with increasing sand content (Albrechtsen and Winding, 1992). High microbial biomass is generally associated with finer particles (silt and clay fractions) for reasons including greater surface area for adhesion and colonization, reduced predation by bacterial grazers, and higher nutrient availability due to greater aggregation of organisms (Perryman *et al*., 2011). Nutrient

limiting conditions have also been shown to limit biomass (Groffman *et al*., 1996, Gsell *et al*., 2004) and although we didn't specifically measure nutrient levels, nutrient limiting conditions are not expected at our sites due to the presence of organic soils which typically have high nutrient availability compared to mineral soils (D'Angelo and Reddy, 1999). Also, nutrient loading is expected to occur from inputs of agricultural and residential runoff (Gsell *et al*., 2004). Another possible explanation for the low biomass observed in urban wetlands could be methodological differences between studies. Sediment samples must be diluted before staining to reduce sediment interference and to obtain countable number of cells. We used the maximum recommended dilution of 1:1000 (Kepner and Pratt 1994). The dilution is also factored into the final cell count calculation. Of the studies that used AODC for biomass estimation in wetland sediments, only Gsell *et al*., (1997) reported using a 1:10 dilution, which is much higher than what we used and may have resulted in higher counts.

We expected high between-site variation of biomass due to differences in % moisture and organic matter between sites. Organic matter and hydrology have been shown to regulate microbial biomass and activity in natural freshwater wetlands, with higher cell counts in wetlands with high organic matter and water table depth (Albrechtsen and Winding, 1992, Groffman *et al*., 1996). However biomass in the investigated urban wetlands did not show much variation and was within the same order of magnitude regardless of differences in organic matter and % moisture content between sites.

Microbial activity as determined by rates of FDA hydrolysis was highest for the CRW which had the highest organic matter content. This result was expected because the

enzymes involved in FDA hydrolysis (i.e esterases, proteases, lipases) are the major enzymes involved in the degradation of organic matter and studies have shown that soils with high organic matter have higher rates of FDA hydrolysis than soils with low organic matter content (Brohon *et al*., 2001).

In summary, microbial biomass at each wetland site was comparable, but lower by an order of magnitude than AODC estimates reported for other wetland sediment types. Although seasonality, runoff pollution and sediment texture may play a role in bacterial densities, the comparable results for different urban wetland types in this study suggest that urban wetlands in general may have lower bacterial densities. Each wetland type receives different amounts of runoff from different sources that could contain varying amounts of sediments and other contaminants such as road salt and various organic and heavy metal pollutants. It could be the combination of these factors responsible for the lowering of bacterial densities. The low biomass observed at each site may also limit the capacity of sediment communities to biodegrade contaminants. In particular organic pollutants, which have been shown to dissipate at a faster rate and more completely in soils with high microbial biomass (Voos and Groffman, 1996).

Chapter 3. Characterization of Microbial Physiological and Phylogenetic Diversity in Urban Wetlands

3.1 Introduction

It is recognized that microbial diversity in wetland environments is critical for the proper functioning and maintenance of the system. The microbial community are the main drivers behind the degradation of organic chemicals and key processes such as decomposition of organic matter, biogeochemical cycling of carbon, nutrients and other elements. The microbial diversity of the system may also influence the resilience of an ecosystem to withstand disturbance from toxicant stress. Although the function of wetland systems is mainly driven by microorganisms, there are a limited number of studies that have focused on microbial communities in freshwater and constructed wetlands (Hartman *et al*., 2008, Truu *et al*., 2009), and even less is known for urban wetlands. A better understanding of the microbial diversity structure and function in different urban wetland types can provide insight into their influence over various ecosystem processes, including the capacity to degrade organic pollutants.

Linking microbial structure with ecosystem functions is an ongoing challenge in microbial ecology due to the technical difficulties associated with characterizing microbial communities. Fully characterizing a microbial community requires the complete enumeration and identification of all microorganisms. Classical methods for bacterial identification and enumeration rely on culture-based methods that exclude the vast majority of indigenous microbes (i.e., less than 1% of microbes) (Kirk *et al*., 2004). Fortunately, PCR-based methods now exist allowing for the identification of a much

larger proportion of the microbial community without culture bias. These PCR-based methods employ primers that target specific genes (e.g., 16S rRNA) which are amplified and fingerprints are generated by separation through different types of polyacrylamide gels. Denaturing gradient gel electrophoresis (DGGE) gels can be used to separate DNA by a linearly increasing gradient of chemical DNA denaturants (formamide and urea). The 16S rRNA gene is usually targeted in diversity studies as this gene is highly conserved among all living organisms and is ubiquitous in all prokaryotes (Head *et al*., 1998). The 16S rRNA gene is composed of both conserved and variable regions. The conserved regions are considered "universal" and serve as annealing sites for PCR primers. These conserved regions are interspersed with variable regions used for phylogenetic differentiation (Nocker *et al*., 2007). This gene is large enough (1550 base pairs) for distinguishing evolutionary relationships and to provide statistically valid measurements (Pace *et al*., 1986). Additionally there does not appear to be lateral 16S rRNA gene transfer between microorganisms (Pace *et al*., 1986).

Although the identification of species through 16S rRNA analysis is useful, these techniques don't provide information about the ecological or functional role of these microbes. Information regarding the functional role of microbial communities in urban wetlands can be obtained by generating community level physiological profiles (CLPP's) based from patterns of sole carbon source utilization. Many approaches can be taken to obtain CLPP's but most often CLPP data are collected using Biolog^{TM} MicroPlates. Biolog^{TM} MicroPlates consist of 96 wells, each containing nutrients, a carbon source and the redox dye tetrazolium violet. Microbial communities are inoculated directly into the plates and if the soil microorganisms can utilize the carbon substrate for growth, the

substrate is oxidized and the tetrazolium violet dye is reduced to a violet formazan which can be measured spectrophotometrically at 590 nm (Stefanowicz, 2006). The rate of colour development in each well reflects species activity and density of cells in inocula, while the diversity of colour intensity gives information about overall microbial diversity in soil.

Although culture-dependent techniques such as CLPP are useful for understanding the potential ecological behaviour and function of microorganisms in urban wetlands, they have to be complemented with culture independent methods such as PCR DGGE to obtain information about the composition of microbial communities. In this study I use a polyphasic approach combining both physiological (BiologEcoplate™) and phylogenetic (DGGE) analysis to give more information on the composition of microbial communities at these sites. Both of these methods have previously been used to successfully compare both functional profiles and phylogenetic profiles of microbial communities from natural and constructed wetlands sediments (Buesing *et al*., 2009, Hadwin *et al*., Ibekwe *et al*., 2003, Nicromat *et al*., 2006, Weber *et al*., 2008).

The goal of this study was to characterize and compare the physiological and phylogenetic diversity of the microbial communities from each urban wetland type to better understand the microbial ecology in urban wetland systems and to see if differing urban wetland types have unique microbial communities. It was hypothesized that the microbial community structure of each urban wetland type would reflect the particular physicochemical conditions, history of contaminant exposure as well as the types and amount of runoff received at each site. Samples were collected from three locations in a

west to east transect to examine potential differences in spatial distribution within individual sample sites.

3.2 Materials and Methods

3.2.1 Wetland Sediment Samples

Sediments used for Biolog analysis were collected on May 19th/2010, as described in materials and methods section (2.2.4), and analysis performed within a few weeks of collection. Sediment sample used for DGGE analysis were collected November 19th/2010 and a subsample from each of 3 samples collected from each site was immediately frozen at -20 ^{o}C until March 14th when they were thawed just before DNA extraction.

3.2.2 DNA Extraction, PCR and DGGE

DNA was extracted from 0.25g wet weight sediment samples using the Mo Bio Powersoil™ DNA Isolation Kit. DNA was stored at 4 ^{o}C and PCR performed within 36 hours of extraction. Before PCR amplification, DNA was quantified using a Thermo Scientific Nano Drop 1000 Spectrophotometer. PCR amplification of 16S rRNA genes from purified DNA was performed using the primers 341f with a GC clamp (40 nucleotide GC-rich sequence, 5'-CCT ACG GGA GGC AGC AG-3') and 907r (5'-CCG TCA ATT CCT TTR AGT TT-3'). These primers amplify a 550-bp fragment of the 16S rDNA between Escherichia coli positions 341 to 907 which is short enough to be separated by DGGE but long enough for reliable phylogenetic inference (Muyzer *et al*.,

1995). The target area corresponds to the V3-V5 hypervariable regions of the 16S rRNA gene (Sanchez *et al*., 2007).

Each PCR reaction tube contained 5ul of DNA template, 10 uM of each primer, 3 ul H20 and 10 ul HotStarTaq Master Mix (Qiagen©) which contains HotStar DNA Taq polymerase, dNTP' s and buffer. The PCR reaction was run on a PTC-100 Programmable Thermal Cycle (MJ Research, Inc) with the following program: Initial activation at 95 ^{o}C followed by 35 cycles of denaturation at 95 ^{o}C for 1 min, primer annealing at 56 ^{o}C for 1 min, primer extension at 72 ^{o}C for 1 min before the final extension at 72 ^{o}C for 10 min. PCR products were checked on a 0.4% agarose gel to ensure that the samples had amplified and that there was no contamination by running a negative control using sterile water.

Samples were loaded onto a 6% (W/V) polyacrylamide gel in 50x TAE (Tris, acetic acid, EDTA). The polyacrylamide gels were prepared with linear 40% to 70% denaturant gradient, where 100% denaturant contains 40% foramide and 7 M urea. The gels were allowed to polymerize for 45 minutes before loading the gel with PCR product. The buffer was preheated to 60 ^{o}C before use and the gels were run at 70 V for 20 hours on a C.B.S Scientific Co. DGGE unit. DNA fingerprints were visualized by staining gel with 50 ul ethidium bromide (EtBr) and using a C-80 Epi-illumination ultraviolet lamp and Lab Works™ Image Acquisition and Analysis software v.4.6

3.2.3 Community Level Physiological Profiling (CLPP) and Biolog EcoPlates

Biolog EcoPlates™ were used because they are tailored to ecological applications and comprise three replicate sets of 31 environmentally applicable substrates of which at

least nine are considered constituents of plant root exudates (Preston-Mafham *et al*., 2002). The use of more ecologically relevant and structurally diverse compounds provides a more useful test for microbial community analysis and to pick up those organisms usually missed through being swamped by faster growing r-selected species (Campbell *et al*., 1997).

