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Microbial Community Characterization and Pathogen Analysis Within Constructed Wetlands of Varying Scale Designed for Contaminant Removal

Michael R. Mitzel
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Microbial Community Characterization and Pathogen Analysis

Within Constructed Wetlands of Varying Scale

Designed for Contaminant Removal

by

Michael R. Mitzel

Honours Biology and Psychology, Wilfrid Laurier University, 2008

Submitted to the Department of Biology

Faculty of Science

in partial fulfilment of the requirements for the

Masters of Science in Integrative Biology

Wilfrid Laurier University

2010

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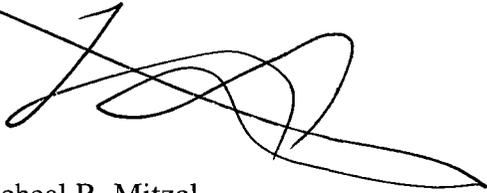
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Abstract

Constructed wetlands (CWs) are complex treatment environments, requiring an integrative research approach to improve our understanding of them. The goal of this thesis was to establish an understanding of the functional and structural characteristics of microbial communities within bench-, field- and industrial-scale environmental treatment systems. The impact of pathogenic and/or antibiotic contaminants on these communities based on their functional and structural profiles using community-level physiological profiling (CLPP) and denaturing-gradient gel electrophoresis (DGGE), respectively, was investigated. Under normal operation, bench-, field- and industrial-scale treatment systems were able to produce similarly behaving structural and functional profiles. Increased retention time was consistently associated with communities with low functional capacity and diverse structural properties when compared to areas that initially received pre-treated or untreated wastewater. This indicates that smaller-scale treatment systems may be able to provide similar environments to those in larger-scale treatment systems. However, the normal community profile was not maintained during antibiotic treatment. Based on the metrics used, functional fingerprints displayed metabolic increases after acute addition of antibiotic, which was then followed by a return to pre-exposure profiles. Conversely, structural fingerprints displayed no acute response, but instead alluded to delayed changes in the proportional abundance of different populations. In addition, the profile was reversed following exposure to untreated industrial wastewater, whereby an increased functional capacity and lowered structural properties were observed. These findings illustrate the dynamic and complex nature of microbial communities in response to selected environmental contaminants.

Acknowledgements

I would like to thank, first and foremost, my thesis advisor Dr. Robin Slawson for her unwavering support and encouragement throughout my masters and undergraduate study. I would also like to thank all the members of the Slawson lab over my time there, specifically Dr. Patrick Duriez, Jan Thomas, Jessica Morrison, Cassandra Helt and Robyn Morrison for their day to day assistance and conversation in the lab. I would also like to thank Dr. Kela Weber for his guidance with Biolog™ analysis and collaborative development of the DGGE analysis, as well as maintenance and operation of the bench-scale mesocosm. I would like to thank my committee members, Dr. Scott Ramsay and Dr. Raymond Legge, for their guidance throughout my MSc candidacy. I would further like to thank the Centre for the Control of Emerging Contaminants (P.I. – Dr. Wayne Parker) for providing the research funding.

    Last, but certainly not least, I would like to thank my parents, Melvin and Sally Mitzel, for their emotional and financial support throughout my academic career.

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1.   Introduction

1.1. Overview

Proper removal of contaminants is a crucial aspect of wastewater treatment. Following

the growing global desire for biological 'green' technology, constructed wetlands (CWs)

have become attractive and more common treatment options, largely due to the low costs

associated with operation and maintenance (Kadlec, 1996), the increase in public green

space (Knight, 1997) and the opportunity for use in rural or developing areas (Kivaisi,

2001). As a result, the complex ecology housed within wetlands is employed for its bio-

remediative capacity despite only scarce understanding of the environmental impact

(Kivaisi, 2001). The natural state of receiving CW microbial communities, the potential

and probable fates of coliform-type/pathogenic organisms and the overall community

changes as a result of exposure have scarcely been investigated (Ottova *et al.*, 1997; Tam,

1998; Khatiwada & Polprasert, 1999; Vacca *et al.*, 2005; Vymazal, 2005a; b). The impact

of influent exposure on the biological communities within these systems, the

sustainability of CW efficiency and the possible impact of CW effluent on the

surrounding environments therefore remain unanswered and concerning questions.

Few studies have investigated the bacterial communities that are responsible for

CW function, and even fewer using multiple characterization techniques (Kivaisi, 2001;

Wagner *et al.*, 2002; Scholz & Lee, 2005; Gutknecht *et al.*, 2006; Malik *et al.*, 2008). As

microbiologists, and biologists in general, turn to the pursuit of a more integrative,

holistic understanding of life, the use of multiple techniques, measuring a number of

characteristics simultaneously becomes a necessary endeavor in understanding complex

microscopic communities. Additionally, research on CWs has typically been localized to

field-mesocosms or pilot-scale systems (e.g., Ibekwe *et al.*, 2003) or bench-top systems (e.g., Hench *et al.,* 2003; Weber *et al.*, 2008), and to date has rarely been done on industrial-scale systems (e.g., Ghermandi *et al.*, 2007; Calheiros *et al.*, 2009).

This thesis aims to integrate multiple community characterization techniques, as well as data obtained from multiple scales, in an effort to obtain a greater understanding of the microbial performance and state of CW systems and their response to normal treatment and perturbation. Integration of genetic and metabolic metrics on CWs of various scales will additionally provide novel information regarding the ability of bench- and field-scale mesocosms to approximate the depth of the microbial diversity found in industrial-scale systems.

1.2. Research Need

Most studies that have been completed on pathogens associated with CWs have focused mainly on coliform reduction efficiencies, or the importance, and un-importance, of widely used macrophytes (Ottova *et al.*, 1997; Soto *et al.*, 1999; Tanner, 2001; Hench *et al.*, 2003). Commonly reported through culture-based methods are high coliform removal efficiencies from water treated by CWs, with a large range of 60 to 99.999% removal (Archer, 2004; Vacca *et al.*, 2005; Wand *et al.*, 2007; Garcia *et al.*, 2008; Morgan *et al.*, 2008). However natural wetlands and, similarily, CWs have been observed to maintain a background fecal coliform content of 10^2 to 10^3 colony forming units (CFUs) per 100 mL (Kadlec *et al.*, 2000). This may allude to the possibility of a maximum total coliform removal achievable through the mechanisms (e.g., microbial competition, predation, UV exposure, etc.) available in CW systems (Ghermandi *et al.*, 2007). These observations clearly indicate the power of CWs to reduce coliform loads, but may also indicate that

certain coliforms, along with any environmentally competitive pathogens they are typically associated with, may be able to find survive and persist within the receiving wetland communities. It is imperative that further understanding of these systems be gained, as both the processes and processors involved in bioremediation of both biological and chemical contaminants remains largely unknown despite the increased use of CWs (Kadlec & Knight, 1996; DeJournett et al., 2007). Without investigation into the microbial ecology of CWs and the function of bacterial populations in relation to pathogen removal, it will remain difficult to reliably assess ecological or human health impacts that could emerge from extended exploitation of complex biological, or 'green', technology.

Research on constructed wetlands is commonly focused on chemical removal (Kadlec, 1999; Chen et al., 2006), and rarely on the pathogens and receiving biological communities within the systems (Vacca et al., 2005). Additionally, to date no single study has considered a general comparison of the bacterial communities in an established industrial-scale treatment system, a field-scale CW mesocosm, and a bench-scale CW mesocosm. Lack of available comparisons between these scales with regard to biological contaminant removal and community structure and function continues to make it difficult to assess the ability of mesocosms to approximate large, field-scale, fully functioning CWs with regard to capacity and sustainability (e.g., Logan et al., 2005).

The rarity of collaboration between biologists investigating microbial communities and engineers responsible for the design and operation of CW systems may also be responsible for the scarcity of microbiological research regarding CWs. Additionally, the focus on these smaller treatment systems has likely arisen through the

relative difficulty in doing research on such large, complex systems, compared to easily accessible pilot- or bench-systems that can be carefully controlled and are protected from environmental events (i.e., rainfall, snow, etc.). Current social trends toward sustainable environmental practices, along with ever increasing waste-water treatment demands will undoubtedly continue to focus more attention on renewable and cost-effective treatment strategies; placing growing importance on the optimal and sustainable function of CWs. Resultantly, the microbial characteristics, the possibility of these systems to act as reservoirs for human pathogens and what role, if any, pathogens play within the ecology of these systems will require elucidation.

1.3. Research Objectives and Approach

The overall goal of this research is to profile the structural and functional characteristics of predominant microbial communities in CWs at multiple system scales in order to better understand the similarities or differences that may exist in the microbial communities of differing sizes. The working hypothesis for this investigation is that pathogen or contaminant presence will impart a discernable impact on the metabolic (i.e., functional) and genetic (i.e., structural) profiles of the receiving CW microbial communities. Additionally, it is hypothesized that the observed genetic and functional diversity will differ with system scale. The general approaches taken to examine this hypothesis include:

> The use of functional and structural microbial community-monitoring techniques in investigations of bench-, field-, and industrial-scale CW or alternative wastewater treatment systems under normal, undisturbed conditions. This was

done in an attempt to better understand the ability of controlled studies to predict

microbial communities found in functioning large-scale environmental sites.

➢ The use of functional and structural microbial community-monitoring techniques

in investigation of bench-, field-, and industrial-scale CW or alternative

wastewater treatment systems under perturbed conditions. This was done in an

attempt to better understand the ability of microbial communities in controlled

environments to respond in comparison to field-scale sites through these

monitoring techniques.

The results of this research will contribute to further the understanding of the

complex bacterial community structure and its response to the presence of a deleterious

contaminant or pathogen presence within CWs of varying sizes. This information may be

used to help optimize CW systems beyond mechanical augmentation, improve CW

treatment efficiency, and identify indicators for preservation of the health and

sustainability of CWs. This research also aims to provide a methodological foundation

for improved functional and structural microbial community assessment of CWs and their

outputs to receiving water bodies.

2.   Background

2.1. What is a Constructed Treatment Wetland? Why Do We Need Them?

Alternative environmental treatment systems are complex environments exhibiting a variety of characteristics possessed by both terrestrial and aquatic ecosystems. Wetlands, by definition, are composed of a large variety of habitats with common hydro-geological properties resulting in a consistently wet, or even submersed, soil bed with flora that is distinct from the dryer surrounding areas (Lewis, 1995). Normally termed artificial, in the 1980's and earlier, or constructed wetlands (CWs) more recently, these treatment systems are similar to man-made (i.e., created) or natural wetlands, but are created for the sole purpose of wastewater treatment (Hammer, 1992). To create a CW, available land can be augmented with modern primary treatment devices (e.g., sedimentation/flocculation tanks) and engineered through modifications in soil composition, macrophyte abundance or diversity, as well as land grading to create a more functional treatment environment through modified hydrology resulting in increased retention time (Kadlec & Knight, 1996; Scholz & Lee, 2005). In the most basic sense, however, a CW can be created by maintaining a discharge of wastewater onto any suitable land (Cooper & Boon, 1987), although the repercussions of this manner of construction may be environmentally detrimental. CWs may also be based in natural wetlands, with some land modifications to maintain a consistent hydrological regime with controlled retention times. However, these haphazard CW construction practices have largely disappeared in both Canada and the United Sates following the North American Waterfowl Management Plan of 1986; which resulted in the North American Wetlands Conservation and Emergency Wetlands Resources Acts of the same year (Environment Canada, n.d.).

Although classification of natural wetlands is often based on macrophyte type and abundance (Lewis, 1995), this is not commonly done with CWs. Instead, CWs tend to be classified based on the direction of water flow and the presence of surface water, categorized as either surface flow, horizontal sub-surface flow or vertical flow. Most industrial-scale CWs currently in operation contain properties of all three types, as each type imparts unique benefits and limitations (Vymazal, 2005a). It should be noted that the industrial-scale system investigated in the current investigation is a constructed treatment lagoon, and not technically a CW. Lagoons differ quite visibly from sub-surface flow CWs, but may be similar in appearance to surface flow CWs. The emergent vegetation found in surface flow CWs and the lack of UV radiation in the subsurface flow CWs are key differences. However, the consistently wet or submerged soil and standard vegetation was present in all three scales of treatment system.

Because wetlands are solar-powered systems, they operate with much lower cost and maintenance requirements than modern wastewater treatment plants (Lewis, 1995; Kadlec & Knight, 1996). Additionally, wetlands can be used to treat a much wider variety of contaminant sources (Gopal, 1999; Scholz & Lee, 2005), which makes them an important option for sustainable source water replenishment strategies. CWs also provide a benefit over wastewater treatment plants by serving as aesthetically pleasing natural recreational areas or wildlife conservation areas that can be frequented by the general population (Knight, 1997). Remediated wastes can serve as a source of nutrients or fertilizer for the surrounding vegetation and eliminate the need for commercial fertilizers to maintain plant growth (Knight, 1997; Kivaisi, 2001). It is important to note that the use of untreated or poorly treated wastewater as the CW influent may provide a potential

route of exposure for transmissible water-borne pathogens if the treatment environment is accessible by the general public (Krishnan & Smith, 1987). The potential for this type of human health impact is one of many reasons further understanding of the microbial ecology of CWs is needed (Vymazal, 2005); especially considering the limited knowledge regarding the prevalence and virulence of bacterial pathogens (Brettar & Hofle, 2008).

From data that is available it is clear that pathogens are being reduced during treatment (Archer, 2004; Ghermandi *et al.*, 2007; Wand *et al.*, 2007; Garcia *et al.*, 2008; Morgan *et al.*, 2008; Calheiros *et al.*, 2009). However, it is unclear if this is a permanent disinfection or merely an artifact of some non-lethal physical removal (e.g., sedimentation; Karim *et al.*, 2004). The ability of pathogens to enter a viable-but-not-culturable (VNBC) state when under environmental stress (Signoretto & Canepari, 2008) is also concerning. As these systems continue to be used to treat effluent containing pathogenic microorganisms that may be novel or display resistance to multiple antibiotics (Chaturvedi *et al.*, 2008), the fate of pathogens in CWs becomes a prevalent human health concern.

2.2. Microbial Communities in Constructed Wetlands

The biotransformations achieved using wetlands have long been exploited as a method to improve water quality, especially when treating wastewater containing a high degree of biological and/or chemical contaminants (Gopal, 1999). One of the driving forces of these transformative ecosystems is the microbial community, a dynamic and mysterious assemblage of microorganisms which includes bacteria, viruses, protozoa, fungi, algae and any other microscopic organisms (Atlas & Bartha, 1998). Although viruses,

protozoa, fungi or other microscopic organisms were not excluded from the analysis conducted here-in, the use of the term 'microbial community' primarily refers to an amalgamation of various bacterial populations. The use of 16S rDNA primers in the molecular methods employed, however, specifically targeted the prokaryotic organisms.

Every microbial community is influenced heavily by the various physico-chemical, also known as 'bottom-up' (McQueen et al., 1986; McQueen et al., 1989), factors of its environment, such as the availability of organic carbon and other nutrients, water activity and turbidity, O_2 availability, oxidation-reduction potentials, temperature and pH (Kadlec, 1999; Mitsch & Gosselink, 2000; Scholz & Lee, 2005). The impact of these physicochemical factors is accentuated in CWs, which provide diverse environments in which all of these factors are highly variable both over time and spatially (Lewis 1995), changing drastically within even a single square micrometer. From a microbiological perspective, these physico-chemical factors, in combination with dynamic hydrological and climatic factors, impart each wetland community with unique structural composition (i.e., who lives there?) and functional abilities (i.e., what do they do?) that dictates the biogeochemical cycling and remediation of contaminants within the CW (Lewis, 1995; Kadlec, 1999). Each of these factors can vary greatly throughout the microenvironments of the wetland, providing complex boundaries or barriers to multiple populations (Colwell & Leadbetter, 2007); populations that may be vast in size, with upwards of an estimated 10^{11} total members per gram of soil or 10^8 per milliliter of water (Atlas & Bartha, 1998), and are most often only defined by the sum of their biochemical abilities and restrictions or their phylogenetic qualities or genetic similarities (Head et al., 1998).