The extraction of microbial cells from sediments was achieved with mechanical agitation using a vortex mixer in a 0.8% (w/v) saline dispersal medium. Briefly, 5 g of sediment were added to a falcon tube containing 35 mL 0.8% NaCl solution and vortex at high speed for 3 minutes. Tubes were centrifuged at 500 g for 2 minutes to remove sediment particles and decanted in a sterile flask. Sediments were re-suspended in 35 mL 0.8% saline solution and the procedure repeated. Cell extracts were diluted 10 fold and 125 ul aliquots of the soil dilution were inoculated directly into each well of the Biolog Ecoplates using an 8 channel pipettor. Plates were placed in polyethylene bags to reduce desiccation and were incubated in the dark at 21 ^{o}C. A 1:10 dilution was chosen as preliminary experiments revealed that dilutions greater than 10^{-1} had long lag phases before colour development (> 2 days), and by the end of one week incubation, overall colour development was too low for analysis.

3.2.4 Plate Reading and Biolog Statistical Analysis

Optical Density (OD) at 590 nm (colour development plus turbidity) and 750nm (Turbidity only) were measured on a BIORAD™ spectrophotometer at time zero and every 24 hours for one week. Turbidity was corrected by subtracting the 590 nm values from the 750 nm values. OD values for the control well (C) were then subtracted from

each individual well (R). If negative values were obtained they were left as 0. Overall colour development in Biolog Ecoplates was expressed as average well color development (AWCD) calculated as:

$$\sum_{i=1}^{31} \frac{1}{31}(Ai - Ao)$$

where A_i represents the absorbance reading for well i and A_0 is the absorbance reading of the blank well. As recommended by Garland (1997) a single time point absorbance was selected for analysis. This time point was chosen by plotting AWCD over time and selecting the time point that was in the linear part of the curve. Color development in biolog plates follow an asymptotic sigmoidal curve so it was important to select a time point where the microbial communities are actively growing and have not reached saturation, but that gave sufficient lag time for slower growing microbes the chance to be detected. Data of each individual well (k) was then normalized by dividing corrected absorbance values by the AWCD (Garland, 1997).

$$\overline{A} = \frac{Ak - Ao}{\sum_{i=1}^{31} \frac{1}{31}(Ai - Ao)}$$

3.2.4.1 Principle Component Analysis

PCA was performed to characterize the relationship of substrate utilization profiles between study sites. PCA is an eigen analysis that projects multivariate data onto new principle components axis which account for as much of the variance of the original data set as possible. Each PC extracts a proportion of the variance in the original data

with the first PC extracting the greatest amount of variance. The PCA was extracted from a variance-covariance similarity matrix since this approach is followed when using the same type of data (i.e., substrate utilization) (Ramette, 2007). Normalized OD data was collected for 9 plates. Each plate contains 3 internal triplicates giving a total of 27 objects to be ordinated. PC axis scores were then correlated with individual variables (substrates) to see which substrate(s) are responsible for explaining most of the variation of a given PC. Analysis was performed using PAST 1.28 statistical software.

3.2.4.2 Cluster Analysis

As recommended by Weber *et al*., (2008) cluster analysis was performed to verify and validate the results obtained from the PCA analysis. An unweighted pair-group method using arithmetic averages (UPGMA) clustering analysis was performed using the bray-curtis index as a distance measure for transformed colour response data. The banding patterns obtained from DGGE of 16S rRNA bands were also analyzed by cluster analysis using computer software Gel Compare II v 6.5. A UPGMA clustering analysis was performed using the Jaccard index as a distance measure for banding patterns.

3.3 Results

3.3.1 Biolog Community Level Physiological Profile analysis (CLPP)

Biolog was used to generate a CLPP based on patterns of sole carbon source utilization. Three subsamples were taken at each site along a parallel west to east transect to examine potential differences in the spatial distribution of microbial community

metabolic profiles within study sites. Each subsample was analyzed in triplicate for a total of 9 data points per site.

The PCA ordination of carbon substrate utilization profiles did not distinctly separate each study wetland (Figure 4). The first PC explained 37.7% of the variation in the data and the second PC explained 13.2% of the variation in data. Data points from each site overlapped, with the CW site showing the tightest clustering of data points followed by the RW, representing a homogeneously distributed metabolic profile at these sites. In contrast, the CRW site had the greatest scatter of data points representing a heterogeneously distributed metabolic profile. The three CRW1 outliers with higher coordinate values on the first PC correspond to the subsample collected from the western most part of the site (Figure 4).

UPGMA cluster analysis was performed to investigate whether the same grouping effect seen using PCA could be seen in the cluster diagram. A similar grouping effect found with PCA ordination was observed in the cluster diagram (Figure 5). The CW and RW site clustered together with the CW site showing a tighter grouping. The CRW1 outliers observed in the PCA also clustered separately from the rest of the samples in the cluster diagram (Figure 5).

The separation of data points along PC axis can be related to differences in carbon source utilization by examining the correlation of each substrate variable to the PC axis scores. Substrates with r values above 0.7 were considered the most important in differentiating among communities (Table 2). Any r value below 0.7 would mean that utilization of these substrates was relatively similar and thus were not variables that

helped in differentiating samples. The only major separation in data points were those outlier points corresponding to a CRW1 subsample taken at the western most part of the site. Analysis of PC1 (Figure 4) indicates that the microbial communities of this CRW1 subsample utilized carboxylic acids (D-Galacturonic acid, Itaconic Acid) and amino acids (L-Asparagine) to a greater degree, and carbohydrates (B-Methyl-D-Glucoside) and polymers (D-Glycogen) to a lesser degree than microbial communities from any other sample. On the basis of PC2 analysis, the microbial communities of the CRW1 subsample utilized the carbohydrate D-cellobiose to a lesser extent than microbial communities from any other sample.

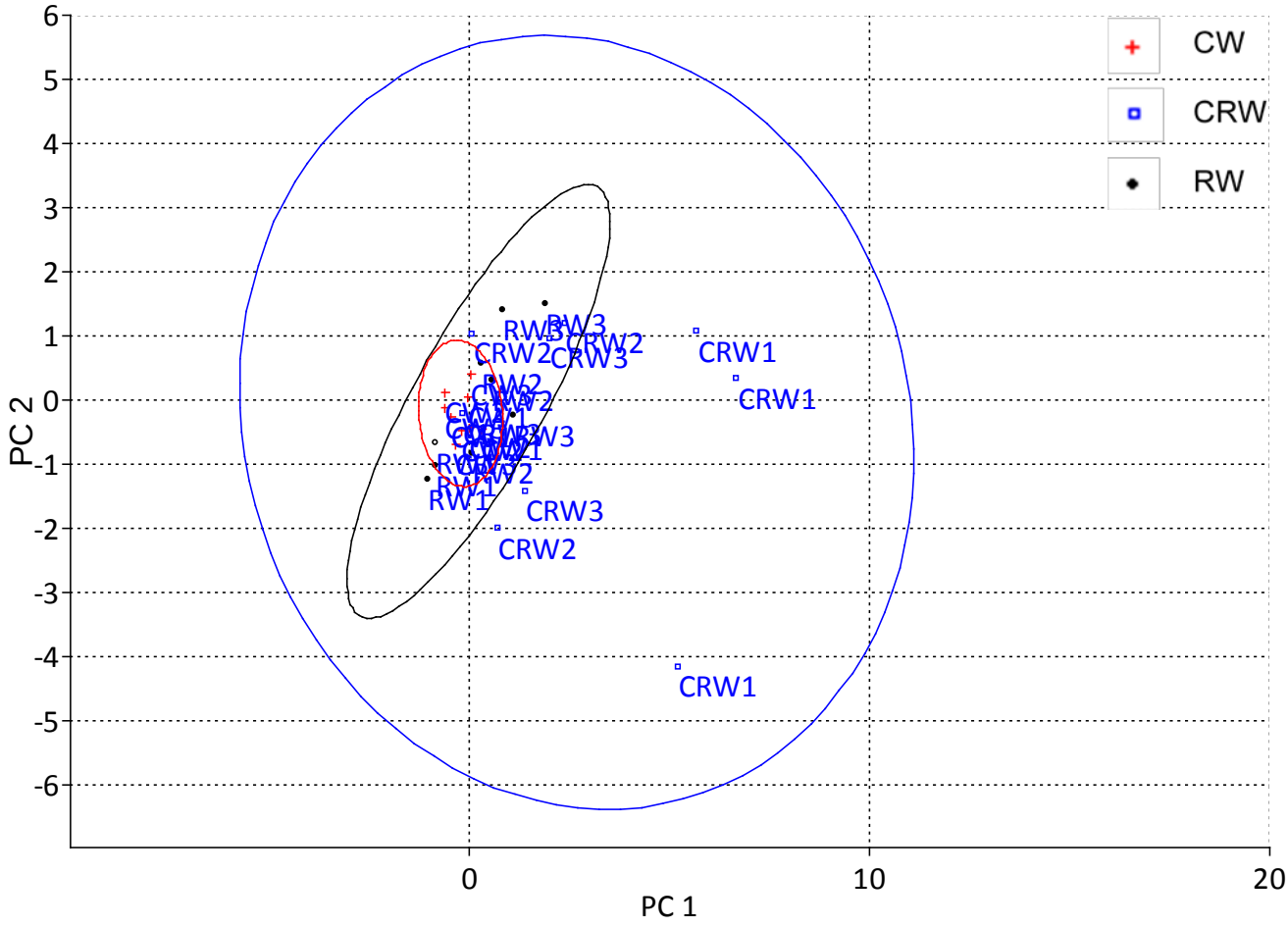


Figure 4.Ordination produced from PCA of transformed colour response data for each wetland replicate with 95% ellipses. Output generated using Past 1.28 statistical software