2.3. Microbial Community Characterization

The complexity of the ever-changing CW environment and its mysterious microbial

inhabitants does not readily lend itself to investigation. Some experts estimate that the

microbial community in a single gram of soil may contain up to 1000 distinct bacterial

species (Rossello-Mora & Amann, 2001) and a single milliliter of water up to 50 distinct

bacterial species (Eiler & Bertilsson, 2004). The total global microbial diversity within

soils and sediments can support an estimated 10,000 taxonomically-unique species

(Torsvik *et al.*, 2002), while aquatic environments have been estimated at approximately

160 taxonomically-unique species (Ritz *et al.*, 1997). When considering the countless

possible interactions among these species, or with other, non-prokaryotic organisms,

attempting to understand the ecology of these systems becomes a truly difficult

endeavour. Because of this diversity and the resulting ecological complexity, researchers

often describe a microbial community in terms of measurable characteristics (i.e., genetic

structure or carbon metabolism), commonly referred to as a 'characterization'. Many

different community characterization techniques have been used on various

environmental samples to generate information regarding properties of a given

community at a given time, usually termed a 'fingerprint' or 'profile'. These techniques

are generally considered to be either biochemical-based and culture-dependent or

molecular-based and culture independent (Kirk *et al.*, 2004). These are reviewed in the

following subsections.

Regardless of the techniques used, however, microbial community

characterization requires reliable and accurate detection, quantification and identification

of environmental microorganisms. This is a difficult task which often requires a

combination of time consuming culture-dependent (i.e., functional, physiological or metabolic) and culture-independent (i.e., structural, metabolic or genetic) methods (Brettar & Hofle, 2008). Comprehensive characterizations can also become expensive in terms of reagents, machinery and qualified personnel requiring extensive collaboration to collect funding, conduct research and properly interpret results (Scholz & Lee, 2005; Brettar & Hofle, 2008).

Despite the difficulties in creating an accurate description of the microbial populations within CWs, a better understanding of the biological forces driving treatment and remediation in this environment remains a crucial facet in working toward efficient and sustainable water treatment strategies (Kadlec & Knight, 1996; Hahn, 2006). Technological advances in recent years have helped to improve molecular biological techniques in order to better assess the chemical and biological complexity of samples from CWs, providing a sensitive and more reliable method for investigation of the structural and functional black box that is a wetland microbial community (Forney *et al.*, 2004).

2.3.1. Culture-Dependent Techniques

2.3.1.1. Classical Culture Methods

Traditional culture-based methods are founded on the assumption that certain living cells are able to grow and replicate on supplied biochemical substrates in specific physico-chemical environments. Due to their cost effectiveness, the lower level of expertise needed, and the large range of media available, culture methods remain among the most popular when measuring fecal contamination and the presence of pathogens (Gilbride *et al.*, 2006; Morgan *et al.*, 2008). Evidence suggests culture-based methods may be unable

to detect viable pathogenic bacteria in environmental samples, especially if cultivation of those bacteria is particularly laborious (Alexandrio *et al.,* 2007) or if these organisms have entered a viable-but-not-culturable (VNBC) state (Signoretto & Canepari, 2008). For this reason, molecular methods are thought to provide improved assessment of pathogenic environmental organism (Straub & Chandler). However, when dealing with complex sample environments, it is not always possible to use molecular techniques because of either the presence of inhibitory compounds which can be difficult or damaging to remove, or an inability to collect quality genetic material (Malik *et al.,* 2008). The separation of certain microbial groups with specific metabolic or physiological functions, independent of genetic lineage, may also be of interest and this is not discernable through molecular techniques (Forney *et al.,* 2004). For these reasons, culture-based methods remain an important set of tools for microbiological examination.

The major limitation of culture methods is that viability cannot be persistently equated with culturability, as culturable organisms are thought to comprise only a small fraction (< 1%-15%) of the total viable microbial population in environmental samples (Oliver, 2005). The medium used to culture bacteria provides a defined set of growth conditions, which may not be sufficient for the growth of fastidious or highly specialized organisms and significantly underestimate diversity in samples (Oliver, 2005). Interference by background microbiota, difficulty in discriminating between pathogenic and non-pathogenic strains, long cultivation steps, and expensive serological confirmatory tests are further limitations of culture-based methods (Rompre *et al.,* 2002).

2.3.1.2. Community-Level Physiological Profiles (CLPP)

Creation of a CLPP is done through the use of BIOLOG™ ECOplates™ (by Biolog Inc.), which supply 31 different types of carbon sources and measure the ability or rate of a given microbial community to metabolize them. This method allows for a relatively quick and cost-effective analysis of the metabolic capacity, or the carbon source utilization pattern (CSUPs), of the microbial community under consideration. There is little need for isolation, enrichment, amplification, or processing of the sample outside of dilution, centrifugation and re-suspension (Calbrix *et al.*, 2005). Since CLPP requires comparatively less labour than many other characterization methods, it can be used intensively to provide detailed information with regard to temporal and spatial variation (Garland, 1997). BIOLOG™ ECOplate™-generated metabolic profiles are used to compute diversity indices or are subjected to multiple types of statistical analysis, which allows for information gained to be compared to other measurements with relative ease (Zak *et al.*, 1994; Weber *et al.*, 2007).

BIOLOG™ ECOplates™ contain 96 wells, three blank along with 31 carbon sources in triplicate (Weber & Legge, 2010). CSUPs are given by the differential metabolism of these carbon sources and expressed in terms of richness (i.e., the number of responsive wells), evenness (i.e., the variation in response among wells) and diversity (i.e., the pattern of richness and evenness among wells). Calculated estimates of diversity, referred to as diversity indices, can be generated through various equations (Brower & Zar, 1984), although the Shannon index (H'; Shannon, 1948) is among the most, if not the most commonly reported index generated from CSUPs in literature. Because of the dominance of H' in the literature, as well as the relationship of the operational definitions

of richness and evenness generated from the CLPP method and other diversity indices

(which usually refer to species and not substrates), H' is the only index calculated in this

study. The maximum value for BIOLOG™ ECOplate™ generated Shannon's H' is 3.434

(i.e., the natural logarithm of 31; the total number of substrates) and occurs when all

wells are metabolized equally; although values between 1, considered to be low diversity,

and 3, considered high diversity (Weber & Legge, 2010).

The major limitation of CLPP is that it is not able to provide a reliable picture of

the community structure. This limitation arises because it is unknown whether or not

carbon utilization is due to a single species or is a result of cooperation among microbes.

Similarly, it is unclear whether a lack of metabolism represents the result of competition

between microbes or signifies the absence of organisms that can utilize the given

substrate (Garland, 1997). However, Garland (1997) asserts that this limitation may be a

benefit in disguise, as the community profile gained through other methods does not

describe the interactions between its members. Another disadvantage of CLPP is the

amount of time required for profile development. Even though inoculation of the plates

can take seconds or minutes, two to seven days may be required for proper development

of the reactions in the BIOLOG™ wells (Garland, 1997). The need for incubation over

this time period imposes culture-based constraints as communities react to the

environment within each well, which may cause death or over growth; altering the

unamended state of the community. Furthermore, proper inoculation of BIOLOG™

ECOplates™ requires understanding of sample properties (i.e., background carbon

presence, cell density or pH) which may not always be known prior to sample to

collection, or may change during the course of a study. This may result in differential

processing of samples from the same location taken on different days or novel processing that the researcher was not prepared for. Nevertheless, CLPP has become a prevalent technique for microbial community characterization of CWs and continues to be applied to a range of different CWs treating a diversity of contaminants (Weber *et al.*, 2008; Zhang *et al.*, 2010).

2.3.2. Culture-Independent Techniques

2.3.2.1. Polymerase chain reaction (PCR)

PCR is a technique used to reproduce millions of copies of a desired DNA sequence with a high degree of reliability. The amplified DNA product can then be used to detect and enumerate organisms through the use of labeled primers, or provide phylogenetic information through sequencing and comparison to genetic databases (Malik *et al.*, 2008). The most common target used to gather species-level information about bacterial communities is 16S rRNA genes (Gilbride *et al.*, 2006; Sanz & Kochling, 2007; Malik *et al.*, 2008). 16S rRNA genes are highly conserved in bacteria and can be used as effective chronometers (i.e., measures of taxonomic divergence) for the inference of phylogenetic relationships, as differences in the ribosomal structures these genes code for are understood to have early delineation in the lineage of a given prokaryotic taxa (Woese, 1987; Muyzer & Ramsing, 1996).

Limitations of PCR include requirement of a high level of technical expertise to optimize PCR reactions and troubleshoot any problems that may be encountered. PCR amplification efficiency is highly dependent on the quality of the DNA provided and is severely hampered by the presence of inhibitory substances such as organic acids or clay particles (Sanz & Kochling, 2007), which are common in constructed wetland samples.

Differential amplification of target genes can introduce biases into PCR-based diversity assessments, as common sequences may hide uncommon ones (Sanz & Kochling, 2007; Malik *et al.*, 2008). Sequences with lower G+C content also separate more efficiently during the PCR denaturation phase and may therefore preferentially amplify during the extension phase (Wintzingerode *et al.*, 1997). These biases should always be kept in mind when using PCR to generate a community profile.

2.3.2.2. Denaturing gradient gel electrophoresis (DGGE)

DGGE is used to separate previously amplified 16S rRNA gene fragments of the same length (commonly 200-500 bp) with variable base pair composition. As the amplified 16S rRNA moves through a charged polyacrylamide gel and is slowly denatured over a urea/formamide gradient a section of it 'melts' (i.e., the double stranded DNA becomes single stranded) and stops moving due to the presence of a denaturant-resistant GC-clamp included on the primer that holds the now single-stranded section of DNA together (forming an immobile 'T' shape; Sanz & Kochling, 2007; Malik *et al.*, 2008). The different chemical properties of the composite nucleic acids determine the mobility, or 'melting point', of each gene fragment, making it possible for DGGE to separate amplified products that differ by a single base pair; creating a 'genetic fingerprint' (Muyzer *et al.*, 1993). The distance travelled in the DGGE gel by any given segment of DNA is considered to be largely a function of its G+C (i.e., guanine and cytosine) content (Muyzer & Ramsing, 1996; Muyzer & Smalla, 1998); with low G+C content bands stopping early, and high G+C travelling further in the gel.

Following DGGE separation of PCR products, each band can be excised from the gel, purified, and sequenced to produce a phylogenetic tree or create fluorescent primers.

However, this can become incredibly time-consuming and expensive. A more cost effective approach to DGGE fingerprint analysis is to create qualitative and quantitative data sets based on band intensity and distance moved (i.e., banding patterns) and analyzed these using gel analysis software and statistical packages (Tourlomousis *et al.* 2010). Diversity indices can also be generated in a manner similar to that done for CLPP, although DGGE-based indices are expressed in terms of richness (i.e. the number of bands in a lane), evenness (i.e. the variation in intensity among bands) and diversity (i.e. the pattern of richness and evenness within a lane). Using these modified operational definitions the same diversity-determining equations can be employed (Brower & Zar, 1984). Although the *H'* (Shannon, 1948) again dominates the literature, the use of other indices, like Hulbert's probability of an interspecies event (PIE; Hurlbert, 1971) may be more appropriate than *H'* considering the taxonomic nature of DGGE data. PIE is originally described as the probability of two randomly observed individuals in a given environment or area belonging to different species (Hurlbert, 1971), while *H'* originally referred to the uncertainty of predicting the next letter in a message or communication (Spellerberg & Fedor, 2003). Ibekwe *et al.* (2007) calculated *H'* (Shannon & Weaver, 1963, as cited by Ibekwe *et al.*, 2007) with no real rationale for the choice to use *H'* made clear. Both *H'*, due its repeated appearance in literature and previous use in DGGE, and PIE, due to the close relationship between the interpretation of DGGE data and the original definition of the equation, are calculated in this study.

DGGE is the method of choice when the desired information does not have to be as thorough as that gained from 16S rRNA clone libraries, but is still precise and extensive enough to determine the dominant members of microbial communities (Sanz &

Kochling, 2007). The chief advantage of DGGE is that it enables rapid monitoring of spatial and temporal changes within microbial community structure, providing a molecular fingerprint with regard to the dominant microbial species. It also comes with relatively low cost for a high degree of information compared to other profiling methods, such as restriction enzyme-based fingerprints (Malik *et al.*, 2008).

Limitations of DGGE include co-migration of different sequences, heteroduplex formation, or the presence of single-stranded sequences, which may confound banding patterns or increase background fluorescence. To overcome these types of problems that can be difficult to diagnose, bands from DGGE gels are commonly referred to as operational taxonomic units (OTUs; Sneath, 2005) as opposed to distinct species, although the latter can still be found in literature.

As the nucleic acids extracted from the samples require amplification using PCR, due to both the relatively low abundance of DNA in environmental samples and the need for the addition of a denaturant-resistant GC-clamp, the same limitations and biases associated with PCR are also prevalent with DGGE (Muyzer *et al.*, 1993). This may lead to the exclusion of a rare community member that may still play vital ecological roles despite their low number. For this reason DGGE should be interpreted only as a measure of those community members which are dominant, not a complete inclusive community measure (Fromin *et al.* 2002).

Another prevalent consideration is that DGGE uses only genetic material; making it incapable of distinguishing viability and unable to provide information regarding the metabolic function of the community beyond inference based on previously described or inferred phylogenetic relationships or sequence analysis. Considering the capacity for

transformation of free DNA, the possible use and transfer of plasmids and the potential

for ambiguity in prokaryote phylogentic relationships (Woose, 1987; Rossello-Mora &

Amann, 2001; Cohan, 2002; Doolittle & Papke, 2006; Doolittle & Zhaxybayeva, 2009),

even the most extensive DGGE-based investigation leaves a number of important

questions unanswered. Despite these limitations, the possible application of DGGE

separated products and the rapid production of genetic diversity indices created from

banding patterns makes this a powerful fingerprinting tool to detect structural changes in

bacterial communities; one that continues to garner significant attention for use on CWs

and microbial communities in general (Jin & Kelley, 2007; Moura *et al.*, 2009; Liu *et al.*,

2010; Maeda *et al.*, 2010) and has an incredible potential when used in combination with

other microbiological techniques that may, in part, address one of the previously stated

limitations.

2.4. The Role of Integrative Biology in Microbial Community Characterization of Constructed Wetlands and Pathogen Monitoring

CWs provide preferential conditions for pathogen removal, as the diverse habitats

encountered in them allow physical (e.g., UV exposure, desiccation), chemical (e.g., pH,

osmotic pressure) and biological (e.g., predation) factors to be exploited simultaneously

(Greenway, 2005). However, it is currently unclear what the fate, behaviour or impact the

exposure of pathogenic microorganisms or potentially deleterious contaminants (e.g.,

antimicrobials, acid-mine drainage, pharmaceuticals, etc.) may have on the receiving

indigenous microbial community. It also remains unclear whether unwanted

contaminants are removed due to sustainable mechanisms, or if removal imparts

detrimental changes in the microbial community structure or its level of metabolic function.

Wake (2008) states that "questions and problems that benefit most from an integrative approach… cut across traditional disciplinary boundaries". It should be clear that applied research on CWs will not only benefit from integrative approaches, but requires them. To move toward a better understanding of CW microbial ecology, an integrative approach consisting of collaboration between engineers, biologists and chemists is necessary; due mainly to the complexity and multidisciplinary nature of CW design, operation and monitoring. Legal professionals may also be involved, as industrial CWs operate on private property and any published results may attract unwanted attention from regulatory authorities or the general public. Further collaboration with medical professionals is also required if human pathogens are under investigation.

Interdisciplinary collaboration can be considered by some to be one of the primary principles of integrative biology; in addition to the application of multiple analytical techniques, analysis of multiple levels of biological organization, and use of hierarchical research strategies (Wake, 2003; Wake, 2008). The importance of each of these integrative principles in generating applicable understanding of biological systems is not lost on investigators concerning the diverse communities in CWs. With these tenets in mind, there is no doubt the integrative biologist will have an incredibly important role in building an understanding of CWs.

2.5. Recent Advances in Understanding the Microbial Communities of Constructed Wetlands

As CWs become evermore prevalent as tertiary wastewater treatment methods (Gopal, 1999; Scholz & Lee, 2005), there continues to be more focus placed on understanding of these systems and the life within them. Microbial community characterization in CWs has received a fair amount of attention in the literature following the turn of the century (Vymazal, J. 2005a; Spiegelman *et al.*, 2009), however, there is still much that remains unknown. Some of the recent advances in understanding the properties of CWs, as well as remaining gaps in this understanding, are presented using four recent and important studies as examples.

Hench *et al.* (2003) investigated the fate of coliforms and pathogens in mesocosms designed to approximate CWs. Using standard culture-based methods, Hench *et al.* (2003) observed a significant reduction of between two and four orders of magnitude of fecal coliforms, and at least one order of magnitude removal for the various pathogens investigated. Werker *et al.* (2007) attempted to further investigate the fate and behaviour of selected pathogens in CW mesocosms using culture-based methods and an inoculum of labeled (i.e., nalidixic acid-resistant) *Escherichia coli*. Werker *et al.* (2007) observed similar removal efficiencies as Hench *et al.* (2003), but reported that the removal rate slowed significantly, eventually leveling off, as the influent load decreased to around 10^4 CFUs/100mL. This finding raises questions about the generally accepted 1st order dynamics of pathogen removal (Vymazal, 2005b), as well as a redefinition of the proposed removal mechanisms (Khatiwada & Polprasert, 1999) to include grazing. Additionally, Hench *et al.*, (2003) also observed that removal efficiency decreased by an

order of magnitude over the second year of study, which also raises concerns about the sustainability of effective pathogen removal in CWs.

Werker *et al.* (2007) and Hench *et al.* (2003) not only provided insights into the potential ability of CWs to remove pathogens, but also raised important questions regarding the sustainability and optimization of CWs as effective treatment systems. However, the impacts the pathogens have on the receiving community were not assessed in either of these studies.

In a third study, Weber *et al.* (2008) assessed the impact of acid mine drainage on the receiving microbial community of multiple planted and unplanted bench-scale systems using BIOLOG™ ECOplates™. Through principal component analysis of the metabolic fingerprints (i.e., CSUPs), Weber *et al.* (2008) were able to assess the robustness and the degree of change shown by the interstitial CW microbial community. The metabolic fingerprint of the CW system was shown to be significantly affected after exposure to the acid-mine effluent, though the communities in the planted mesocosms showed greater stability (Weber *et al.*, 2008). These results clearly showed an impact on the metabolic profile of the receiving community, but it remains unclear whether this was accompanied by a significant structural change. In other words, was the metabolic change observed caused by the rise and fall of different populations within the community as a result of influent exposure, or alternatively, the result of the same organisms using different metabolic pathways?

Ibekwe (2003) investigated a pilot-scale CW system using molecular methods (PCR-DGGE) to characterize both the universal bacterial community and the ammonia-oxidizing population from a pilot-scale system used to treat dairy wastewater. Ibekwe *et*

al. (2003) were able to observe a high-degree of diversity in *Nitrosospira* spp. and *Nitrosomonas* spp., which was consistent with a significant removal of ammonia; a known ability of these taxonomic groups. Though this study focused only on one specific, functional group within the wetland community, it was able to provide useful information regarding the structural link to the community's function.

Each of these four studies used a different characterization method to investigate the bacterial community from a unique perspective. Additionally, all four studies were done in bench- or field-scale systems. It is unclear to what degree these smaller-sized models are able to simulate large, industrial-scale treatment systems.

In an attempt to provide information relating to the important and unanswered questions regarding CW microbial communities, this study will consider all three scales to investigate potential relationships between them in any of the metrics employed. Combining community profiling methods (i.e., DGGE & CLPP) will also allow the current study to achieve a more complete understanding of the characteristics or changes of community structure and function that may be associated with pathogen or antimicrobial presence and removal.

3.   Study Sites, Experimental Plan and Associated Methodology

3.1. Site Descriptions and Sampling Protocols

3.1.1. Bench-scale Constructed Wetland Mesocosm

The bench-scale constructed wetland (CW) mesocosm site was located at the University

of Waterloo and operated under the supervision of Dr. Kela Weber. All details regarding

the bench-scale mesocosm site description were provided by Dr. Kela Weber (personal

communication, September 27th, 2010). The experimental setup consisted of four

mesocosms planted with common reeds (*Phragmites australis*) and seeded with an

activated sludge inoculum from a local municipal wastewater treatment plant. The

mesocosms were designed in the same manner as those used previously (Weber and

Legge, 2010; Weber *et al.* 2010). The construction of each mesocosm was completed

using schedule 80 (wall thickness of 1.5 cm) polyvinylchloride (PVC) columns (90 cm by

25 cm diameter) filled to approximately 80 cm with pea gravel (average equivalent

spherical diameter of 2 cm, 80% limestone) and operated to 70 cm with water, which at

starting was a void volume of approximately 12 L for all mesocosms. Water was

circulated with a 1/200 HP, 3200 rpm, March series-1 (1A-MD 1/2) centrifugal pump

(March Manufacturing Inc., Glenview, IL). The water inlet was situated 5 cm below the

water level, and the water outlet was situated 5 cm from the bottom of the PVC column

(see Figure 3.1.).
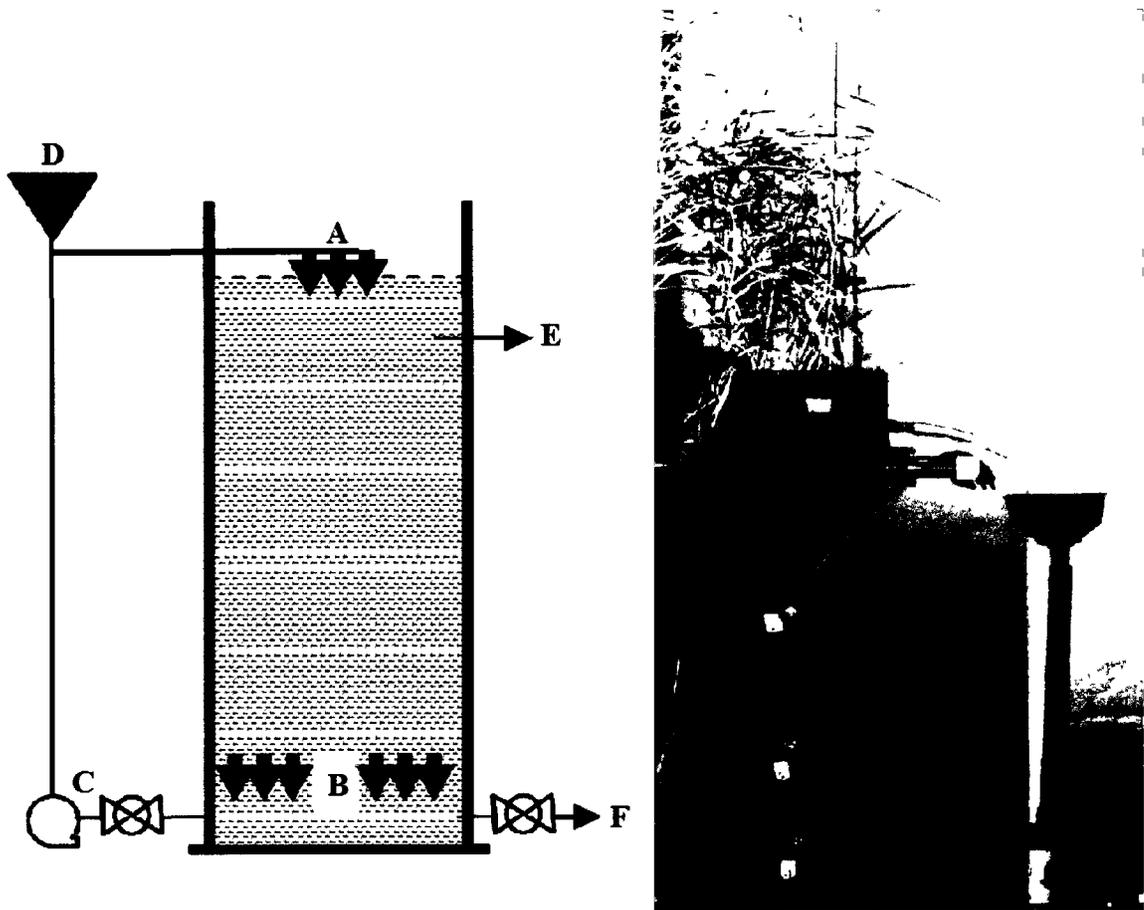
Figure 3.1. Schematic (left) and photograph (right) of bench-scale CW mesocosm[*]. In the schematic, water is fed into the mesocosm (A) and allowed to percolate through the pea gravel bed to be collected at the bottom (B) and is re-circulated via a small centrifugal pump (C). An atmosphere exposed port serves as an injection and sampling (D) point. Drainage ports are located near the top to prevent overfilling (E), and near the bottom (F) for mesocosm drainage.

Bacterial community seeding of the mesocosm was done by adding fresh limestone gravel alternating with 160 mL of inoculum at depths of 10 cm, 40 cm and 65 cm. *P. australis* were collected from a local marsh, cultured in pots with peat moss and transferred to the mesocosms, along with a small amount of peat moss, into the top section. Three small *P. australis* plants approximately 30 cm high were used initially to plant each mesocosm and initial root depth was approximately 20 cm in all four cases. The mesocosms were maintained under laboratory conditions with a relative humidity of

[*] Schematic information and photograph provided by Dr. Kela Weber, used with permission.

40-60% and temperature between 24-28°C. All mesocosms were exposed to artificial illumination of 14,000 lm with a 15:9 hr photoperiod and plants were sprayed daily with tap water during the photoperiod to avoid drying.

The mesocosms were operated in a constant recycle mode where water was circulated at approximately 2.4 L/min, resulting in an average cyclic hydraulic retention time of approximately 4-5 min. Each week the mesocosms were filled with the simulated wastewater and operated under constant recycle for the remainder week, at which point the mesocosms were completely drained before being refilled with new simulated wastewater for the following week's operation. Simulated wastewater solution was based on the descriptions of solutions used by Kargi and Karapinar (1995), Droste (1996) and Wang $et\ al.$ (2008) and consisted of 1 g/L molasses, 0.049 g/L urea, 0.0185 g/L $NH_4H_2PO_4$, yielding a glucose concentration of 0.588 g/L from the molasses, a carbon-oxygen demand (COD) of approximately 500 mg/L and a COD:N:P ratio of approximately 100:5:1. To encourage $P.\ australis$ survival, a nutrient solution according to Hoagland and Arnon (1938) was also added to the simulated wastewater. The nutrient solution was mixed in regular tap water and fed to the wetlands to result in calculated interstitial concentrations of 28.75 mg/L $NH_4H_2PO_4$; 151.5 mg/L KNO_3; 236 mg/L $Ca(NO_3)_2 \cdot 4H_2O$; 123.25 mg/L $MgSO_4 \cdot 7H_2O$; 9.175 mg/L FeNaEDTA; 0.715 mg/L H_3BO_3 ; 0.4525 mg/L $MnCl_2 \cdot 4H_2O$; 0.055 mg/L $ZnSO_4 \cdot 7H_2O$; 0.0125 mg/L $CuSO_4$ and 0.005 mg/L $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$.

All four mesocosms were given an initial one week running period after which two of the four systems were exposed to ciprofloxacin at a concentration of 2 µg/mL for a 5 day period; a concentration that should have a clear effect on environmental organisms

(Thauvin-Eliopoulos & Eliopoulos, 2003). After this 5 day period the interstitial ciprofloxacin was removed by draining the mesocosms. The mesocosms not exposed to ciprofloxacin were considered control duplicates and are identified here-in as control or 'No Cipro', while the corresponding duplicate mesocosms that received ciprofloxacin are labelled 'Cipro'. Both control and 'No Cipro' are used interchangeably, as 'No Cipro' mesocosms a control for the ciprofloxacin perturbation, but also a model of the natural development of these types of systems.

Samples for use in DNA extraction were collected in duplicate from the drainage outlet (see 'F' in Figure 3.1) in 50 mL pre-sterilized, DNA/RNA-free disposable conical-bottom screw-cap centrifuge tubes (Axygen, Fischer Scientific, Whitby, ON) and transported in a cooler filled with ice from University of Waterloo to Wilfrid Laurier University - Waterloo campus. Samples were collected on Nov. 16th, 23rd, 30th (2009); Jan. 4th and 18th (2010). Samples were processed within 3 hours of collection and the protocols performed are presented in the following section. Samples taken on Nov. 23rd (i.e., the same day as the ciprofloxacin addition) were collected immediately prior to addition of the antibiotic.

3.1.2. Field-scale Constructed Wetland

The field-scale CW investigated is located on the property of the Haliburton Fish Hatchery in Haliburton, ON., and was constructed in 2008 as a subsurface flow cold climate treatment wetland receiving fish-farm waste. The CW is bisected and planted with common cattails (*Typha latifolia.*; planted 2008) and wild rice (*Zizania palustris*; planted 2008) that died and was replaced with lake sedge (*Carex lacustris*; planted 2009) to create two parallel flow paths through the CW. Septic effluent enters the wetland at a

rate of approximately 2500 L/day through a single pump which passes wastewater into

both sides of the wetland. Once through the wetland, the treated water enters a combined

chamber, and is pumped into a phosphorous filter comprised of iron slag. From here,

phosphorous filter effluent flows into a holding pond (see Figure 3.2).

Hydraulic retention time for the waste effluent is approximately 38 hrs in the

wetland (Dr. Gord Balch, personal communication, Dec. 1st, 2010) and 13 hrs in the iron-

slag phosphorous filter (Laura Sansford, personal communication, Dec. 2nd, 2010).

Samples were collected in 500 mL sterilized glass bottles and shipped from Haliburton,

ON to Wilfrid Laurier University on ice via Fedex to arrive the following day by noon. A

total of six samples were collected on each sampling date; specifically Feb. 8th, 22nd, 24th;

Mar. 1st, 10th, and Apr. 12th (2010). Sample locations are identified in the schematic

presented in Figure 3.2. and are here-in referred to as 'Septic', taken from the influent to

the septic pump; 'Cattail' and 'Sedge/Rice', taken from the first access port on the cattail

and sedge planted sides, respectively; 'Final Pump/Combined', taken from the combined
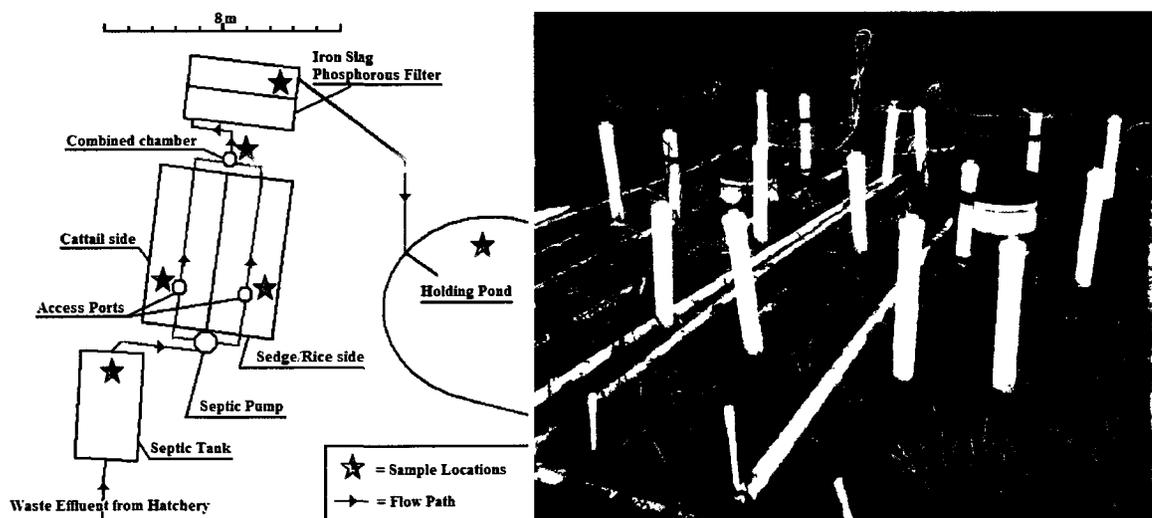
Figure 3.2. Schematic (left) and photograph (right) of the field-scale CW[*]. Wastewater flow is indicated by the blue arrows. Sample locations are denoted by red stars with blue outline.

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[*] Schematic information provided by Dr. Gord Balch (Fleming College). Photo taken by Michael R. Mitzel.

pump chamber at the end of the CW; 'Slag Filter', taken from the effluent of the iron slag phosphorous filter, and 'Pond', taken from the receiving water body (i.e., holding pond).

A mass of 12.5 g of oxytetracycline was added as a slurry each morning, for a total of five additions beginning Feb. 22nd until Feb. 26th (2010), at the exit of the septic tank in an attempt to create a nominal concentration of 5 mg/L based on the average flow of 2500 L/day mentioned earlier. This exposure was done to represent the effect of an antibiotic contaminant slug, in accordance with a collaborative research plan under Dr. Brent Wootton (Principal investigator, Ontario Ministry of the Environment, 2010).