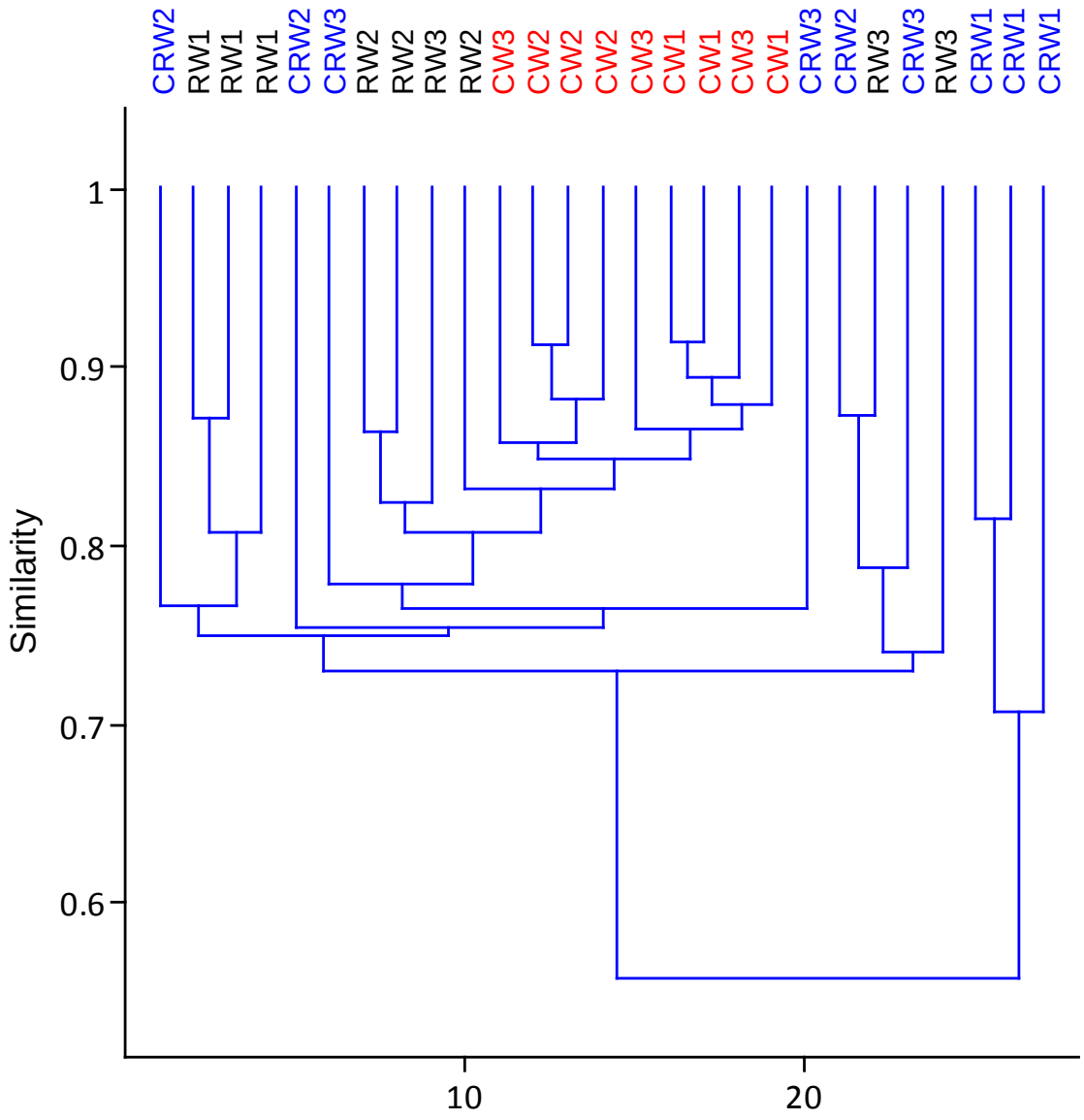


Figure 5.Cluster Diagram from Biolog transformed colour response data for each study wetland. Constructed Wetland (CW), Contaminated Remnant Wetland (CRW), and Remnant Wetland (RW).

Table 2. Correlation of carbon source variables to PC's for analysis of all wetland samples

PC1		
Carbon Source	**R**	**R^2**
Carbohydrate • **B-Methyl-D-Glucoside**	**-0.72**	**-0.54**
Carboxylic Acid • **D-Galacturonic acid** • **Itaconic Acid**	**0.83** **0.76**	**0.7** **0.58**
Amino Acid • **L-Asparagine**	**0.9**	**0.82**
Polymer • **Glycogen**	**-0.74**	**-0.54**
PC2		
Carbon Source	**R**	**R^2**
Carbohydrate • **D -cellobiose**	**0.86**	**0.75**

r, Pearson correlation coefficient

r^2, Regression coefficient

3.3.2 Microbial Community Structure (DGGE)

Gel Compare computer software was used to generate a cluster diagram of the 16S rRNA gene profiles obtained from DGGE analysis. The DGGE patterns showed relatively consistent but not identical banding patterns among the 3 subsamples (i.e., CRW1, CRW2, CRW3) taken across a west to east transect for each urban wetland type (Figure 6a). The cluster diagram indicates that overall, all sites share 50% resemblance. The number of bands per lane varied from 5 to 12 with 3 common bands among each sample (Figure 6a). There were 3 additional bands that were only common among CRW and RW sites for a total of 6 common bands between these sites (Figure 6a).

Despite the relative consistency in banding patterns within site subsamples, the CRW community fingerprint was different enough to be grouped separately from the CW and RW sites (Figure 6b). Subsamples CRW1 and CRW3 clustered together with 90% similarity and CRW2 produced almost twice as many bands (12) and formed its own branch (Figure 6b). The community fingerprints obtained for the RW and CW could not be distinguished from each other and they clustered together as two separate sub clusters with 60% resemblance (Figure 6b). The fingerprint obtained for the CW2 subsample was omitted from analysis since only two very faint bands were produced.

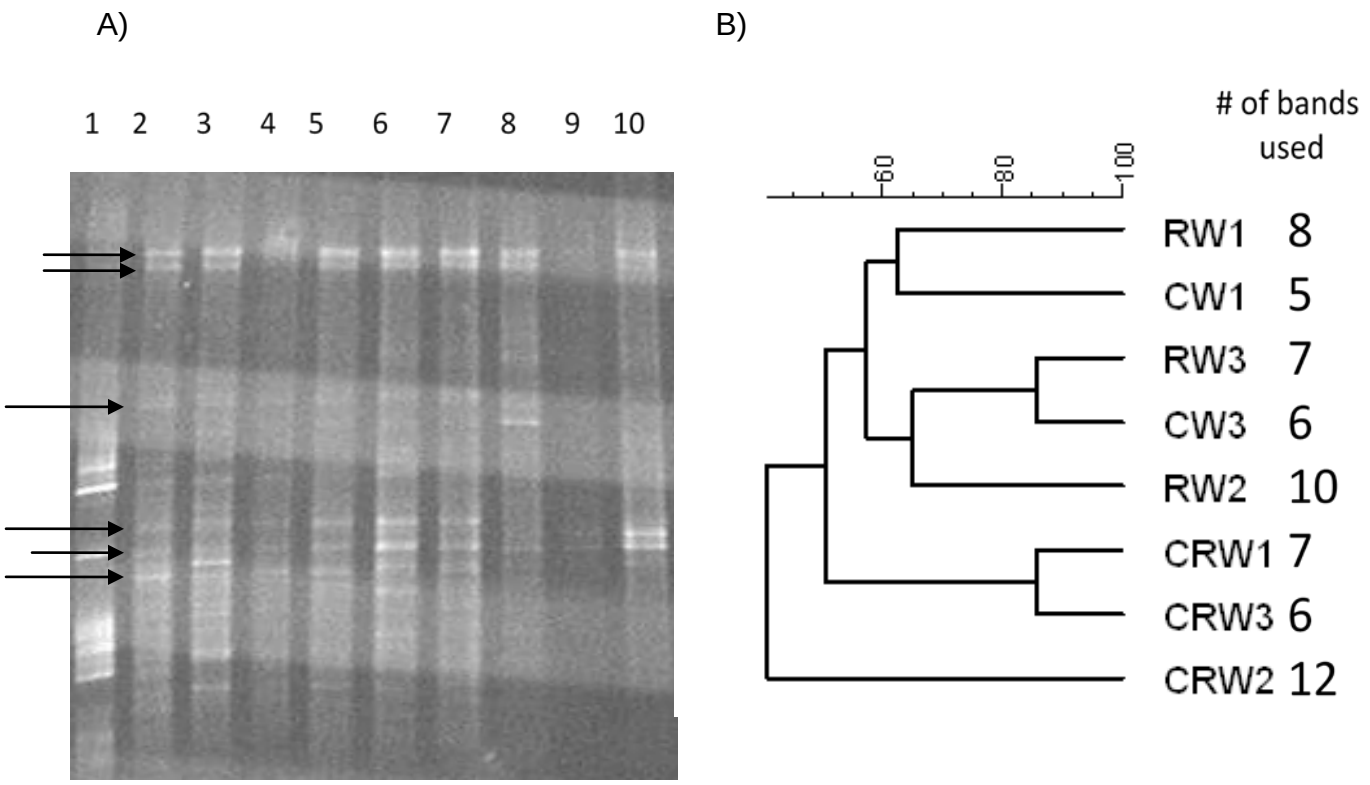


Figure 6. A) DGGE gel displaying banding patterns of 16S rRNA gene sequences obtained from Total DNA extracted from each wetland study site. Lane 1: Ladder 2: CRW1 3:CRW2 4:CRW3 5:RW1 6:RW2 7:RW3 8:CW1 9:CW2 10:CW3 B) Cluster analysis of 16S rRNA fingerprint obtained from total DNA extracted from each study wetland. Small arrow → indicates bands found in all sites. Large arrow ⟶ indicates bands found only in CRW and CW sites.

3.4 Discussion

Biolog Ecoplates were used to compare physiological profiles of heterotrophic microbial communities from sediments of different urban wetland types. Physiological profiles were based on utilization patterns of ecologically relevant and structurally diverse carbon sources. The three urban wetland types investigated in this study could not be distinctly separated based on physiological profiles. The heterotrophic communities at these sites had similar metabolic capacities as seen by the data overlap in the PCA analysis and through similar groupings observed in cluster analysis (Figure 4 and 5).

By taking subsamples across transects in each wetland we examined whether physiological profiles within individual sites varied along a spatial gradient. The microbial community metabolic profiles of the CW and RW wetlands appear to be homogeneously distributed within these wetlands due to grouping of data points from different subsamples. However, the CRW microbial communities appear to have a more heterogeneously distributed metabolic profile. The subsample (CRW1) taken at the western most part of the CRW grouped separately from every other sediment sample (Figure 5). The CRW1 sediment subsamples were drier (88% moisture) and not completely saturated like samples taken from the central and eastern part of the site (223% and 323% moisture).

This is likely due to a small downhill gradient that allowed more water to accumulate in these areas. Differences in moisture content may have selected for microbial communities with a different phenotypic potential. This higher within-site heterogeneity for the CRW compared to RW and CW may also be explained in part by

differences in hydrological regime, where CRW does not experience the same amount of flooding and flush-out as the other sites due to its distance from roadways and direct tributary feeds. The CW is more frequently and thoroughly flooded after rainfall events because these wetlands are designed to capture and drain residential road runoff. The RW also experiences flooding due to its close proximity to a roadway and tributary inflow. Flooding can result in a homogenous distribution of microbial populations as water bridges are created that link microbial populations within different sediment microhabitats that are normally spatially isolated when soils are dry (Zhou *et al*., 2002). Other studies have successfully used Biolog to track spatial changes in physiological profiles within individual sites. Garland and Mills (1991) were able to distinguish among subsamples taken along a coastal lagoon transect where open water samples grouped separately from inland marsh samples. Griffiths *et al*., (2003) showed that metabolic profiles changed with increased depth in a grassland soils.