3.1.3. Industrial-scale Treatment Lagoon

The industrial-scale treatment lagoon site was constructed in 1992 and operates from spring until winter (i.e., April through December) of every year. This treatment lagoon system was built for the purpose of treating pre-treated effluent from a poultry slaughterhouse kill-floor located in southern Ontario that processes approximately 300,000 birds per week. The treatment lagoon system consists of three lagoons, in series, which receives wastewater at a load of approximately 400-500 litres per min for 8 hours per day. During normal operation of the lagoon treatment system, pre-treatment of the kill floor effluent is done using ferric chloride as a flocculent inside a large flocculator within the processing plant. The effluent is subsequently screened and the resulting clear and colourless post-flocculated effluent is pumped out of the processing plant and into the first lagoon. The post-flocculated effluent is aerated as it enters the first lagoon though an inlet pipe located at the centre of the bottom of the lagoon, positioned to pass influent directly through the aerator. Water from the first lagoon flows passively through an underground passageway constructed with concrete and steel into the second lagoon;

entering below the water line at the side nearest the first lagoon. From the second lagoon, water again flows passively through a similar underground concrete-steel passageway and into the third lagoon. Storm water from the property is collected and added to the lagoon system through channels that empty into the second passageway. The site also contains two over-flow lagoons that are used in parallel with the third lagoon, which receive effluent from a bypass junction in the second concrete-steel passageway if necessary. At the conclusion of the operation period the water held in the third lagoon, as well as any of the over-flow lagoons, is pumped into a concrete finishing house where it is held until it is discharged into a nearby stream. Sample locations are denoted by the red stars with blue outline in Figure 3.3 and are here-in referred to as 'PostFloc', taken from the exit port of the flocculator; 'Lagoon 1', taken from the littoral zone of the first lagoon; '1 to 2', taken from the first underground concrete-steel passageway accessed through a service entryway; 'Lagoon 2', taken from the littoral zone of the second lagoon, directly between the entry port from the first lagoon and the exit to the third; '2 to 3', taken from the second underground concrete-steel passageway accessed through a service entryway; 'Lagoon 3', taken from the littoral zone of the third lagoon opposite the entry port from the second lagoon.

Water samples were taken on June 24th; Sept. 21st; Nov. 17th (2009); July 5th and Sept. 28th (2010). Samples were collected in duplicate using sterile 250 mL (Nalgene, Fischer Scientific, Whitby, ON) bottles and transported in a cooler on ice by car from the poultry processing plant to Wilfrid Laurier University - Waterloo Campus.
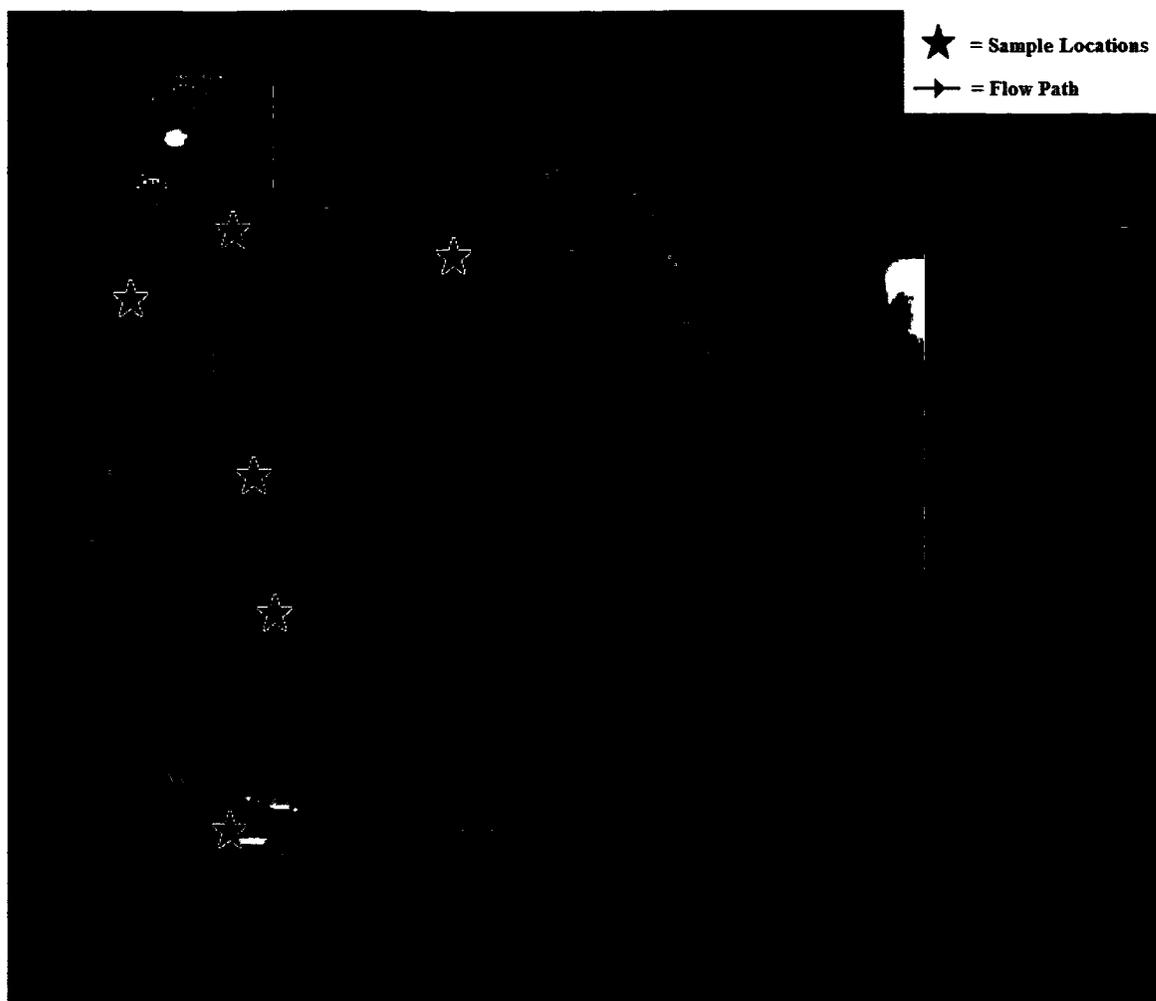
Figure 3.3. Photograph of the industrial-scale treatment lagoon. Wastewater flow is indicated by the solid blue lines with direction of flow indicated by the blue arrows. Overflow paths are denoted by the dashed blue lines. Sample locations are denoted by red stars with blue outline. The surrounding area has been edited to preserve anonymity of the site location by request.

Important site-related notes include the addition of an unknown amount of a commercial wastewater digestion product, ACTIVE 8™ (Naschem Inc., Mississauga, ON), by plant operators between the 'PostFloc' and 'Lagoon 1' samples, which began sometime between the June 24th and Sept. 21st (2009) samples. Additionally, the morning of the day prior to sample collection on Sept. 21st (2009), the flocculator was bypassed due to a clog in pipes, resulting in untreated kill floor effluent being pumped into the first lagoon. For this reason, the 'PostFloc' sample was unavailable on this day. To attempt to counteract any negative effect, plant workers added 12,500 kg of ferric chloride to the

third lagoon. Also, approximately six or seven days prior to collection of the sample on

Sept. 28th (2010), another clog in the pipes leading up to the flocculator caused an

unknown amount of untreated kill floor effluent spill into the rainwater collection tank

and enter directly into the third lagoon. This continued for three days, until the flocculator

pipes could be cleared. Addition of ferric chloride similar to that done on Sept. 21st

(2009) was done the day prior to collection of the Sept. 28th (2010) samples, to counteract

the addition of untreated effluent to the third lagoon. These notes will be reiterated during

discussion of the related results.

3.2. Laboratory Methods

Culture methods

Enumeration of heterotrophic plate count (HPC) was carried out on the field- and

industrial-scale samples within 8 hours of sample arrival. HPCs for the field-scale

samples were done on all samples collected using R2A agar (BD Difco, Fisher Scientific,

Whitby, ON) after creation of a dilution series from 10^0 to 10^6 using 9 mL dilution blanks

containing sodium-free dilution buffer (APHA, 1998) of which 100 µL from each

dilution was spread, in duplicate, onto R2A plates. HPCs from the industrial-scale were

done in the same manner as the field-scale, however, LB agar (BD Difco) and 1/5th LB

was also used, alongside R2A, to provide a range of richness in the growth media. Due to

logistical concerns (i.e., incubator space), the lack of change in HPCs and the intensive

labour required investigation of the HPCs were not continued beyond June 24th. HPCs

were counted after 5 to 7 days of incubation at 25°C in accordance with the standard

method (APHA, 1998).

E. coli was enumerated for all three scales within 8 hours of sample collection using 47

mm 0.45 μm cellulose-mixed-ester filters (Millipore, Fisher Scientific, Whitby, ON)

placed on mFC basal-medium (BD Difco) with 1% 5-bromo-4-chloro-3-indolyl-β-D-

glucuronide (BCIG; Inverness Medical Inc., Ottawa, ON) and incubated for 24-48 hrs at

44.5°C in a water incubator in accordance with the standard method (APHA, 1998). 10

μL, 100 μL, 1 mL, 10 mL of each sample was filtered and colonies displaying blue

colouration were considered to be *E. coli*. 100 μL of *E. coli* (ATCC® 25922™, Inverness

Medical Inc.) and *Enterococcus faecalis* (ATCC® 49532™, Inverness Medical Inc.)

were also filtered for use as positive and negative controls regarding media presentation,

respectively. Spread-plates and filters containing between 25 and 300 colonies were

considered countable. In the absence of any countable plate for a given sample, an

estimate was created using the pattern of growth displayed on all plates or filters for the

corresponding sample. Enumeration of faecal coliforms (FCs) were also undertaken for

the industrial-scale site, using the same filtration procedure done for the *E. coli* with the

filters placed on mFC agar with 1% Rosolic acid (BioShop Inc., Burlington, ON) and

incubated for 24-48 hrs at 44.5°C in a water incubator in accordance with the standard

method (APHA, 1998). Blue colonies were considered to be FCs.

   Isolation of *Salmonella enterica* was also carried out for the field- and industrial-

scale samples following a procedure previously assessed for reliability (Bartram, 2007).

10 mL of sample was added to 1x buffered peptone water (BPW; BD Difco) and

incubated at 37°C and 120 rpm overnight. 1 mL of the resulting culture was added, in

duplicate, to freshly made 9 mL tetrathonite broth containing 2% iodine solution (TB; BD

Difco) and incubated at 37 °C and 160 rpm overnight. 1 mL of *E. coli* (ATCC #25922,

Inverness Medical Inc.) grown in nutrient broth (NB; BD Difco) overnight was also

added to 9 mL of TB as a negative control for this broth, while 1 mL of *S. enterica* ser.

typhimurium (ATCC® 13311™, Inverness Medical Inc.) grown similarly was carried

through the entire extraction procedure as a positive control for the entire isolation

procedure. 100 μL of TB was added to the centre of a plate containing approximately 20

mL of modified semi-solid Rappaport-Vassiliadis media (MSRV; BD Difco), in

duplicate, and incubated at 42°C overnight to select for motility. 100 μL of *Pseudomonas*

aeruginosa (Ward's Natural Science, Rochester, NY) grown in NB overnight was also

added to the centre of an MSRV plate as a negative control for this media, while the

isolation control provided the positive control. Using sterile toothpicks, 3 separate streaks

from the outer edge of the MSRV plates were taken and streaked onto MacConkey's agar

(MAC; BD Difco) and incubated at 37 °C overnight. A fresh colony of *E. coli* (ATCC®

25922™, Inverness Medical Inc.) was also streaked onto a MAC plate from each batch

made as a positive control, while the isolation control acted as the negative control for

this media. Two selected colonies appearing clear and colourless on MAC were

transferred to a new MAC plate and streaked for purity. Each of the resulting 12 per site,

or fewer, prospective *S. enterica* isolates was then streaked onto lysogeny broth agar (LB;

BioShop) plates for storage.

Once the prospective *S. enterica* isolate was grown on LB, a single colony was

used to stab and streak a triple sugar iron (TSI; BD Difco) agar slant and double stab a

lysine iron agar (LIA; BD Difco) slant. Both TSI and LIA slants were incubated for 24-

48 hrs at 37°C and assessed for expected presentation (see Appendix B, Table B.1. for

description of the expected biochemical presentation). *Klebsiella pneumonia* (ATCC®

700603™, Inverness Medical Inc.) and *Providencia rettgeri* (ATCC® 9250™, Inverness

Medical Inc.) were used as controls for LIA, and *P. aeruginosa* (Ward's Natural Science)

and *E. coli* (ATCC #25922, Inverness Medical Inc.) were used as controls for TSI. The *S.*

enterica ser. *typhimurium* (ATCC® 13311™, Inverness Medical Inc.) used as the

extraction control served as a control for both TSI and LIA. A full loop, or whatever

remained, of this same isolate from LB used to inoculate the TSI and LIA slants was also

used to inoculate 2 mL of filter sterilized urea broth, which was subsequently incubated

for 24-48 hrs at 37°C. *K. pneumonia* (ATCC® 700603™, Inverness Medical Inc.) and *S.*

enterica ser. *typhimurium* (ATCC® 13311™, Inverness Medical Inc.) were used as

positive and negative controls, respectively, for urea broth.

Colony PCR for Confirmation of S. enterica

All *S. enterica* isolates that produced characteristic presentation on TSI, LIA and urea

broth (see Appendix B, Table B.1.), along with some selected isolates that did not, were

used in colony PCR to confirm the culture-based findings. Template DNA was produced

by suspending an individual colony in 100 μL of 10 mM Tris-HCl (pH 8.0; Sigma-

Aldrich, Oakville, ON) in a 1.7 mL sterile microcentrifuge tube with a hole poked

aseptically in the lid using a sterile 22 gauge needle prior to suspension of culture. The

resulting cell suspension was boiled at 95°C for 5 min and centrifuged at 13,200 g for 5

min. PCR was performed with 5 μL of the supernatant using the primers sal-F (5'-

CGTTTCCTGCGGTACTGTTAATT-3') and sal-R (5'-

AGACGGCTGGTACTGATCGATAA-3') from Lee *et al.* (2006) and obtained from

Sigma-Aldrich. PCR mastermix, based on Lofstrom *et al.* (2004), for this genus-specific

primer set was prepared such that each 25 μL reaction contained 1x Go-Taq™ Flexi

(Promega, Fisher Scientific, Whitby, ON) Green PCR Buffer, 0.5 μM of each primer, 1.5

μM MgCl, 1.5U Go-Taq™ Flexi (Promega), 200 μM dNTP (Promega) and 11.1 μL of

Milli-Q water. PCR was performed using a BioRad™ I-cycler iQ PCR machine (Bio-Rad

Laboratories, Richmond, CA) under conditions consisting of an initial denaturation step

of 94°C for 5 min, followed by 35 cycles of 94°C for 20 sec and 65°C for 1 min, followed

by a 7 min, 72°C extension step. PCR concluded with a 4°C hold until storage at -10°C. *S.

enterica* ser. *typhimurium* (ATCC® 13311™, Inverness Medical Inc.) and *E. coli*

(ATCC® 25922™; Inverness Medical Inc.) were used as the positive and negative

controls, respectively. A PCR blank containing 3 μL of the same Milli-Q water used in

creation of the mastermix was also included.

Colony PCR reaction success was measured by loading 10μL PCR product into

2% agarose gel in 1x TAE buffer. Gels were run for 60 min at 100V, stained with

ethidium bromide solution for 15 min and visualized using BioRad™ GelDock™ XR

(Bio-Rad Laboratories) with amber filter to confirm the presence of an intense 82 bp

band. As these primers were adapted from a real-time protocol, only the intensity of the

expected band denotes either a positive of negative reaction (see Appendix A, Figure A.2.

for an example gel).

CLPP

CLPP for the field-scale site was done within 8 hours of sample arrival using Biolog™

ECOplates™ (Biolog Inc., Hayward, CA), inoculated following considerations presented

in Garland (1997), Garland *et al.* (2007), Calbrix *et al.* (2005), Weber *et al.*, (2007) and

Weber and Legge (2010). Prior to inoculation, samples were spectrophotometrically

analyzed at a wavelength of 420 nm as a means to assess background carbon levels

(Weber & Legge, 2010). If the value was greater than 0.2, the sample was diluted one order of magnitude and analyzed again. Dilution to fulfill this guideline was required for all of the septic tank samples and the Feb. 22nd iron-slag phosphorous filter sample. Original or diluted samples were also spectrophotometrically analyzed at 590 nm to ensure no extreme differences in the absorbance of the inoculated load, as this is the wavelength used to measure plate development. No major differences (i.e., greater that 0.1 OD) were found between any samples at this wavelength. Biolog™ ECOplates™ were inoculated with 150 µL of sample per well, with care taken not to cross contaminate any wells; incubated at 22°C in the dark; analyzed using a SpectaMax 190 (Molecular Devices, Sunnyvale, CA) spectrophotometer and data collected using SoftMax Pro ver. 3.1.2 (Molecular Devices) every 24 hr.

CLPP for the industrial-scale site was done within 8 hours of sample collection using Biolog™ ECOplates™, inoculated following considerations presented in Garland (1997), Garland *et al.* (2007), Calbrix *et al.* (2005), Weber *et al.*, (2007) and Weber and Legge (2010). Due to differences in pH between the sample locations, ranging from 3.6 in the 'PostFloc' to 9.8 in 'Lagoon 3', a cell wash was performed prior to inoculation of the Biolog™ ECOplates™. The cell wash was completed by adding 20 mL of sample to a 50 mL sterile centrifuge tube and centrifuged at 500x g for 10 min. The supernatant was transferred to sterile centrifuge tube and spun at 15,000x g for 20 min. The resulting supernatant was decanted; the pellet re-suspended in 10 mL of sterile 0.85% NaCl; the solution centrifuged again at 15,000x g for 20 min, and the supernatant decanted again. The washed pellet was re-suspended in 5 mL of 0.85% NaCl and 1 mL spectophotometrically analyzed at 600 nm. Using the resulting OD, a portion of the

remaining 4 mL of cell solution was added to a total volume of 16 mL to create an

inoculating solution with 0.01 OD_{600}, or the maximum OD_{600} possible using the entire

remaining 4 mL of cell suspension. Biolog™ ECOplates™ were inoculated, incubated,

analyzed and data collected and exported following the same procedure described for the

field-scale CW.

DNA Extraction

10 mL of the duplicate sampled bench-scale interstitial water or the original seeded

sludge was filtered onto a 22 mm 0.22 μm polycarbonate filter (Millipore), soaked in un-

buffered PCR-grade Milli-Q (Millipore) water, within 3 hours of sampling. Each filter

was placed into a PowerSoil (Mo Bio Laboratories Inc., Solana Beech, CA) bead tube

using sterile forceps, ensuring the surface containing the filtrand was facing the middle of

the tube and accessible to the tube contents. DNA was extracted following the protocol

supplied by the manufacturer.

100 mL of interstitial water from the field-scale CW was filtered onto a sterile 47

mm 0.22 μm polycarbonate filter (Millipore), soaked in unbuffered PCR-grade Milli-Q

(Millipore) water, within 3 hours of sample arrival. Each filter was carefully folded over

itself to conceal the side containing the filtrand and placed into a PowerSoil (Mo Bio

Laboratories Inc.) bead tube using sterile forceps. The filter was then cut into small

pieces using a new, sterile No. 11 blade (Feather, Fischer Scientific, Whitby, ON) on a

sterilize No. 3 handled scalpel for approximately 5 minutes in the bead tube. DNA was

subsequently extracted following the protocol supplied by the manufacturer.

For industrial-scale samples collected in 2009 and 2010, 1 mL of sample was

centrifuged at 13,200 x *g* for 5 min in sterile 1.7 disposable centrifuge tubes, the

supernatant decanted and another 1 mL of sample added, centrifuged and decanted. This was repeated until 10 mL of sample had been pelleted and done once for each duplicate sample. The resulting pellet was re-suspended in a PowerSoil™ (MoBio Laboratories Inc.) bead tube solution and DNA was extracted following a modification of the procedure for wet soil samples supplied by the manufacturer and theory presented in Sambrook and Russel (2001).

Additionally, 10 mL of the industrial-scale samples collected in 2010 were filtered onto a 47 mm 0.22 μm polycarbonate (Millipore) soaked in unbuffered PCR-grade Milli-Q (Millipore) water. Filter-cutting and subsequent DNA extraction was performed in the same manner and the field-scale samples within 8 hours of sample collection.

PCR for DGGE

For all of the samples collected from the three scales, PCR was performed using 5 μL of template DNA with the primers 357f (5'-CCTACGGGAGGCAGCAG-3') with a GC-clamp (5'- CGCCCGCCGCGCCCCGCG CCCGTCCCGCCGCCCCCGCCCG-3') added to the 5' end, and 518r (5'-ATTACCGCGGCTGCTGG-3'), modeled after Ogino *et al.* (2001) and obtained from Sigma-Aldrich. Due to the difference in amount of sample filtered, 10 μL the template from the field-scale site was diluted in 90 μL of 10 mM Tris-HCl (pH 8.0; Sigma-Aldrich) prior to addition to the PCR reactions.

The primer set used is considered a universal set and targets the hyper-variable V3 region of bacterial 16S rDNA (Muyzer *et al.*, 1993). PCR mastermix for this universal primer set was prepared such that each 50 μL reaction contained 1x Go-Taq™ Flexi (Promega) Green PCR Buffer, 0.5 μM of each primer, 1.5 μM MgCl, 1.5U Go-

Taq™ Flexi (Promega), 200 µM dNTP (Promega) and 21.3 µL of Milli-Q (Millipore)

water. PCR was performed using a BioRad™ I-cycler iQ PCR machine (Bio-Rad

Laboratories). Touch-down PCR conditions were based on those described in Muyzer *et*

al. (1993) and consisted of an initial denaturation step of 94°C for 5 min, followed by 20

cycles of 94°C, 65°C and 72°C for 1 min each, in which the annealing temperature of

65°C was decreased by 1°C every 2 cycles to a temperature of 56°C on the 20th cycle. Ten

additional cycles of 94°C, 55°C and 72°C for 1 min each followed. PCR concluded with a

7 min, 72°C extension step and was held at 4°C until storage at -10°C. PCR reaction

success was measured by loading 10 µL PCR product into 1.5% agarose gel in 1x TAE

buffer. Gels were run for 60 min at 100 V, stained with ethidium bromide solution for 15

min and visualized using BioRad™ GelDock™ XR (Bio-Rad Laboratories) with amber

filter to confirm the presence of only a 233 base-pair band in sample wells and absence of

any bands in the blank, which consisted of the 5 µL of the same Milli-Q water used in

creation of the mastermix instead of template DNA.

DGGE Conditions

For all of the samples collected from the three scales, DGGE was performed following

the methods and rationale presented by Green *et al.* (2009) with slight modification. 8%

(wt/vol) acrylamide gels containing a linear denaturant gradient ranging from 40 to 65%,

with 100% denaturant defined as a solution of 7M urea and 40% formamide. Gels were

run for 17 hrs at 70 V (1190 V·hr) using a CBS Scientific™ DGGE-2401 machine (CBS

Scientific Inc., Del Mar, CA) set to a constant temperature of 60°C. 15 µL of sample PCR

product was added to each lane, allowing free lanes for the DGGE ladder.

DGGE Ladder Creation

DGGE ladder was created using 8 cloned sequences from various environmental samples

known to move distinctly and consistently through the DGGE gel. These cloned and

purified sequences were obtained from Dr. Josh Neufeld (University of Waterloo) and

amplified individually using the 357f-gc and 518r primer set and PCR reaction conditions

described in the PCR for DGGE subsection (see pgs. 23 & 24), modified to include 3 μL

of template instead of 5 μL. The remaining 2 μL volume was replaced by increasing

Milli-Q water to 23.3 μL. The ten PCR reactions were pooled post-PCR to create 500 μL

of DGGE ladder and this was used in all DGGE gels, which was diluted by adding 500

μL 10 mM Tris-HCl (pH 8.0; Sigma-Aldrich). 10 μL of the ladder was added to a central

lane and both outside lanes.

DGGE Image Acquisition

Gels were stained with 1x SYBR Gold solution (10,000x stock diluted in 1x TAE;

Invitrogen, Burlington, ON) for approximately 1 hr. Gels were then placed in a BioRad™

Gel Doc™ XR (Bio-Rad Laboratories) and flooded with deionized water before being

photographed with BioRad™ SYBR Gold filter (Bio-Rad Laboratories). The resulting

image was captured using Quantity One® software (Bio-Rad Laboratories), ensuring the

gel was exposed for less than the time required to produce saturated pixels. The image

was then exported to an 8-bit .tif file, excluding overlays, and saved at the scan resolution

(2879 dpi) and size of 1360 x 1024. See Appendix A, Figure A.1. for an example DGGE

gel.

3.3. Data Analysis

HPC and E. coli Analysis

HPCs and *E. coli* enumeration were converted based on dilution factor, expressed as CFU per mL and averaged across all countable plates for each sample location at each sampling event. HPCs were enumerated at both 5 and 7 days of incubation, but only the count from the 7th day was used in analysis. *E. coli* was enumerated at 20, 24 and 48 hrs of incubation, but only the 24 hr count was used as numbers did not change at 48 hrs.

S. enterica Analysis

Due to inclusion of two enrichment steps in the isolation protocol, *S. enterica* isolation is considered to be qualitative only. Also, only lactose negative isolates were considered as being prospective. Prospective isolates were considered those that displayed typical or near-typical presentation (i.e., lacking one or two of the expected biochemical characteristics) on LIA and TSI. Prospective isolates were confirmed as positive or negative based on the results from colony PCR. Isolation results were coded as positive if one or more positive *S. enterica* isolate was found in a given sample location on a given event, or negative if none were found.

CLPP Analysis

The metric for use in analysis of the field-scale CW Biolog™ ECOplates™ was chosen to be a time point of 48 hours after incubation and the metric for use in analysis of the industrial-scale Biolog™ ECOplates™ was chosen to be a time point of 72 hours after incubation, following rationale presented in Weber and Legge (2009). As BIOLOG™ ECOplate™ contains each well type in triplicate, the average of the three blank wells was used in the correction of each well. Matrices of the raw and blank-corrected data were

constructed with each of the carbon sources treated as a variable, the sample location treated as an object and absorbance of a particular well used as values in matrix construction. Negative well responses were coded as zeros during data treatment according to Weber *et al.* (2007). The blank-corrected matrix using the average of the triplicates was transformed using a Ln+1 and Taylor power-law transformation to check if either enhanced normality, measured through skewness and kurtosis of the variables, or homoscedasticity, assessed through the variance ratio (Legendre & Legendre, 1998; Weber & Legge, 2010) of the data. A qualitative matrix was also created, coding the number of wells with a blank-corrected absorbance greater than 0.25 OD_{590} as a 1, and the corrected absorbance less than 0.25 OD_{590} as a zero. Each qualitative vector was summed and used as a measure of substrate richness, which was also used in calculation of the CLPP-based diversity indices.

First described by Zak *et al.* (1994), the carbon source utilization patterns (CSUPs) provided by BIOLOG™ ECOplates™ can be used to calculate a number of metrics and have been found to provide reliable data for multivariate analysis. the Shannon's *H'* (Shannon, 1948) and the result considered a measure of the diversity of carbon-based substrate utilization for a given community. *H'* from a CSUP can be calculated as:

$$H'_{CLPP} = -\sum p_i \ln(p_i) \qquad\qquad \text{(eq. 1)}$$

Where:
        H'_{CLPP} - substrate diversity
        p_i - ratio of the activity of a particular substrate to the sum of activity of all substrates

One Dimensional Community Functional Divergence

A one dimensional community functional divergence measure was calculated according

to Weber and Legge (2009) using the triplicate-averaged blank-corrected CLPP data. The

Euclidean distance measure was used in this study as a measure of dissimilarity of the

CSUPs gathered for any one location at a time point, in comparison to the original day 0

CSUP for that location. For the field-scale CW data the last sample prior to antibiotic

exposure, collected Feb. 22nd, was used as the day 0 from which divergence was

calculated. Additionally, the Euclidian distance from each previous time point was also

calculated as a measure of the amount of total change over time. Euclidean distance can

be calculated in n dimensions, however, in this case the Biolog™ ECOplates™ provide

an $n=$ 31 (i.e., 31 different carbon sources).

Given the two points:

$$P = (p_1, p_2, ..., p_n) \,\&\, Q = (q_1, q_2, ..., q_n)$$

Where (for the field-scale CW):
  P= functional profile at day 0, 8, 15, or 49
  Q= functional profile at day 8, 15, 29, 49, or 73 from start-up

The Euclidean distance was calculated as:

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

(eq. 2)

One dimensional community functional divergence measure was also calculated

for the industrial-scale lagoon and the Sept 21st, 2009 sample was used as the day 0 from

which divergence was calculated. As there was no available post-flocculation sample

available on this day, substrate divergence was not calculated for this location.

DGGE Analysis

DGGE images were loaded into GelComparII (Applied Maths, Austin, TX) software

following the manufactures instructions. Bands were detected using the automated band

detection algorithm provided in the program, set to the minimum cut-off that detected

only the expected bands in the ladder-containing lanes. The resulting band detection

output was examined using the original image to ensure only bands clearly visible to the

unaided eye in the original picture were selected and that bands were not placed in areas

which contained peaks due to dust or other image inconsistencies. Bands that appeared to

the unaided eye but were over looked by the band search algorithm were excluded to

ensure consistency regarding the treatment of background intensity. Densitometric peaks

for each banding pattern in each lane were exported into text files conferring both band

movement (i.e., number of pixels from the top of the gel; R_f) and relative band intensities

(i.e., intensity of each band compared to total intensity of each lane, for each separate

lane). This output was transferred to Microsoft® Office Excel™ 2007 for anchor

normalization and matrix construction.

Sample band movement was anchored to the first and eighth ladder bands, (i.e.,

the bands with the largest and smallest R_f valves within each 40-65% gel), following

theory presented in Tourlomousis *et al.* (2010). Although the ladder was made with 10

bands, only eight were expected, as the 9th and 10th bands denatured between 65 and 70%

denaturant. Gels where 9 or more standard bands appeared were excluded from analysis.

In all cases the ladder was run on the furthest left lane, the centre lane, and the furthest

right lane, with movement anchoring completed separately for the right side and left side

of the gel. This ensured that for sample bands on the left side of the gel the average

movement of both the centre ladder and the left-most ladder positions were used to set the 1 to 1000 movement scale. This process was similarly repeated for the right side of the gel using the right-most and center lanes. Some sample fragments were found to lie outside the ladder movement range, both below and above the ladder on the gel, so a correction factor of 1500 was added to all movement values to prevent any negative integers in the data set. This resulted in all final movement values lying between 0 and 2500. Each band was then classified into different movement groupings in blocks of 50 (i.e., 1-50, 51-100, 101-150 etc.) giving a total of 50 groupings. Each of the 50 groupings was then treated as a variable, with the sample site treated as an object and, for what will be referred to herein as 'quantitative' , relative band intensities used as values in matrix construction. A qualitative matrix was also created, coding the presence of each band as a 1, and the absence as a zero. Each qualitative vector was summed and used as a measure of genetic richness, or the number of operational taxonomic units (OTUs), which was also used in calculation of the diversity indices to be described.

The quantitative matrix was transformed using a Ln+1 and Taylor power-law transformation to check if either enhanced the normality, measured through skewness and kurtosis of the variables, or homoscedasticity, assessed through the variance ratio (Legendre & Legendre, 1998; Weber & Legge, 2010).

Diversity indices were also calculated using the relative band intensity as a measure of sequence proportion in the original sample (Ibekwe et al., 2007). Both the Shannon index of diversity (H'; Shannon, 1948) and probability of interspecific encounters (PIE; Hurlbert, 1971) were calculated.

H' can be calculated as:

$$H'_{DGGE} = -\sum p_i \ln(p_i)$$ (eq. 3)

Where:

H'_{DGGE} = genetic diversity

p_i = ratio of the intensity of a particular band to the sum of bands in a given lane

PIE can be calculated as:

$$PIE_{DGGE} = \left[\frac{S}{S-1}\right]\left[1 - \sum p_i^2\right]$$ (eq. 4)

Where:

PIE_{DGGE} = probability of two repeated samples producing different species

p_i = ratio of the intensity of a particular band to the sum of bands in a given lane

S = genetic richness (i.e., number of identifiable bands in sample lane)

Rank-abundance Plots

Rank-abundance plots were created using the average of the duplicate relative band intensities plotted in order of decreasing intensity for each of the R_f movement group (i.e., group of 50 pixels) at each time point. Graphs for each system location or mesocosm treatment type are seperated to assist visualization of changes in the community profile.