The microbial communities of the CRW1 subsample utilized specific carboxylic acids and amino acids to a greater extent, and specific carbohydrates and polymers to a lesser extent than the microbial communities from any other sample. These differences reflect variations in the number of microorganisms that are able to utilize a particular substrate and become stained with tetrazolium violet (Graland and Mill, 1991). Therefore at this particular sample location there was a greater number of microorganisms able to utilize specific carboxylic acids (D-Galacruronic Acid, Itaconic Acid) and amino acids (L-Asparigine) within the cell, and fewer microorganisms able to utilize specific carbohydrates (B-Methyl-D-Glucoside) and polymers (Glycogen). Plant roots are known to exude various amino acids, carboxylic acids, carbohydrates and polymers and

therefore differences in plant density at this sample location may have affected the availability of specific substrates to that particular group of microorganisms thereby altering dominance of specific members within the microbial community (Grayston *et al*., 1998).

It is important to mention that technical and methodological limitations associated with Biolog could have influenced these results and are well documented in the literature (Garland, 1997, Konopka *et al*., 1998, Preston-Mafham *et al*., 2002, Stefanowicz, 2006). A limitation of particular importance is differences in initial inoculum density which may affect overall well colour development even when the same species are present. In this study we normalized for differences in inoculum density by dividing the response of each well by the overall AWCD (Garland and Mills, 1991, Garland, 1997, Weber *et al*., 2008). However I was mainly interested in comparing the response of microbial communities of different habitat types and not interested in monitoring dynamics in composition over time. Therefore, normalizing the effects of inoculum density was really not necessary (Garland, 1997).

It is interesting that in DGGE analysis, the CRW1 subsample clustered with the CRW3 sample at 90% similarity, yet these sites had different phenotypic potential based on Biolog analysis. These samples were also the farthest away from each other along the sampling transect. Although DGGE identifies dominant populations in a microbial community it provides no functional information about these microbes. It is possible that the 6 bands common in all CRW subsamples may not represent the culturable fraction of the microbial community that is assessed in Biolog analysis. It is also recognized that Biolog results can be strongly influenced by rare members of a microbial community that

are present at too low a density in the environment to be detected by DGGE, but that after sufficient lag time produce enough density to be detected (Hadwin *et al*., 2006). These findings illustrate the importance of combining both functional and phylogenetic diversity analysis to get a better picture of the structure and function of microbial communities.

The groupings of CRW subsample in the cluster analysis of both physiological and phylogenetic profiles seem to support each other in distinguishing the CRW from the other wetland types. Considering how all three urban wetland types have similar physicochemical properties, the main factors distinguishing the CRW from the other wetland types are history of previous contaminant exposure and differences in runoff inputs. The DGGE clustering could not distinguish between the CW and RW sites. These two sites had 3 common bands with the RW sample producing between 7-10 bands and the CW sample producing 5-6 bands. The relative similarities in banding patterns between samples resulted in the CW and RW clustering together as two separate sub clusters. These sites could also not be distinguished based on physiological profiles suggesting that the microbial communities at these sites were similar and homogeneously distributed. The fact that the RW and CW could not be distinguished based on either physiological or phylogenetic analysis is interesting considering the differences between these two wetland types (e.g., the CW is manmade and designed to receive and drain excess storm water runoff).

It is possible that urbanization of wetlands has the effect of homogenizing microbial communities. The CW and RW are more likely affected by road runoff than the CRW which is not directly adjacent to any roadway and the least subject to urban runoff

which this may also help to explain the differences obtained in both genetic and physiological analysis at this site. The construction of roadways is known to alter the hydrology of urban wetlands resulting in excess inputs of road runoff containing excess pollutants and this may be the driving factors behind homogenization by urbanization (Boyer and Polasky, 2004, Ehrenfeld, 2000). However there are no studies in literature comparing microbial community distribution in urban and natural wetlands. Although the microbial communities at the CW and RW sites were similar, they are not identical. Small variation in microbial diversity can be influenced by a myriad of factors including sediment composition, plant communities, hydrology, seasonality, depth, nutrients, organic matter content and prior land use including exposure to pollutants (Bosio *et al*., 2006, Ahn and Peralta, 2009, Nicomrat *et al*., 2006, Torsvik and Ovreas, 2002).

The highest number of distinct DGGE bands observed in any of the wetland samples was 12, suggesting an overall low diversity at all urban wetland sites. A possible reason for this may be that all three sites were mainly composed of sandy sediments which have been associated with a low microbial diversity compared to the silt and clay fractions normally associated with higher diversity (Sessitch *et al*., 2001). The presence of pollutants is another factor normally associated with reduced microbial diversity (Hadwin *et al*. 2006, Kim *et al*., 2009, Nicromat *et al*. 2006, Ravit *et al*., 2003, Trevors, 1998). For example, Nicomrat *et al*. (2006) only obtained 7 bands form DGGE of 16S rRNA obtained from microbial communities in wetland sediments receiving acid mine drainage, whereas other studies using DGGE to characterize wetland microbial communities have obtained up to 32 bands for non impacted wetlands (Hadwin *et al*., 2006). The wetland sites investigated in this study receive substantial inputs of urban

runoff that could potentially contain a variety of contaminants, in particular the CRW which has been historically contaminated with organic and heavy metal pollutants and may explain the low genetic diversity that was obtained.

Other reasons for the observed low diversity include not having adequate amounts of bacterial template DNA for PCR amplification of less numerous members of the microbial community. It is also possible for DNA from dominant species to create intense bands that mask DNA from closely related but less numerous microbial community members (Muyzer *et al*., 1993). Here DGGE was performed using a bacterial primer set that amplified a relatively short 550 bp fragment that may have caused closely related species to co-migrate as a single band (Muyzer and Smalla, 1998). Electrophoretic mobility (i.e., melting behaviour) is mainly determined by amplicon GC base-pair content which may be similar even when DNA sequences vary (Nocker *et al*., 2007). It is also possible for the 16S rRNA gene to occur in multiple copies in bacteria, as a single species can have different operons coding for 16S rDNA (Muyzer and Smalla, 1998). Furthermore only dominant populations that represent at least 1% of the total community can be detected by DGGE (Muyzer and Smalla, 1998) and it could be that diversity is underestimated and more bacterial species are present but at less than 1% of the total community.

In summary both physiological and phylogenetic profiles revealed that the CW and RW had homogenously distributed microbial communities compared to the CRW site. Urbanization may be a significant factor influencing the homogenization of microbial communities and appears to be driven by excess road runoff containing a variety of contaminants (e.g., heavy metals, organic pollutants, road salt). The groupings

of CRW samples in the cluster analysis of both physiological and phylogenetic profiles seem to support each other in distinguishing the CRW from the other wetland types and this likely reflects its history of contaminant exposure. Overall the genetic diversity at each site was low and may be explained by a combination of factors including the presence of coarse sandy sediments and exposure to contaminants in runoff which has the effect of reducing overall diversity and selecting for few species adapted to pollutant stress.

Chapter 4. Microbial Community Biodegradation and Tolerance of Pollutants in Urban Wetlands

4.1 Introduction

Urban wetlands receive non point source runoff from a variety of residential, agricultural and industrial sources. Even in cases were runoff enters upstream rivers, streams or infiltrates groundwater, urban wetlands are hydrologically connected and will eventually intercept these sources of water and potentially mitigate the contaminants associated with urbanization (Groffman *et al*., 2003). It is well known that natural and constructed wetlands have intrinsic physical, chemical and biological retention and degradative processes useful for treating organic chemical compounds (Imfeld *et al*., 2009, Faulwetter *et al*., 2009). However knowledge of the functioning of urban wetlands with respect to the removal of organic contaminants in runoff is currently limited (Kohler *et al*., 2004, Maillard *et al*., 2011, Thurston, 1999), and most studies have limited their investigation to constructed storm water wetlands and have ignored natural remnant wetlands which are also found in urban environments.

The pollutant attenuation function of urban wetland systems could have significant importance in terms of reducing potential toxicity of organic contaminants to animals and humans. Especially after rainfall events, pesticides can enter aquatic environments at concentrations potentially toxic to aquatic organisms (Stanley *et al*., 2009). In terms of human health, some pesticides have been identified as carcinogenic (e.g., Alachlor, Heptachlor) while others such as 2,4-dichlorophenoxy acetic acid (2,4-D) may cause specific organ damage to the liver and kidney (Garabrant and Philbert, 2002).

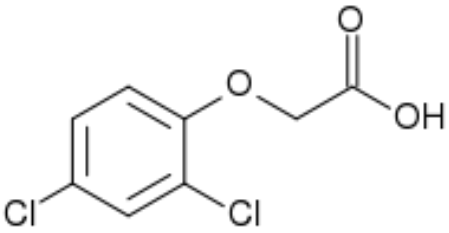
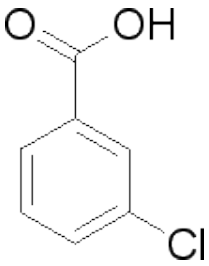
In this study we used 2,4-D and 3-chlorobenzoic acid (3-CBA) as model chlorinated aromatic compounds to assess the degradation potential in different urban wetland types. The structure and chemical properties of these compounds are presented in Table 3. Worldwide, 2,4-D is a commonly used herbicide and although in Ontario a provincial herbicide and pesticide ban took effect in April 2009, class 9 pesticides (which includes 2,4-D) are still permitted for commercial use on golf courses or for residential use to protect families from noxious weeds such as poison ivy (Ontario MOE, 2011). 2,4-D is one of the most mobile pesticides and is readily mobilized by rainfall events where it either runs off directly into adjacent surface waters or leaches through soils in to groundwater systems (Nesbitt and Watson, 1980). 3-CBA is a bacterial transformation product of chlorinated organic compounds such as PCBs and is commonly found in landfill leachate (Wyndham and Straus, 1998).

The removal of toxic organic compounds in wetland systems can be evaluated by monitoring the disappearance of soluble phases of the parent chemical in the water column (Reddy and D'Angelo, 1999). These types of experiments simulate runoff pollution and account for removal by multiple pathways including microbial transformations, sorption, volatilization and photodegradation. Microbial degradation activity can be specifically evaluated by mineralization assays of C^{14} radiolabeled substrates and quantification of evolved $^{14}CO_2$. Both types of experiments were conducted in the present study using sediments collected from three different urban wetland types.

In cases where toxic organics are overloaded to wetlands, microbial activity and degradation may be greatly curtailed. Therefore toxicity tests on microbial activity can

provide information about whether or not representative organic and metal contaminants have the potential to adversely affect the ability of microbes to biodegrade organic pollutants. The objective of this study was to assess the biodegradation potential of three different urban wetland communities using 2,4-D and 3-CBA as model chlorinated organic pollutants. Microbial enzymatic inhibition assays were also conducted to assess the potential toxicity of organic and metal pollutants on microbial activity.