Averaged Banding Pattern

A plot of the averaged banding pattern from the DGGE profiles was created in order to visualize both the changes in relative abundances of the observed OTUs, while conserving the information regarding the R_f movement group (i.e., G+C content) of the OTUs.

One Dimensional Community Genetic Divergence

A one dimensional community genetic divergence measure was also calculated for all three scale according to Collins *et al.* (2000) and Weber and Legge (2009), modified to

replace 'substrate' divergence with 'species' divergence. The Euclidean distance was calculated using the untransformed quantitative data set, for a given mesocosm, to determine the dissimilarity of each time point to the original day 0, as a measure of overall divergence. Euclidean distance can be calculated in n dimensions, and for the DGGE-derived data the groups of band R_f values provided an $n= 50$. Euclidean distance was calculated using Equation 2.

For the bench-scale mesocosms, the sample take on Nov. 16th was considered the day 0 from which divergence was calculated. For the field-scale CW, the last sample prior to antibiotic exposure, taken on Feb. 22nd, 2010, was used as the day 0 from which divergence was calculated. Divergence calculated between the Feb. 8th and 22nd samples was used as a measure of undisturbed genetic divergence. For the industrial-scale lagoon, the June 24th, 2009 sample was used as the day 0 from which divergence was calculated. Additionally, the Euclidian distance from each previous time point from each investigated treatment system was also calculated (i.e., if the bench-scale, Nov. 16th from Nov. 23rd, Nov. 23rd from Nov. 30th, etc.) as a measure of the amount of total change over time. For all sites the first sample used was that considered to be day 0 in the previous calculation of functional divergence.

Multivariate Statistical Techniques

Principal component analysis (PCA) for the bench-scale mesocosms was completed with the Taylor-transformed averages (b= 1.3363) from the DGGE data set using Statistica 8.1 (Statsoft, Tulsa, OK). Field-scale CW PCA was completed using the quantitative Taylor-transformed, blank-corrected (b= 1.104) CLPP means and Taylor-transformed (b= 1.396) DGGE data set. PCA for the industrial-scale lagoon was completed for Taylor

transformed (b= 0.9606), blank-corrected CLPP and Ln+1 transformed DGGE data sets.

PCs were extracted from the covariance matrix of the data with any zero vector spaces

excluded. Only visualization around the first 3 PCs for each analysis are presented in the

plots shown. For general background of the interpretation and a description of

characteristics of the PCA performed see James and McCulloch (1990), Legendre and

Legendre (1998), and Jung and Marron (2009).

Tree cluster analysis using untransformed CLPP and DGGE means from all three

sites was also done with the aid of Statistica 8.1 (Statsoft, Tulsa, OK), with linkage

distances calculated by the unweighted pair-group method (UPGMA) using Euclidian

distances (Jolliffe, 2002).

Data Presentation

The bacterial enumerations and CLPP-derived indices calculated in this study are

represented as time course data in the form of line-graphs. The DGGE-derived indices

calculated in this study are represented as time course data in the form of bar-graphs and

line graphs, depending on which gave clearer visualization of trend behaviour. For the

bench-scale CW site, the average of the mesocosms exposed to ciprofloxacin and those

not exposed to ciprofloxacin are shown in the different plots for each metric calculated.

Error bars for the bench-scale are included in the time course plots and denote two

standard deviations (one on either side of the data point). For the field- and industrial-

scale sites time course data is separated into spatially-based objects based on the location

within the wetland that the sample was taken. Error bars expressing standard deviation in

the field- and industrial-scale DGGE-derived plots was not possible due to the low

number of observations (i.e., 2). Error bars expressing standard deviation in the field-

and industrial-scale CLPP-derived plots were left out to ease visualization.

4.   Bench-scale Constructed Wetland Mesocosm Results and Discussion

In order to address part of the overall goal of this research, to profile the structural and functional characteristics of predominant microbial communities in CWs at multiple system scales, genetic structure in the bench-scale CWs was monitored using DGGE. The functional profiles of these CWs were assessed by Dr. Kela Weber and are not included in this document. The impact of an antibiotic contaminant (i.e., ciprofloxacin) was also investigated to better understand the response of bench-scale CWs microbial communities to perturbation.

4.1. Structural Fingerprinting

DGGE fingerprinting was completed on all samples taken, resulting in a total $n= 42$; 5 time points by 4 constructed wetland (CW) mesocosms plus the sludge inoculum, sampled in duplicate. Figure 4.1 displays the trends of the genetic richness, defined as the number of bands above background observed in DGGE gels. Bands are commonly referred to as operational taxonomic units (OTUs; Sneath, 2005). The use of the term OTU maintains that DGGE bands may not be indicative of a specific taxon, but instead groups possessing similarity within some characteristic. In this case that characteristic is the melting point of V3 region 16s rDNA; a melting point which differs with sequence identity and the region that confers phylogenetic information (Woese, 1987). For the purpose of this discussion, a band will be considered a unique OTU, thereby denoting a wider variety within the community with increasing band number.

From the samples collected prior to addition of the ciprofloxacin, there did not appear to be a difference in the number of detectable OTUs between the four mesocosms,

although all four appeared to have an increased number of OTUs from the initial sludge inoculum. This indicates that there may have been genetic contribution to the mesocosm community from the *Phragmites australis* associated bacteria, or any bacteria that may have been associated with construction materials. The 'Cipro' samples taken immediately following ciprofloxacin removal showed little difference from the control condition. The lack of differences following acute antibiotic exposure was surprising, as susceptible organisms should have been affected based on the concentration used (Thauvin-Eliopoulos & Eliopoulos, 2003). However, the 'Cipro' CW contained on average 7 OTUs more than the 'No Cipro' mesocosms on Jan. 4th. Additionally, samples taken on Jan. 18th continued to display more detectable OTUs in the 'Cipro' mesocosms than the 'No Cipro' mesocosms.
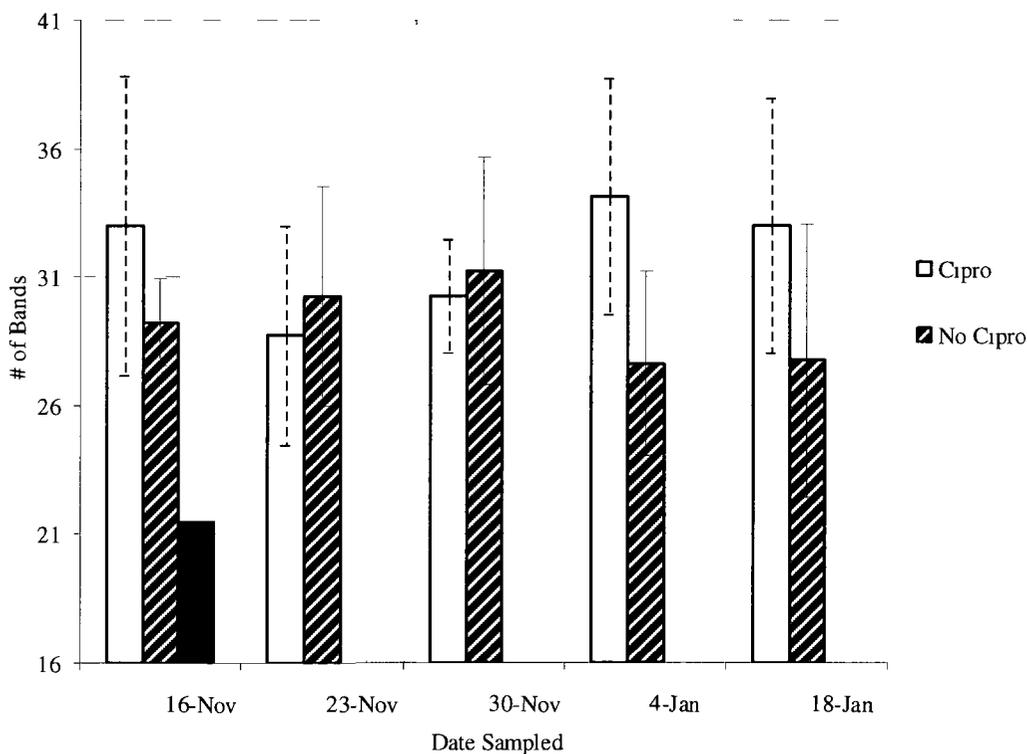
Figure 4.1. DGGE-based community richness (# of bands) from the bench-scale CW. Error bars indicate 1 standard deviation on either side of the mean. The grey box is added to assist visualization ciprofloxacin exposure. The sludge inoculum is denoted by the solid black bar.

The apparent lack of immediate effect of the antibiotic on Nov. 30th and the increase in OTUs on Jan. 4th may have several possible explanations. The primary mechanism of ciprofloxacin removal is thought to be photodegradation (Cardoza *et al.*, 2005), which would have been entirely absent due to the construction design of the CW mesocosms. If not photodegraded, ciprofloxacin is known to bind preferentially to fine organic matter and it is unknown if the antibiotic remains active despite sorption (Belden *et al.*, 2007). Additionally, ciprofloxacin has an expected half-life in the environment nearly 3 times that of its half-life in humans (i.e., 12 hrs vs. 4 hrs), and has been observed to persist for 16 hrs, the entire length of the study, at nearly the entirety of the amount added in dark water systems (Belden *et al.*, 2007). Therefore, one possible reason for the lack of decrease in OTUs observed immediately following antibiotic removal and the subsequent increase one month later may be due to an increased detachment from the gravel-root substrate by community members, resulting in higher representation in the sampled interstitial water. The compensatory effect on richness estimates of the increased detachment from the CW mesocosm matrix into the sampled interstitial water may have therefore hidden the antibiotic-related loss of susceptible OTUs following acute exposure on Nov. 30th. Sampling of the attached biomass may be able to clarify the observed effects; however this would result in sacrifice of the mesocosm and a cessation of further monitoring.

Similarly, by Jan. 4th, any residual antibiotic should have become further bound or degraded by the CW. Continued detachment from areas still containing ciprofloxacin that may have remained active due to the lack of light available to the systems may be involved. Attempts to survive and colonize the nutrient-rich interstitial water by the

remaining organisms may be responsible for the observed increase in OTUs above that of the 'No Cipro' condition. Populations that would have otherwise remained un-sampled, as part of the CW mesocosm matrix, or at levels below detection would now be present in the interstitial water of the 'Cipro' condition. The convergence of the OTUs estimates visualized in the final sample may have been indicative of the microbial community in the 'Cipro' condition beginning to colonize the previously unfavourable gravel-root matrix; while the now established, attached populations in the control condition have begun to shed older members into the interstitial water. This convergence may also be due to the extinction of populations that were unable to colonize the antibiotic-laden matrix and also unable to survive while suspended due to increased competition present in the 'Cipro' interstitial water.

To further explore the behaviour of the bench-scale CW mesocosm, two diversity indices were calculated; the Shannon index of diversity (H'; Shannon, 1948) and the probability of interspecific encounters (PIE; Hurlbert, 1971). Both of these are represented graphically in Figure 4.2. Diversity calculated from a genetic fingerprinting method can be interpreted as a measure that considers both the number of distinct OTUs and the relative proportion with which they are observed; where communities with a greater number of more, evenly represented phylogenetic groups are consider more diverse than those with fewer, less evenly represented groups.

Like the estimates for the number of OTUs, there was an apparent difference in genetic diversity between the initial sludge inoculum and the mesocosms prior to antibiotic exposure for both indices. Additionally, there was no apparent difference with regard to either genetic diversity estimate for the samples taken immediately following

removal of the antibiotic, or even after one month following. The observed genetic

diversity may be due, in part, to the same detachment mechanisms suggested previously,

which would have maintained an increased proportion of the CW microbial community

in the interstitial water despite the presumed loss of susceptible organisms from the

matrix. However, the lack of differences in genetic diversity was also a result of the

decreasing diversity trend in the 'No Cipro' mesocosms observed over the first 49 days of

development. Although the microbial community in the control mesocosms would not

have been subjected to antibiotic stress, the competition present may have caused an

accentuated lag-like phase, as the various populations would have been struggling to

colonize niches within the 'No Cipro' CW matrix; resulting in increased biosynthesis of

cellular products to assist adhesion, and depressed investment given to replication

(Brading *et al.*, 1995; White, 2003).

The only observable difference between the two conditions using genetic diversity

indices was found between the PIE values for Jan. 18th, indicating that there may have

been a notably more genetically diverse community present in the control CW

mesocosms by the end of the investigation period. This trend toward more variety in the

number and relative proportion of OTUs on Jan. 18th was also seen through *H'*, although

not as pronounced using that index. Intuitively, this difference can easily be attributed to

the antibiotic-related loss of susceptible organisms. However, when considered along

with the lack of differences in genetic richness estimates, the response of the CW

community to the ciprofloxacin contaminant becomes more complex.

The 'Cipro' mesocosms displayed a decreasing trend in genetic diversity

estimates despite increases in number of observed OTUs; while the undisturbed

A

B
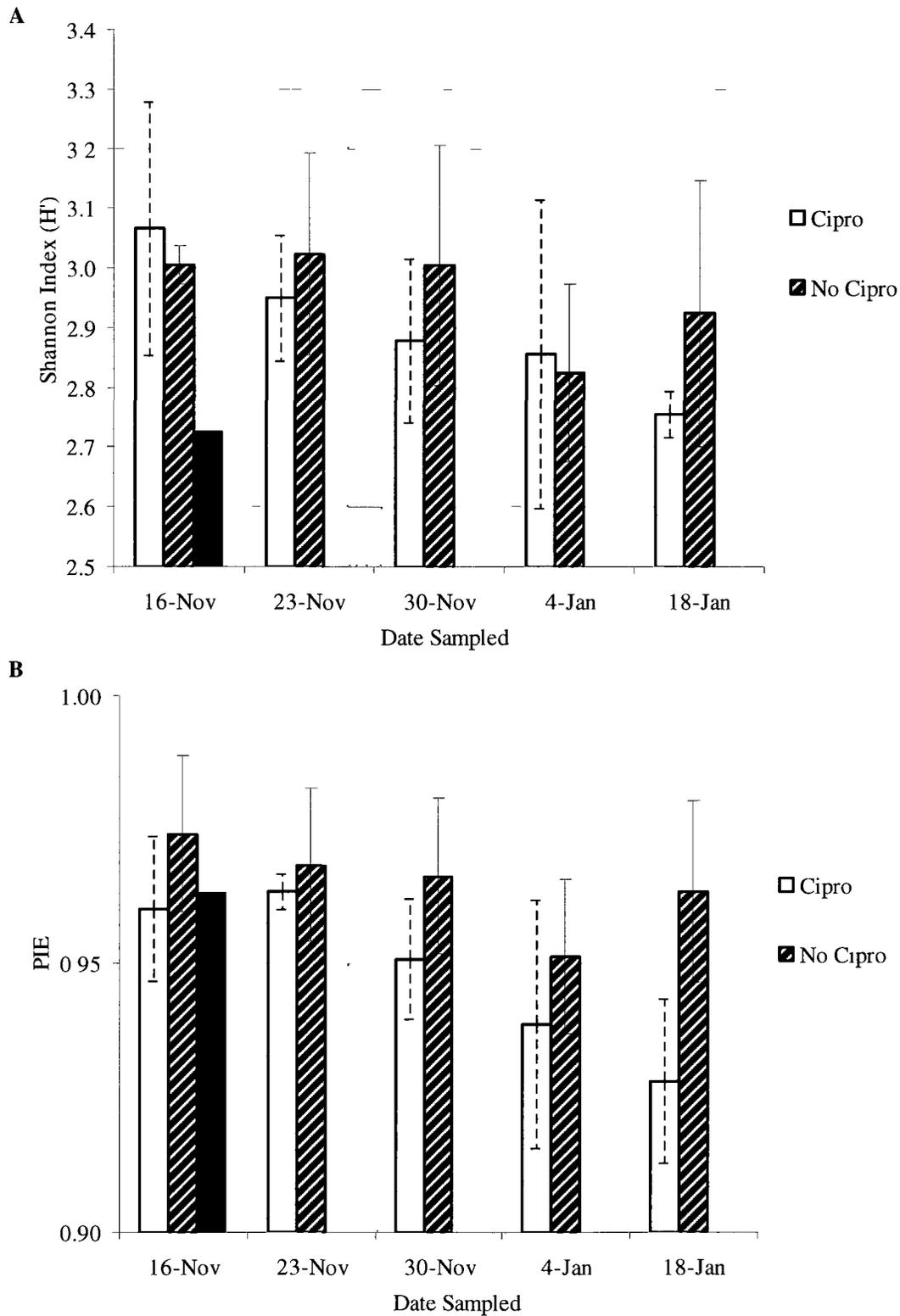
Figure 4.2. DGGE-based community diversity from the bench-scale CW. A) Shannon index (H'); B) Hurlbert index (PIE). Error bars indicate 1 standard deviation on either side of the mean The grey box is added to assist visualization of exposure to ciprofloxacin. . The sludge inoculum is denoted by the solid black bar.

mesocosms displayed both decreasing diversity in the representation and number of phylogenetic groups over the first two months. Colonization of the CW gravel-root matrix in the 'No Cipro' control condition would largely favour the organisms which are biofilm pioneers, have increased surface hydrophobicity, require close-association to a biofilm or produce pili, capsules, slime-glycolipoprotien or other adherents that allow attachment to the substrates present in the mesocosms (Brading *et al.*, 1995). These types of microorganisms, which would not necessarily be adapted to suspended conditions in the open water and would quickly disappear from the sampled interstitial fraction in favour of a more suitable niche; leaving the organisms preferring suspension (i.e., organisms that are motile or do not produce adherents that will allow adhesion to mesocosm substrates) to dominate the sampled community, thereby resulting in lower number and relative proportion of OTUs in the genetic fingerprint. The attached community may have remained below detection in the interstitial water until the biofilms became aged; as the rate of bacterial shedding in a developing biofilm is less than that in mature biofilms (Brading *et al.*, 1995). Therefore, the increasing trend in both number of observed phylogenetic groups and the relative similarity of their representation seen in the final interstitial sample, taken after two months of development, may be a result of increasing biofilm shedding in the control CW mesocosms. Continued sampling would be required to confirm this explanation.

Conversely, in the 'Cipro' mesocosms, guilds belonging to the early biofilm colonizers would have been allowed to inhabit niches during the first week of undisturbed development. However, upon ciprofloxacin addition, some microorganisms (i.e., those not yet deeply rooted in the week old biofilm) may have been forced into

resuspension to avoid the antibiotic, while others would undergo an increased stress-response to deal with it or, if neither was possible, cell death. Any of these responses could result in perturbation to the normal progression of development in the community. As mentioned earlier, the combination of the negative antibiotic-related cell death and compensatory detachment-related gain of both the amount and types of previously attached organisms is likely the reason why little acute response to antibiotic exposure was observed in the genetic fingerprints. The continued decline in diversity, along with the rise and fall of richness estimates, may be indicative of a re-colonization of the 'Cipro' CW matrix once the antibiotic was degraded or no longer present, or by prevailing microorganisms or tolerant survivors continuing on with previously delayed CW community development following cessation of the perturbation. Continued investigation of the microbial community in this system may elucidate whether this antibiotic-based perturbation during development is being overcome by the surviving community.

It should be noted that *P. australis* growth continually deteriorated following antibiotic exposure, eventually undergoing chlorosis and eventual death by day 100 from start-up (Dr. Kela Weber, personal conversation, September 27th, 2010). Additionally, CW porosity was found to be much less in the 'No Cipro' condition until plant death; at which point 'Cipro' porosity began to decrease substantially (Dr. Kela Weber, personal communication, September 27th, 2010). The initial decrease in porosity shown by the 'No Cipro' CWs provides evidence for the natural development of a biofilm matrix via increased attachment.

The use of richness and diversity estimates for phylogenetic community structure, although easily testable and comparable, provide a complex picture of the community changes and can be subjected to multiple interpretations depending on the approach used to analyze them. An example of this subjectivity is provided within this chapter, as PIE produced a large visual difference on Jan. 18th beyond that of the error bars, while *H'* did illustrated only a minor difference. In order to clarify this discrepancy, as well as provide a more detailed illustration of the changes within community structure that resulted from both exposure to ciprofloxacin and the natural genetic development of a bench-scale CW, rank-abundance plots were created (see Figure 4.3). Relative band intensity of the OTUs was used to describe abundance.

Based on the rank-abundance curves in Figure 4.3, there did not appear to be any major differences with regard to the overall population structure detected in the four mesocosms prior to ciprofloxacin exposure. The original sludge inoculum displayed a relatively even dominant population structure, with 8 OTUs contributing to greater than 5% of the relative-abundance. The evenness of the population appeared to decline continually over the first week of mesocosm development. Like in the previous metrics, there did not appear to be a discernable impact of acute antibiotic exposure. There did, however, appear to an impact visible one month after removal of the contaminant as the 'Cipro' depicted much less evenness in comparison to the 'No Cipro' mesocosms. Jan 18th, maintained the decrease in evenness, and displayed the clear appearance of a single, very dominant OTU, seen by the change in the size of the 1st ranked OTU between the 'Cipro' and 'No Cipro' conditions on the final day. The most dominant OTU in the 'Cipro' mesocosms was also nearly twice the size of the most dominant OUT in the
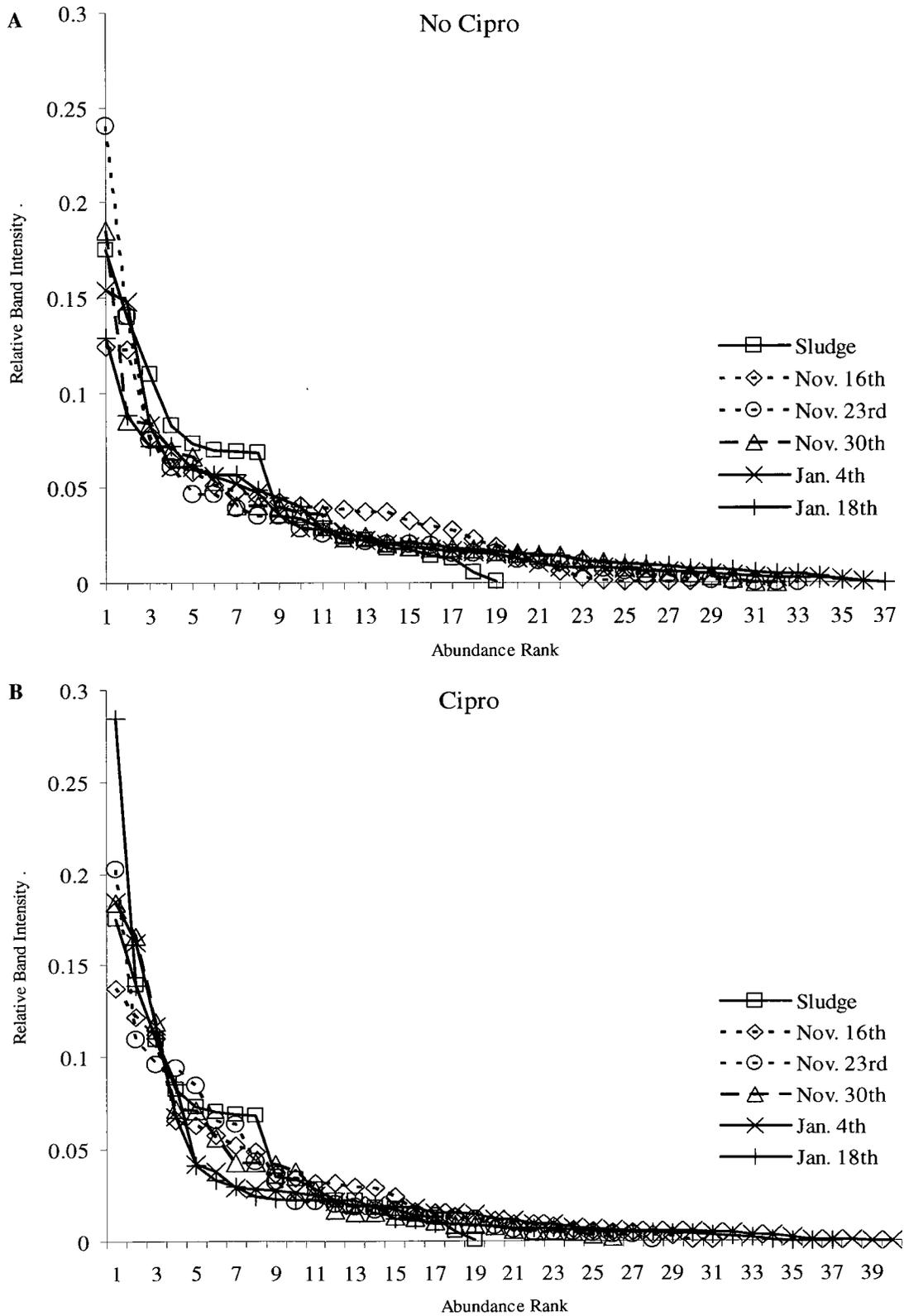
Figure 4.3. DGGE-based rank-abundance curves for the bench-scale CW. A) The 'No Cipro' condition and B) The 'Cipro' condition. Ciprofloxacin was administered between the Nov. 23rd and 30th samples.

control. There also appeared to be a slight decrease in the size of the 4th through 8th ranked OTUs, as there appears to be a much sharper decline in the rank-abundance curve of the 'Cipro' mesocosm compared to the 'No Cipro' in the final two samples collected. This indicates that there was an clear effect of ciprofloxacin exposure on the evenness of the microbial community structure.

Additional information regarding the microbial community structure may also be contained in the G+C content possessed by the groups that appeared to change in Figure 4.3. To visualize this difference, Figure 4.4 depicts the averaged relative abundances from the genetic profile, maintaining the movement of these locations in the DGGE gel. The combination of OTUs with similar G+C content in ribosomal DNA into the same groups accounts for some of the error associated with band movement in DGGE gels and accounts for the fact that groups with similar G+C content have been considered to be phylogenetically similar (Wayne *et al.* 1987).

Based on the averaged banding pattern plots, the sludge used for inoculation of the CWs appears dominated by two large peaks, each comprising over 15% of the lane intensity, and three or four moderate peaks which were all between 5 to 10% of the lane intensity each. After the first week of development all four mesocosms developed similarly. The immediate appearance of a novel and abundant OTU near the 15th movement group, as well as novel bands with higher G+C content after a day suggests a large influence of bacterial populations not associated with the sludge inoculum on the initial community composition of the bench-scale CW. By the end of the first week, on Nov. 23rd, the disappearance of the previously novel high C+G bands, along with the appearance of new low G+C bands, suggests a waxing and waning of different populations above and below
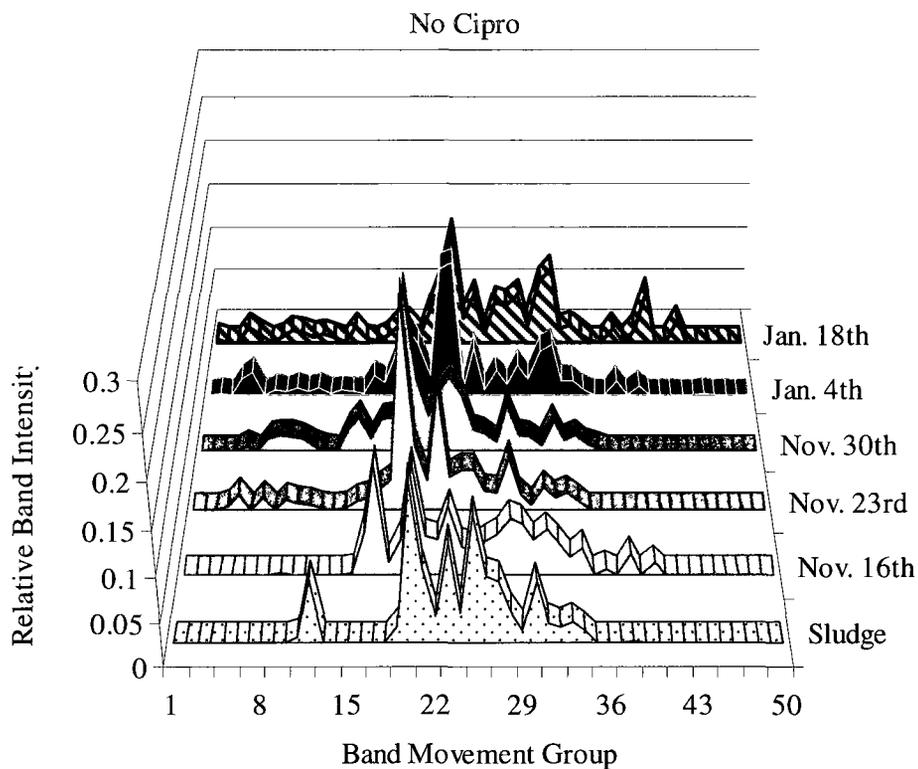
A

No Cipro

B
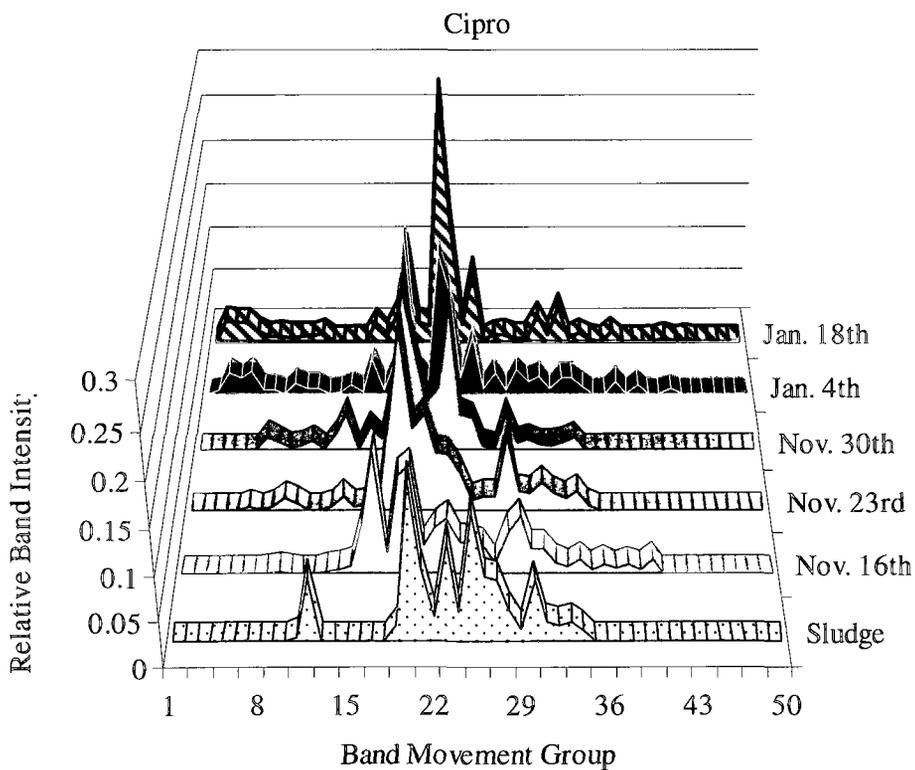
Cipro

Figure 4.4. DGGE-based averaged banding pattern for the bench-scale CW. A) The 'No Cipro' condition and B) The 'Cipro' condition. Ciprofloxacin was administered between the Nov. 23rd and 30th samples.

detection thresholds among the moderately common OTUs during initial development of the mesocosm; indicating that phylogenetically distinguishable groups play distinct roles in development of the CW mesocosm and that the representation of these groups in the interstitial water varies temporally; a well documented phenomenon in other types of biofilms (Brading *et al.*, 1995).

Immediately following ciprofloxacin exposure on Nov. 30th, both experimental conditions appeared very similar, with the dominant OTUs remaining within similar movement groups. There is, however, a slight difference at this point regarding the visual evenness between the two conditions, as the 'No Cipro' CWs displayed a minimal but widespread increase in abundance of all the observed OTUs. Both conditions, however, still portrayed a community dominated by the two phylogenetic groups with moderate C+G content, located around the 20th and 22nd movement groups. It should be noted that these OTUs were dominant even in the original sludge sample, indicating that the sludge inoculum was able to provide considerable contribution to the CW mesocosms despite the differences in observed OTUs seen in Figure 4.1.

By Jan. 4th, the reappearance of low and high G+C OTUs in both CW conditions can be observed. The 'No Cipro' condition displayed a noticeable decrease in relative intensity of one of the two dominant OTUs at this point, coupled with an increase in the intensity of bands near the 30th movement group. The Jan. 18th sample produced two visually different communities, supported by the difference in PIE values and rank abundance plots presented above. Although the 'No Cipro' and 'Cipro' conditions displayed a community with moderately abundant OTUs and the most prominent OTU in similar R_f groups, the relative intensity of this OTU in the 'Cipro' condition was nearly

double that of the same OTU in the control condition. Additionally, the 'No Cipro' CW displayed a very even population as ten OTUs contributed between greater than 5% of the relative community abundance and six contributed more than 3%; in contrast to the six and three contributing similar proportions, respectively, from the 'Cipro' CW. It is evident from the average banding patterns that there are differently structured communities with regard to the size of the non-dominant OTUs found within the two treatment groups.