Table 3. Structural and select chemical properties of 2,4-D and 3-CBA

	2,4-Dichlorophenoxyacetic Acid	3-Chlorobenzoic acid
Chemical structure		
Molecular Weight (g/mol)	221.04[a]	156.57 [e]
Solubility (mg/L) (25 °C)	900 [b]	1000 [e]
Partitioning Coefficient (PC)	32 [c]	2.71 [e]
Octanol/Water Partitioning Coefficient (Kow)	2.81 [d]	N/A
Vapor Pressure (mmHg)	$1.4x10^{-7}$ [a]	$1.7x10^{-3}$ [e]

A- Data retrieved from Walters, 1999

B- Data retrieved from Gaultier *et al*., 2009

C- Data retrieved from Tansel *et al*., 1998

D- Data retrieved from Jemba *et al*., 2006

E- Data retrieved from 3-CBA MSDS from www.ChemicalBook.com

4.2 Materials and Methods

4.2 1 Wetland Sediment Samples

Sediments used for UPLC biodegradation experiments of 2,4-D and 3-CBA were collected November 19th/2010 and as described in materials and methods section (2.2.4). 2,4-D experiments were conducted three days after sample collection on November 21/2011 and 3-CBA experiments were conducted January 3rd/2011. Sediments used for the ^{14}C radiolabeled 2,4-D and 3-CBA mineralization experiments were collected April 12/2011 and they were performed on April 14/2011.

4.2.2 2,4-D and 3-CBA Sediment/Slurry Biodegradation Experiments

Experiments were preformed in sterile 250 mL Erlenmeyer flasks with cotton ball stoppers wrapped in cheesecloth to allow gas exchange. Sediment moisture content was normalized by centrifuging samples at 4000 rpm for 5 min and decanting the water layer. Each treatment consisted of 60 g sediment and 40 mL sterile water for a 3:2 sediment water slurry ratio with a total volume of 100 mL. To ensure degradation was a result of microbial activity abiotic controls using autoclaved sediments were prepared. Sterile water controls were also prepared to assess potential removal via abiotc mechanisms such as volatilization and photodegradation. All treatments were performed in triplicate. Treatments were allowed to acclimatize for 48 hours before pollutant addition. After acclimatization, 1 mL of a 10 000 ppm 2,4-D or 3-CBA stock solution dissolved in water was added to each 100 mL treatment for a final concentration of 100 ppm. An aliquot (2 mL) was taken for quantification of 2,4-D or 3-CBA at time 0 and every 14 hours for two weeks. The sample was centrifuged at 10,000 rpm for 5 min and filtered (0.2 µm) to

remove particles and was quantified by ultra performance liquid chromatography (UPLC). Sediment pore water collected form original samples were also measured for background concentrations of 2,4-D.

4.2.3 UPLC of 2,4-D and 3-CBA

Samples were analyzed for 2,4-D and 3-CBA by reverse phase chromatography on a Waters Acquity ultra-performance liquid chromatography (UPLC) system quipped with a Waters Acquity UPLC BEH C18 Column (2.1 x 100 mm, 1.7 µm). The mobile phase was a 35:65 mixture of Acetonitrile: Phosphate buffer (pH 3) with a flow rate of 0.4 mL min^{-1}. 2,4-D and 3-CBA were detected using Waters photodiode array (PDA) at 224 nm and were identified based on retention times. Concentrations of 2,4-D and 3-CBA in each treatment were determined by interpolation from a 5 point standard curve constructed with 2,4-D and 3-CBA at concentrations between 10 ppm and 150 ppm dissolved in water.

4.2.4 ^{14}C Radiolabeled 2,4-D and 3-CBA Mineralization Experiments

Sediment subsamples from each site were mixed and 2 g (wet weight) sediment samples were added to sterile scintillation vials. All treatments were prepared in triplicate. Each 2 g treatment received 350 ul of ring labelled ^{14}C-2,4-Diclorophenoxyacetic acid (0.96 Bq ul^{-1}) or ^{14}C-3-Chlorobenzoic acid (1.28 Bq ul^{-1}) from 100 ppm stock solutions for a final concentration 17.5 ppm and a total activity of 338 Bq (2,4-D) or 452 Bq (3-CBA). To ensure mineralization was a result of microbial activity abiotic controls were prepared by autoclaving sediments (20 min at 121 ^{o}C).

To verify the integrity of the stock solution we included sterile water controls, positive controls consisting of known 2,4-D and 3-CBA degraders (*Alcaligen eutrophus* JMP 134 and *Burkholderia phytofirmans* R 172 respectively) and treatments consisting of a bacterial strain not expected to degrade either contaminant (E.coli K12). Each treatment consisted of 2 mL minimal mineral medium and 200 ul of either, *E.coli* K12 at 1.3 x 10^8 cells/mL, JMP 134 at 1.4 x 10^9 cell mL^{-1} J and R172 at 5.06 x 10^8 cell mL^{-1}. Bacterial numbers were determined by plate counts. Cultures of JMP 134, R 172 and *E.coli* K12 were grown for 48 hours at 30 ^oC on R2A agar plates and prepared with a minimal mineral medium. Suspensions of JMP 134, R 172 and *E.coli* were prepared in 2 mL minimal mineral medium and cell numbers quantified by dilution plating on R2A.

To trap ^{14}C-carbon dioxide, a small 1000 ul recovery vial containing 100 ul of 1M sodium hydroxide (NaOH) was placed inside each sealed treatment vial and incubated in the dark at 21 ^oC. NaOH traps were sampled weekly and replaced with clean NaOH. The NaOH was mixed with 10 mL Ecolume^{TM} liquid scintillation fluid and counted for 1 min in a Perkin Elmer TriCarb 2900 TR scintillation counter.

4.2.5 FDA Hydrolysis Acute EC_{50} Toxicity Tests

The FDA method has been determined to be a suitable test for assessing toxicity of organic and inorganic chemicals to microbial activity in both soil and activated sludge samples (Haigh and Rennie, 1994, Leszczynska M and Oleszkiewic, 1996). Details of the experimental protocol are described in chapter 2 section (4.2.5) Effects of organic and inorganic chemicals of FDA hydrolysis (i.e microbial activity) was determined by dissolving either 2,4-D or copper sulfate ($CuSO_4$) at a log series of concentrations

suitable for toxicity tests and adding these to 1 g sediment in a total volume of 7.5 mL water. $CuSO_4$ was chosen as a model metal compound since copper is an important metal commonly found in urban runoff (Perryman, 2011). Sediment blanks without FDA addition were prepared for background correction and sterilized controls were also prepared. The inhibitory effects of the test chemicals on FDA hydrolysis were calculated as follows:

% inhibition = 100 x (1- [μT1/ μT2])

Where: μT1 = rate of hydrolysis in the sample with chemical

μT2 = rate of hydrolysis in control samples

EC_{50} values were calculated by fitting % inhibition data to a four parameter logistic curve and extrapolating EC_{50} values.

4.2.6 Statistical Analysis

Data was first tested for normal distribution by the Shapiro-Wilk's test and for equal variance using the Brown and Forsythe's test for homogeneity of variances ($p \leq 0.05$). Statistically significant difference among all treatment means were analyzed by one way repeated measure analysis of variance. If there was significant differences ($p \leq 0.05$) pairwise comparison of means was performed using the Holm-Sidak test. All Data was analyzed with SigmaPlot 11.0 software package.

4.3 Results

4.3.1 2,4-D and 3-CBA Biodegradation from the Water Phase of a Sediment-Water Slurry

After 14 days incubation at room temperature no degradation of 2,4-D was detected from the water phase of a sediment-water slurry in any experimental or autoclaved abiotic control treatments (Figure 7). There was no significant difference between the values obtained for both experimental and control treatments ($p \leq 0.05$) for any of the wetlands. The observed variation is likely due to differences in instrument calibration each time samples were measured (Figure 7). The high sample volume did not allow for all measurements to be performed on the same day. No background 2,4-D was detected in the pore water of any wetland site.

No results were obtained for the 3-CBA biodegradation experiments as 3-CBA could not be detected in the water phase of any experimental sample. An increasingly broad peak was detected in the 3-CBA standard samples prepared in water at an average retention time of 1.8 min and 2,4-D had an average retention time of 2.2 min.

4.3.2 2,4-D and 3-CBA Mineralization

Each urban wetland type displayed varying capacities to mineralize either C^{14}-radiolabeled 2,4-D or C^{14}-radiolabeled 3-CBA. Overall mineralization of 2,4-D for all sites after 21 days was low (<32%) (Figure 8). The RW had the greatest capacity to mineralize 2,4-D at 32% with an observed lag phase of 14 days required before any substantial mineralization was detected. The CW and CRW had lower but similar 2,4-D mineralization capacities at 7% and 9% respectively, with a 14 day lag phase required for

the CRW samples. Overall mineralization of 3-CBA for all sites after 21 days was low (<17%) (Figure 9). In contrast to the 2,4-D experiments, the RW did not have much capacity to mineralize 3-CBA at only 2%. However both the CW and CRW had similar 3-CBA mineralization capacities at 17% and 14% respectively, with a lag phase of 14 days required for the CRW samples.

The data presented in this section were subtracted from the autoclaved sterilized controls. Positive controls using known 2,4-D and 3-CBA degraders (Alcaligen eutrophus JMP 134 and Burkholderia phytofirmans R 172 respectively) readily mineralized the substrates with 91% and 67% conversions respectively (Appendix A). *E.coli* K12 was not expected to degrade either contaminant and was used as a negative control. *E.coli K12* treatments did however result in mineralization so these treatments were investigated to determine whether they were possibly contaminated with other bacterial species. Aliquots of these treatments were plated on R2A which revealed growth of more than just *E.coli* k12 due to the presence of different colony forming species, suggesting that contamination impacted the results.

4.3.3 Microbial Community Tolerance of 2,4-D and $CuSO_4$

The potential toxic inhibitory effects of organic and inorganic chemicals on FDA hydrolysis (i.e., microbial activity) was determined using 2,4-D and $CuSO_4$ as model urban runoff compounds. 2,4-D did not inhibit enzymatic FDA hydrolysis when sediments were treated with concentrations up to 200 ppm. Additions of $CuSO_4$ up to 2000 ppm were required to inhibit FDA hydrolysis with each site showing a varying degree of tolerance (Table 4). The CRW had the highest tolerance with only 33%

inhibition followed by the RW at 77% inhibition and the CW which was most sensitive at 93% inhibition.

Table 4. Acute toxicity tests on microbial activity using $CuSO_4$ to assess effects of metal pollution on enzymatic hydrolysis of FDA. CW: Constructed Wetland CRW: Contaminated Remnant Wetland RW: Remnant Wetland

Sites	EC_{50}	% Inhibition at Highest $[CuSO_4]$ 2000 ppm
CW	480 ppm	93%
CRW	>2000 ppm	36%
RW	1163 ppm	77%

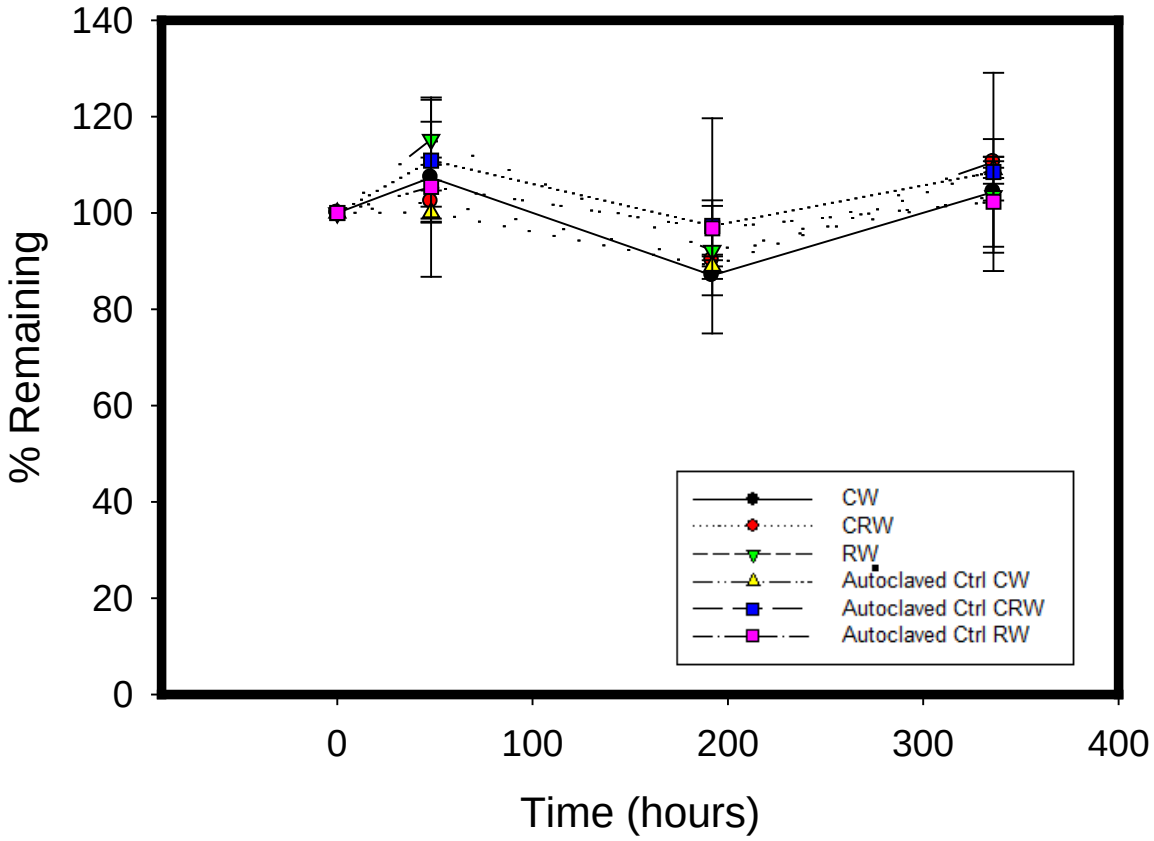


Figure 7. Mean 2,4-D removal from the water phase of a sediment/water slurry over 14 days +/- standard error (SE), repeated measures ANOVA, Holm-Sidak, $p<0.05$

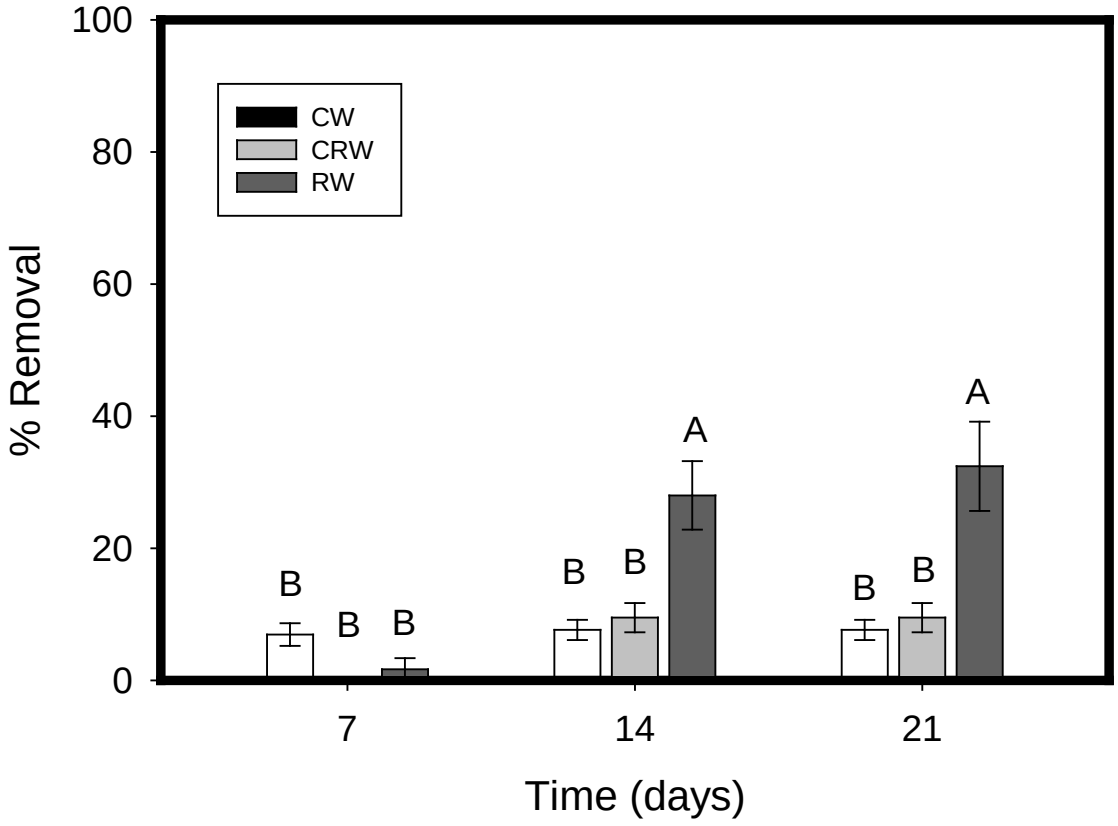


Figure 8. Mineralization of C^{14}-labeled 2,4-D over 21 days of incubation +/- standard error (SE), repeated measure ANOVA, Holm-Sidak, $p<0.05$, A significantly different from B

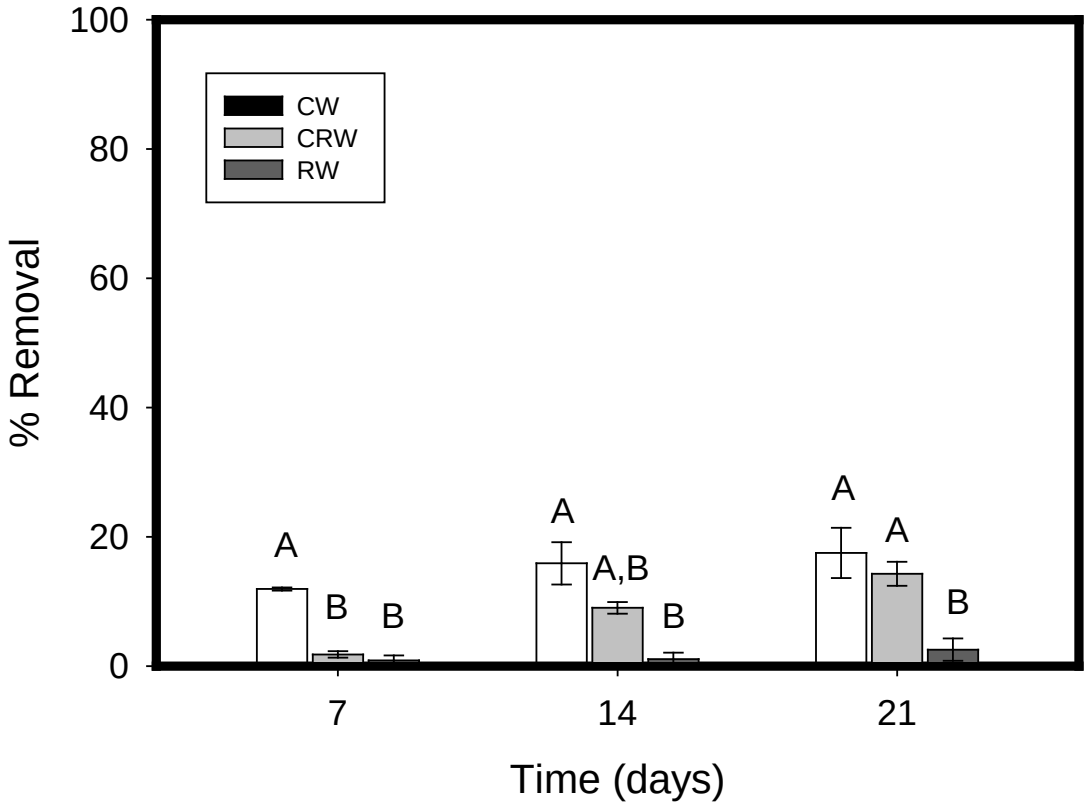


Figure 9. Mineralization of C^{14}-labeled 3-CBA over 21 days incubation +/- standard error (SE), repeated measure ANOVA, Holm-Sidak, $p<0.05$, A significantly different from B

4.4 Discussion

Two laboratory biodegradation experiments using UPLC and C^{14} mineralization assays were conducted to assess the functional capacity of urban wetlands to naturally attenuate common chlorinated organic contaminants. When monitoring removal of 2,4-D in the water column of a sediment/water slurry, no 2,4-D was removed after 14 days incubation. Some sorption of 2,4-D to sediments may have occurred but would not have been detected since % removal was based on measurements taken after 2,4-D additions at time zero and sorption would have likely occurred by this time (Gaultier *et al.*, 2009).

2,4-D has a pKa of 2.8 and the pH of the treatments ranged from 7 – 7.2, therefore 2,4-D would have primarily been in its dissociated anionic form and only very moderate sorption is expected to occur due to repulsion from the negative charges of the sediment (Walters, 1999). Generally, 2,4-D sorption is negatively correlated with pH and positively correlated with organic matter content (Gaultier *et al*., 2009).

The lack of degradation observed in these experiments may be explained by several reasons. First, the sediment/water slurries were completely saturated and oxygen content in these treatments was likely low, thereby limiting the aerobic biodegradation of 2,4-D. Increased oxygen supply has been shown to increase rates of 2,4-D biodegradation (Sinton *et al*., 1986). In contrast anaerobic conditions decrease rates of biodegradation. Boyle *et al*. (1999) studied the anaerobic degradation of 2,4-D in different marine and pond sediments and found that only sediments with a history of contaminant exposure resulted in 2,4-D degradation within 80 days and other non contaminated sites did not exhibit any 2,4-D loss even after 160 days.

Secondly, the sediment microbial communities may have had access to easily degradable forms of organic matter present in the sediments and simply did not require the use of 2,4-D as a carbon substrate for growth. Incubation periods longer than 14 days may have resulted in detectable losses of 2,4-D as more easily degradable forms of carbon become depleted. Also, if initial biomass is low, a long lag phase may be required for 2,4-D degrading organisms to sufficiently increase to a level where significant degradation is possible (Sinton *et al*., 1986). Voos and Grauffman (1997) using HPLC detected a 20% loss of 2,4-D in freshwater wetland sediments after 10 days incubation

which increased to 92.4% after 20 days. This supports the idea that a longer lag phase may have been required before degradation could be detected.

The lack of 3-CBA detection in treatment samples may be explained by interference from other molecules co-extracted in the water phase. Additional purification using solid phase microextraction (SPME) could have been used to separate 3-CBA from background compounds and may have made detection possible. The lack of signal may also be attributed to sediment effects such as sorption. However 3-CBA has a very low partitioning coefficient (2.71) and is not expected to sorb significantly to sediments.

Although UPLC biodegradation experiments are useful for detecting transformations of the original parent compound, C^{14}-labeled pollutant mineralization assays detect the complete conversion of substrate to CO_2 by microbial communities (Johannesen and Aamand 2003, Kristensen *et al*., 2001). In this present study C^{14} mineralization assays were conducted to verify if similar results would be obtained as the UPLC experiments. Unlike the UPLC experiments some mineralization of C^{14}-labeled 2,4-D and 3-CBA was detected in the treatment samples although overall, mineralization rates were low. These results at least confirm that the microbial communities of these wetlands possess the capacity for 2,4-D and 3-CBA degradation. 2,4-D and 3-CBA degraders are widespread however they have not been isolated from every ecosystem (Fulthorpe *et al*., 1996, Fulthorpe *et al*., 1998).

The low mineralization values obtained are similar to those obtained by Entry (1999) who assessed 2,4-D mineralization in forested wetland sediments and obtained only 3.6% 2,4-D mineralization after 5 weeks of incubation which increased to 30.7%

after 15 weeks. Low 2,4-D mineralization (<40%) has also been reported for surface sediments from a chalk aquifer (Kristensen *et al*., 2001). These authors suggested that mineralization was limited due to availability of other utilizable carbon and electron donors and also to a lesser extent sorption to sediments. Other possible explanations include differences in bacterial communities with inherently different rates of 2,4-D mineralization or differences in the initial size of the 2,4-D degrading microbial populations (Lipthay *et al*., 2007). In this study a 14 day lag phase was observed for the CRW and RW sites before any substantial 2,4-D mineralization occurred. Lag phases of more than 14 days for 2,4-D by indigenous populations have been observed in both aquifer sediments and freshwater river sediments (Chinalia and Killham, 2006, Liphtay *et al*., 2007). Lag periods can reflect the time required for indigenous 2,4-D degrading populations to increase in numbers to a level where mineralization is detectable or reflect the time required for the genetic selection associated with exchange of mobile catabolic plasmids involved in 2,4-D degradation (Boyle *et al*., 1998). In contrast to saturated sediments, agricultural soils previously exposed to 2,4-D have shown to completely mineralize 2,4-D without a lag phase due to acclimation of microbial communities to repeat pesticide exposure (Cheah *et al*., 1998).

The lag phase observed in our studies suggests that the microbial communities in these urban wetlands are not acclimatized to 2,4-D or 3-CBA exposure. Since 2,4-D is highly mobile the observed lag phase may also suggest that urban wetlands may not play a significant role in removing 2,4-D before it gets flushed away by the next heavy rainfall event. However it is important to note that these experiments were conducted under laboratory conditions and may not reflect what would happen under natural conditions.

The urban wetland sites in this study are all dominated by cattails (*Typha spp*.) and the presence of wetlands plants would likely enhance pollutants removal due to rhizosphere effects including increased biomass and the creation of oxic/anoxic interfaces. My original experimental design included cattail microcosms but due to technical and time constraints these experiments had to be brought into the lab.

The 2,4-D and 3-CBA degradation pathways are well known and they produce a number of intermediates (Perkins *et al*., 1990). In our study we used ring labelled substrates, therefore if transformations of the parent compounds occurred, (such as cleavage of the acetate side chain which is the first step in 2,4-D degradation pathway), these intermediates would not have been detected because we measured complete conversion to $^{14}CO_2$. Although mineralization is low we do not know to what extent the parent compound was transformed and whether or not these metabolites resulted in any toxic inhibitory effects. Furthermore, it is not clear if degradation is a result of a single bacterial species or if many members in a population were involved in co-metabolism as both routes are possible (Fournier, 1980). The observation that the RW mineralized 2,4-D at 32% but appeared to lack the ability to substantially mineralize 3-CBA at 2% is curious, since both compounds can be degraded by the same *tdf* metabolic pathway (Perkins *et al*., 1990). These sediments may contain other organic pollutants delivered through runoff that may influence the co-metabolism of 2,4-D over that of 3-CBA.

Entry (1999) found that excess nitrogen deposition in wetlands reduced rates of both herbicide mineralization and active biomass concentrations. It is possible that co-contaminants present in the investigated urban wetlands are impairing the ability of microbial communities to mineralize other organic pollutants such as 2,4-D and 3-CBA.

Toxicity tests on microbial activity (e.g., FDA hydrolysis) were conducted to investigate whether certain organic or metal contaminants are impairing the ability of urban wetland microbial communities to transform organic pollutants. The assessment of toxic effects is also important as it may reflect impairment of other important microbial functional processes including turnover of organic matter and nutrients. If microbial activity is impeded by pesticides and metals, this may have important ramifications for the functioning of the wetland as a whole and the vital ecosystem services it provides to the watershed.

The enzymes involved in FDA hydrolysis represent a functionally redundant "broad scale" group of enzymes involved in the degradation of organic matter and include proteases, lipases and esterases (Chaer *et al*., 2009). The reason 2,4-D did not inhibit FDA hydrolysis is likely because it was present at too low of a concentration to inhibit these enzymes to any great extent. 2,4-dichlorophenol, a structurally similar compound to 2,4-D, has a reported IC_{50} FDA hydrolysis value of 204.6 ppm in activated sludge (Leszczynska and Oleszkiewic, 1996). However the authors did not report the exact amount of activated sludge used in their study which could have been less than the 1 g of sediment used in this study and hence resulting in higher toxicity.

$CuSO_4$ did inhibit microbial activity, but at very high concentrations (2000 ppm). Copper inhibits the action of enzymes by binding to sulfhydryl groups within the active site or by displacing enzymatic cofactors (Sandrin and Maier, 2003). Copper has been shown to inhibit the aerobic biodegradation of crude oil and other organic compounds (Sandrin and Maier, 2003). Inhibition is mediated by the ionic form of the metal and therefore high concentrations of $CuSO_4$ may have been required due to complexation of

Cu^{2+} ions with SO_4^{2-} and other natural ligands such as OH- and CO_3^{2-}, that once formed may not be able to bind and inhibit enzymes (Flemming and Trevors, 1989). Additionally, the enzymes involved in FDA hydrolysis are also exuded by cells and can be adsorbed onto clay minerals or humic colloids and potentially retain their catalytic capacity and persist in harsh environments (Burns, 1982). Extracellular enzymes are less sensitive to environmental conditions that could adversely affect the physiological state of microorganisms and could explain why high $CuSO_4$ concentrations were required before any inhibition was observed. This suggests that the nature of the sediment environment itself contributes to protecting microbes from toxicity associated with copper in runoff.

The CRW had the greatest tolerance to $CuSO_4$ concentrations, and is likely explained by the fact that this site has been historically contaminated with low levels of metals for decades including copper (Ginou, 2010). The microbial populations at this site have had many generations to develop chromosome, plasmid or transposon-encoded resistance mechanisms to deal with the presence of metals. In the case of copper, the main resistance mechanism involves the use of bacterial ATPase efflux pumps or intracellular sequestration in the cytoplasm in the form of black copper sulphate precipitate (Bruins *et al*., 2000). Copper additions to soil have been shown to enrich particular microbial populations (Lejon *et al*., 2010). Thus, difference in microbial $CuSO_4$ tolerance at each urban wetland site likely reflects differences in microbial community structure with unique resistance-adapted mechanisms, depending on history of previous contaminant exposure.

In summary, each urban wetland types had the capacity to biodegrade chlorinated organic pollutants although to a limited extent. The varying capacities between sites likely reflects differences in microbial communities with different composition and density of 2,4-D and 3-CBA degrading populations. The presence of 2,4-D did not inhibit microbial activity however additions of $CuSO_4$ did have an inhibitory effect. Microbial communities at the CRW with a history of metal exposure had the greatest tolerance to copper sulfate additions reflecting the presence of microbial communities with adapted metal tolerance mechanisms.

Chapter 5: General Conclusions

The objective of this research was to characterize and compare different types of urban wetlands and assess their capacity to attenuate model chlorinated organic pollutants, as well as tolerate common pollutants found in urban runoff. This was accomplished through investigation of the sediment properties and microbial community characteristics. Because microbial transformation is the main driver behind pollutant removal in wetland systems, aspects of the microbial communities in each urban wetland was investigated, including microbial biomass and activity, microbial diversity, ability to biodegrade contaminants and tolerate contaminant exposure. Three different urban wetland types with differing structure and contaminant history were investigated.

Each urban wetland type had the capacity to mineralize model chlorinated organic pollutants although only to a limited extent, confirming the presence of 2,4-D and 3-CBA degrading microorganisms. Differences in mineralization capacity reflect differences in the composition and density of specific 2,4-D and 3-CBA microbial degraders. Each wetland had different tolerances to metal exposure depending on the history of contaminant exposure and nature of sediment structure. Sites with a history of low level metal contamination and with finer textured sediments with high organic matter (i.e., CRW) had the highest tolerance to Cu exposure.

Analysis of the physiological and phylogenetic diversity of each wetland microbial community revealed remarkable similarities in community composition and spatial distribution between wetlands types. Only the CRW could be distinguished from the other sites based on differences in sole carbon source utilization and in 16S rRNA genetic

diversity. The strong similarities of the physicochemical conditions between wetland types suggests that the unique microbial community at the CRW is likely due to its history of receiving point source pollution, as well as differences in hydrological regime. The CW and RW also represent different wetland types such that the CW was artificially created to control velocity and peak flow of stormwater as well as filter out sediments and pollutants, while the RW is the remnant of a natural forested wetland that has been impacted by surrounding residential areas and an adjacent major roadway. Regardless of these differences, the microbial communities at these sites have very similar physiological and phylogentic profiles which may be a result of urban homogenization of these wetland communities. The effect of urban homogenization appears to be driven by inputs of road runoff containing a variety of excess pollutants (i.e., organic pollutants and heavy metals, road salt). Contaminant inputs also appear to reduce overall bacterial density and genetic diversity in urban wetlands compared to other natural wetland types.

Very few studies have investigated the capacity of urban wetlands to attenuate chlorinated organic contaminants or have looked at microbial community diversity and distribution in these systems. The findings of this research provide a better understanding of the structure and function of the microbial communities in urban wetland systems as well as their ability to biodegrade chlorinated organic compounds. Urbanization appears to reduce the microbial diversity and functional capacity of urban wetlands although this conclusion requires further investigation. This research focused primarily on chlorinated organic pollutants while urban wetlands have the potential to attenuate other forms of runoff pollution as it passes through urban wetlands, such as heavy metals, excess nutrients, dissolved organic matter, sediments, and pathogens. Therefore it is still likely

that these systems contribute to overall ecosystem health by improving urban water quality.

5.1 Future Research

The conclusions of this investigation of urban wetlands are only preliminary and more research is required to validate my findings. The concept of microbial community homogenization by urbanization could be investigated more thoroughly by looking at microbial structure and diversity in a greater number of different urban wetland types situated adjacent to roadways. The number of samples taken within sites should be increased and compared to natural undisturbed reference wetlands. Analysis of contaminants found in runoff after storm events should also be performed to reveal the type and level of pollutants entering these systems. A continual monitoring of microbial biomass in urban and natural reference wetlands is required over longer periods of time (i.e., seasonally) to validate the general observation that microbial biomass in urban wetland systems is low.

Outdoor biodegradation experiments including the presence of wetland plants should also be performed under natural fluctuating environmental conditions to take into account rhizosphere effects as well as abiotic processes such as photodegradation. Under natural conditions, these processes may produce breakdown products that are more readily catabolised by microbes and may reveal a greater functional capacity than that obtained under laboratory conditions. The effect of co-contaminants on biodegradation should also be investigated by performing biodegradation experiments using mixtures of contaminants. Cloning and sequencing of DGGE bands would reveal the actual species

that make up the microbial communities in urban wetlands. However to identify those members directly involved in the degradation of 2,4-D stable isotope probing (SIP) using ^{13}C labelled -2,4-D could be performed and labelled DNA sequenced to reveal the identity of those members.

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Appendix A

Mineralization of C^{14}-Labeled Organic Compounds by Controls and in Sterile Water

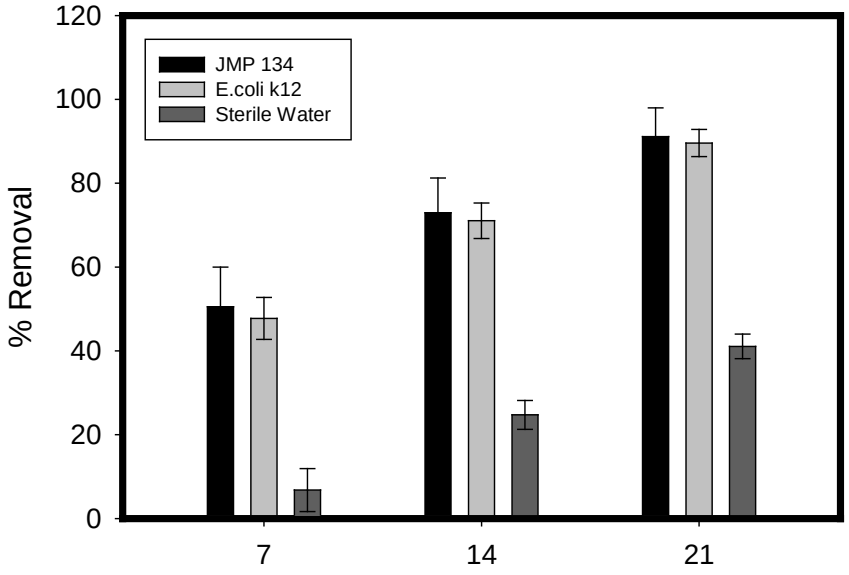


FIgure A 1. Mineralization of C^{14}-Labeled 2,4-D by Controls and in Sterile Water

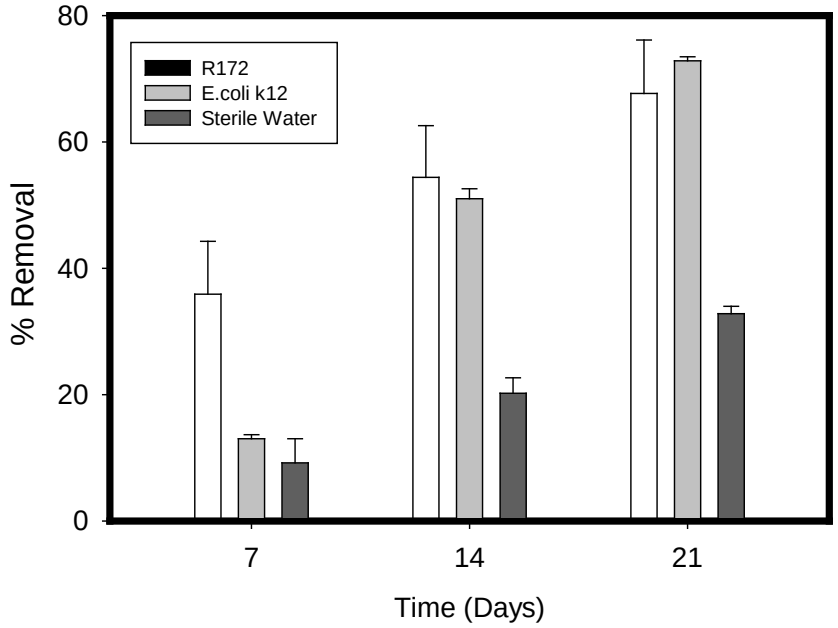


Figure A 2. Mineralization of C^{14}-Labeled 3-CBA by Positive Controls and in Sterile Water

Appendix B

Maps and Pictures of the Urban Wetlands Investigated in this Study

Figure B1. Constructed Wetland (CW) in Early Spring

Figure B2. Contaminated Remnant Wetland (CRW) in Early Spring

Figure B3. Remnant Wetland (RW) in Early Spring

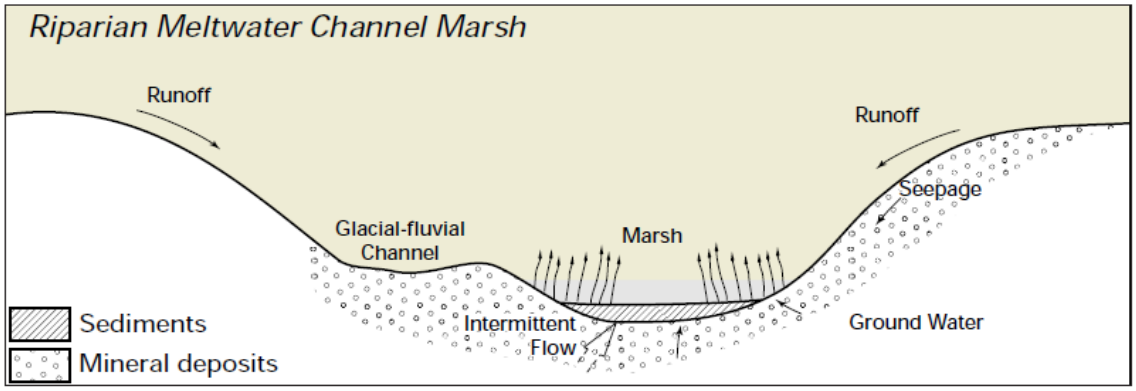
Appendix C

Description of Wetland Types Adapted from the Canadian Wetland Classification System with Specific Details for Riparian Meltwater Channel Marshes.

Wetland Type	Description
Marsh	Characterized by periodic or persistent standing water or slow moving surface water which is neutral to alkaline and generally nutrient-rich. Vegetation is dominated by graminoids, shrubs, forbs or emergent plants
Bog	Characterized as a peatland receiving water exclusively from precipitation and not influenced by groundwater; *Sphagnum*-dominated vegetation
Fen	Characterized as a peatland receiving water rich in dissolved minerals; vegetation cover composed dominantly of graminoid species and brown mosses
Swamp	Characterized as a peatlands that are periodically standing surface water and gently moving, nutrient-rich groundwater, with vegetation dominated by woody plants often more than 1 m high
Shallow Open Water	Characterized as wetlands with free surface water up to 2 m deep, present for all or most of the year, with less than 25% of the surface water area occluded by standing emergent or woody plants. Submerged or floating aquatic plants usually dominate the vegetation.

(The National Wetland Working Group, 1997)

- ***Riparian Meltwater Channel Marsh****:* These marshes develop in abandoned channels or degraded stream channels situated in broad spillway valleys and postglacial alluvial and outwash plains where water flow is ephemeral or discontinuous. The primary water source in the marsh is from surface flow and groundwater inflow. Characteristic features are: (a) situated in broad spillway valleys, and alluvial and outwash plains.



Appendix D

Raw Data for Sediment Physicochemical Properties

	Study Sites		
Factor (replicate)	**CW**	**CRW**	**RW**
pH			
1	7.19	6.99	7.05
2	7.21	7.06	7.07
3	7.26	7.09	7.07
Salinity (ppt)			
1	24	2	2
2	15	7	2
3	19	5	2
Moisture (% dry wt.)			
1	100.02	88.94	72.56
2	107.73	223.45	106.69
3	39.111	323.16	66.45
Organic Matter (%)			
1	9.69	6.63	5.31
2	7.19	23.34	13.05
3	5.58	25.31	5.86
Physical Structure			
Sand (%)	87.3	68.3	78.8
Silt (%)	9	23	17
Clay	3.7	8.9	*4*
Soil Type	Sand	Sandy Loam	Loamy Sand