The differences in PIE values and appearance of the rank-abundance curves and averaged banding patter plots displayed emerging differences in the final community structures between the two experimental conditions with regard to community evenness. However, it is also interesting to look at the amount of this change, the rate at which the community change is occurring and how the rate changes over time. In an attempt to produce a simple metric for comparison that would easily address changes in sample variation genetic divergence curves were calculated in Figure 4.5. The curves represent the total amount of divergence in a single dimension (i.e., the Euclidian distance) between each sample; based largely on Collins *et al.* (2000), Weber and Legge (2009) and through personal communication with Dr. Kela Weber (various dates, 2008-2010). The amount of structural community divergence from the initial community was monitored by measuring change between each sample and the first sample taken on Nov. 16th (see Figure 4.5.A), similarly to Weber and Legge (2009) but using DGGE data instead of CLPP. Additionally, the genetic divergence between each sample and the sample taken immediately previous (see Figure 4.5.B) was calculated. The slope of the
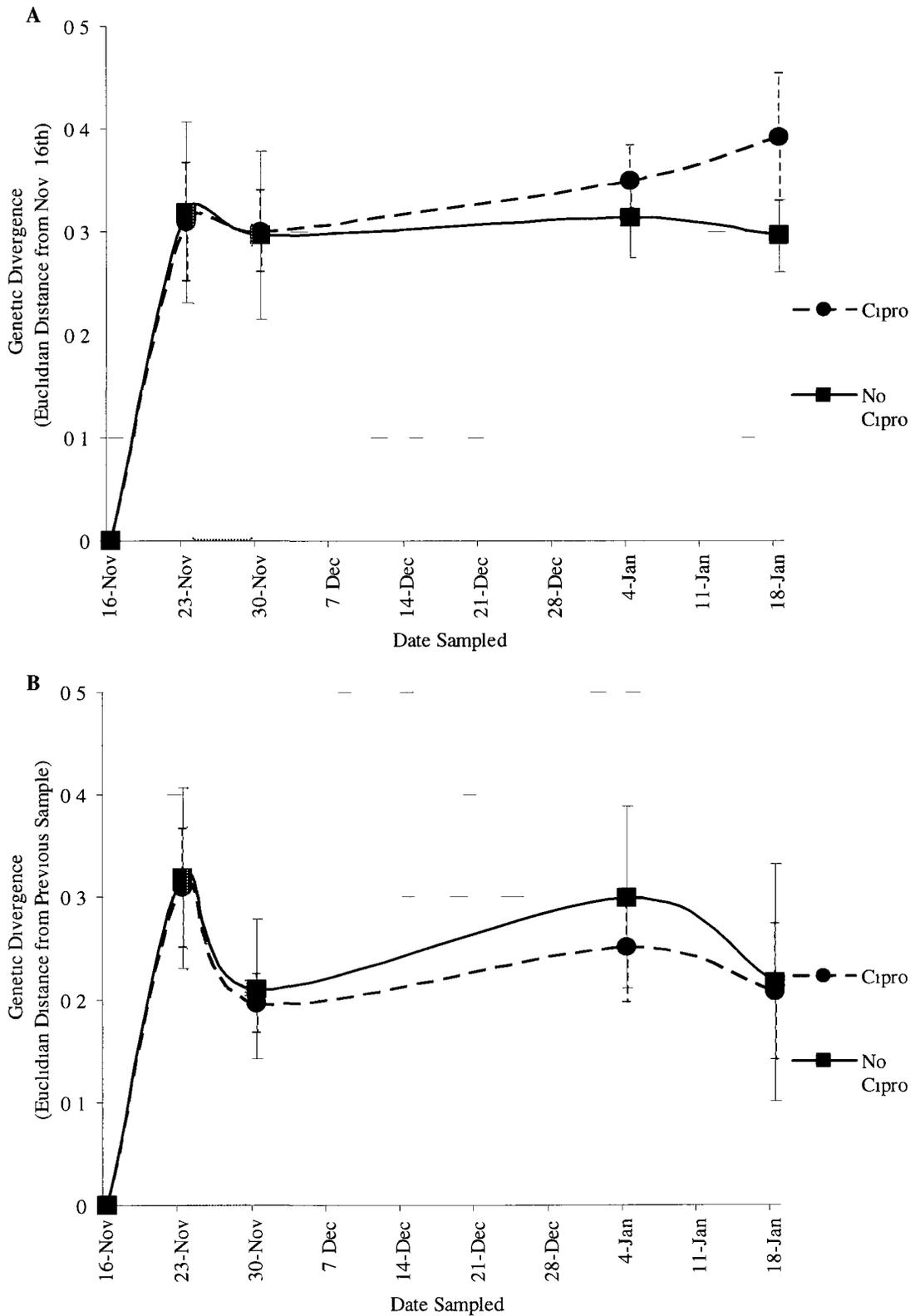
Figure 4.5. Genetic divergence based on Euclidian distances of untransformed DGGE profiles from the bench-scale CW A) Divergence from the first sample taken on Nov 16th and B) Divergence from the previous sample Error bars indicate 1 standard deviation on either side of the mean The grey box is added to assist visualization of exposure to ciprofloxacin

line in Figure 4.5.B would be indicative of the rate of structural or genetic change of the community over that time period.

Figure 4.5.A displayed a very similar divergence from the initial microbial community for both treatments, prior to and immediately following the antibiotic. However, by the final sample on Jan. 18th the 'Cipro' CWs displayed a continued divergence from the initial community, while the control condition appeared to maintain consistent amount of genetic divergence from the initial sample regardless of what OTU group the changes were occurring in (see Figure 4.5). This finding may indicate that the perturbed community is less stable with respect to variation within community structure during steady state function, compared to the un-impacted community structure. Figure 4.5.B showed no apparent differences in the amount or rate of divergence between the two mesocosm conditions over time. However, there did appear to be a decrease in the amount of structural community divergence noticed between Nov. 23rd and 30th noticed in both conditions. This observation from the 'Cipro' condition can be potentially explained as the acute community response to the antibiotic; however an acute effect was not visualized in any of the preceding figures. Furthermore, a similar decrease in divergence was observed in the 'No Cipro' mesocosm.

It may instead be the case that, between the first and second weeks after start-up, the microbial community had reached a stage in which further development became partially dependent on a slowly growing population or guild. This decreased divergence may also be due to continued matrix-adhered biofilm development, leaving a relatively unaltered suspended community to dominate the sample taken. Regardless of the reason for this delayed divergence, when considering both amended and unamended mesocosms,

it seems that this stage of community development is able to proceed despite the presence of a deleterious chemical.

Interestingly, a trend of increasing structural divergence was also displayed by both communities following the large decrease in divergence between the 8th and 15th days from start-up, with a slightly decreased magnitude in the 'Cipro' CWs. These findings may indicate that only the resistant populations present in the start-up community may have been able to colonize the CW matrix in the presence of antibiotic, undergoing similar successive cycles and the 'No Cipro' community regardless of the presence of antibiotic. However, due to the increased selectively of the antibiotic-laden CW environment, the resulting attached populations may be fewer and consequently show a reduced amount of divergence within the suspended community.

By the Jan. 4th sampling event, 49 days from start-up, the control condition had displayed an increased rate of divergence from both the previous sampling period, as well as the 'Cipro' CW on the same day. The difference in divergence rate observed on Jan. 4th may be indicative of the influence of the undisturbed and increasing developed matrix-adhered community on the suspended community. The depressed magnitude of the curve between 15 and 49 days in the 'Cipro' CWs may further indicate the long-term effects of the antibiotic exposure on the plasticity of the CW community; resulting in a less plastic community due to antibiotic exposure. Whatever the exact effect of the antibiotic on the community, it is clear from Figure 4.5 that this effect is not immediate and has pressed the development of the receiving community in a direction unlike that displayed in the control CW. These trends may indicate that antibiotic exposure may have long-term effects on the ability of a community to produce natural structural variation. It

is unclear whether or not this decreased capacity for change should be concerning, or if this has repercussions regarding the sustainability of microbial communities within CWs.

Like the clarification of structural change provided by the rank-abundance plots, principal component analysis (PCA) and tree cluster analysis may be able to shed light on the current pictures of the relative similarities, or dissimilarities between the two experimental conditions, while taking into account the dimensionality of the collected data. PCA is a powerful exploratory (i.e., functions without an *a priori* hypothesis) tool that can provide information regarding the dissimilarity of a large number of cases using their behaviour over a number of variables, or dimensions (Legandre & Legandre, 1998). PCA was done (see Figure 4.5) using band R_f groups as variables and relative band intensity as the quantitative measure.

From Appendix A, Figure A.5 it can be observed that PCA alluded to the earlier finding that the CW communities were considerably dissimilar to the initial sludge inoculum, as well as the high degree of similarity between the two conditions on day 1 and on day 8, reaffirming the negligible difference between the CWs prior to ciprofloxacin exposure. Furthermore, there is continued similarity between the two conditions on day 15, further confirming the lack of acute effect on the microbial community structure displayed in the previous analyses. The 'Cipro' and control conditions at 49 days were still highly similar, displaying no obviously greater dissimilarity to each other than the two conditions on the days prior. However by Jan. 18th, 73 days from start-up, the two experimental conditions displayed a large degree of dissimilarity, further confirming the previously used metrics.

Like PCA, tree cluster analysis also displays the relative dissimilarity of data set.

However, where PCA can hide dissimilarity based on the PCs used to set the

plot axes (e.g., the difference between the closeness of 'Cip_15' and 'NoCip_15' in

Figure 4.5.A and B), tree cluster analysis is thought to be an indicator of the true distance

of similarity or dissimilarity, unrelated to the dimensionality of or perspective taken to

view the data (Legendre & Legendre, 1998). Because there may be better directions than

the dominant PCs to see similarities or clusters within a given data set, the use of a

clustering technique along with PCA is suggested (Jolliffe, 2002). Tree cluster analysis

using Euclidian distances as a distance measure is presented in Figure 4.6.

Tree cluster analysis further confirmed the dissimilarity of the sludge inoculum

from the rest of the CW system. A high degree of similarity between the day 1 samples

was also observed. The samples taken from the control mesocosm before and after the

antibiotic exposure are very similar, and considerably dissimilar from the day 1 samples.

The 'Cipro' exposed mesocosms remained generally similar to each other and the

controls, although they displayed slightly increased dissimilarity in comparison.

By Jan. 4th, the 49th day of operation, increased dissimilarity from the Nov.

samples was observed, although there remained very a similar linkage distance between

the two conditions as the observed prior to ciprofloxacin addition. The final sample taken

from the control condition illustrated continued temporal dissimilarity, although

remaining still linked to the original start-up community. Conversely, the 'Cipro' CWs

retained high similarity to the previous sample. This provides further evidence of a less

malleable community within the ciprofloxacin exposed CWs, indicated by a decrease in

natural genetic variation.
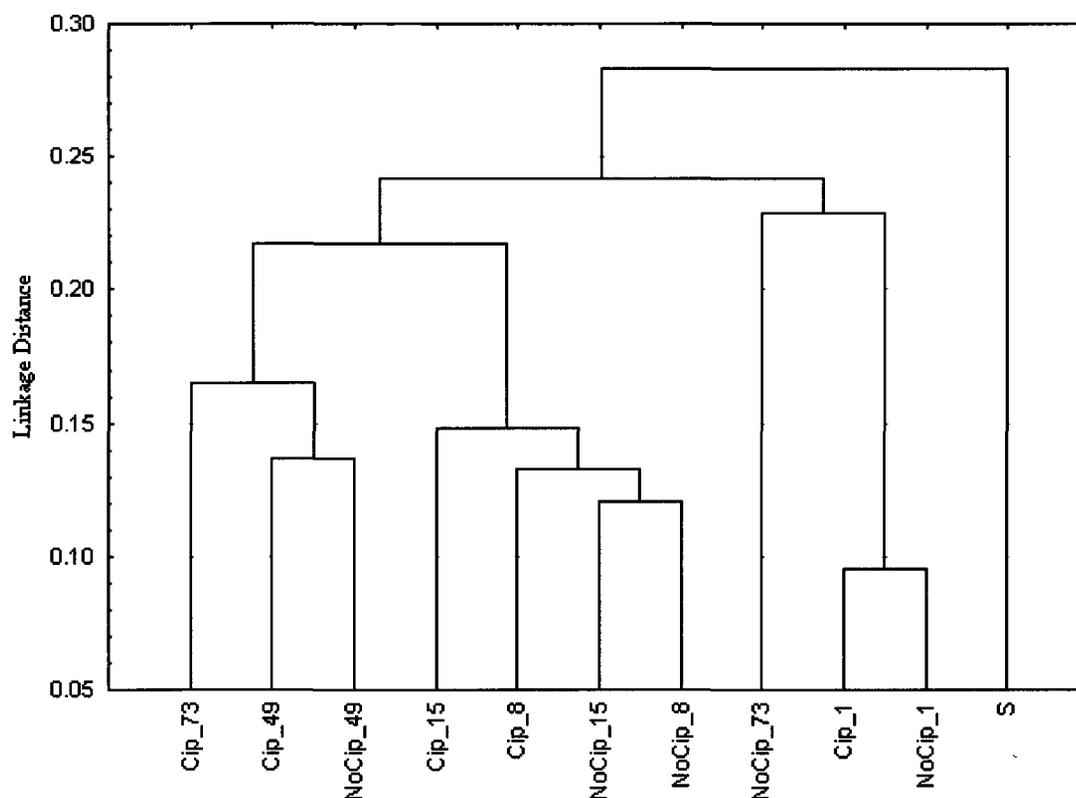
Figure 4.6. DGGE-based tree cluster analysis using the untransformed means from the bench-scale CW. Linkage distance was calculated using UPGMA of Euclidian distances. 'S' refers to the sludge inoculum, while 'Cip' and 'NoCip' are the ciprofloxacin-exposed and unexposed mesocosms, respectively. The number following indicates the number of days from start-up. Ciprofloxacin was administered between day 8 and 15 from startup.

Both PC and cluster analysis were able to produce additional visualization of the increasing dissimilarity within the 'No Cipro' mesocosm. Importantly, this is the same visual difference that was noticed most effectively in the plot of PIE (see Figure 4.2) and the averaged banding pattern (see Figure 4.4.), indicating that these data reduction technique were able to identify the same visual trends observed, despite a low number of samples, in the earlier less mathematically-intensive plots.

Differences between the two experimental conditions and the changes that occurred during development were clearly recognized through exploratory multivariate statistical analysis, genetic divergence and changes in observed number and relative abundance of OTUs over time. Changes observed through the chosen metrics have

indicated a delayed effect of antibiotic perturbation on the structure of the microbial community.

4.2. Conclusions from Bench-scale Characterization

Based on the results previously discussed, the following conclusions from the bench-scale CWs can be made:

➢ The effect of ciprofloxacin exposure appeared delayed in the genetic fingerprints. The lack of acute response may be the result of using an indirect measure (i.e., the interstitial water) for the complex interactions involving detachment of non-resistant, adhered populations within the treated condition and continued colonization of the un-sampled matrix in the unexposed mesocosms.

➢ Ciprofloxacin exposure did not appear to considerably affect the number of OTUs in the interstitial water that were abundant enough for detection with PCR.

➢ Rank abundance and average banding pattern plots indicated a pattern of structural change within the experimental group unlike the structural change in the control condition a month after ciprofloxacin exposure. PCA and tree clustering analysis also allowed visualization the differential relative contributions of OTUs to the community structure.

➢ Multivariate statistical analysis indicated dissimilarity of the interstitial water community structure between the 'Cipro' and 'No Cipro' CWs by 73 days from start-up.

➢ PIE was able to differentiate structural diversity between the two mesocosm types on the final day of sampling. This indicates that PIE may be a more appropriate diversity metric than H' when using techniques providing OTU-based data.

5.   Field-scale Treatment Wetland Results and Discussion

In order to address part of the overall goal of this study, which was to profile the

structural and functional characteristics of predominant microbial communities in CWs at

multiple system scales, a field-scale CW was monitored using CLPP and DGGE, along

with some culture-based methods. Additionally, the impact of an antibiotic contaminant

(i.e., oxytetracycline) was investigated to better understand the ability of a field-scale CW

microbial community to respond to perturbation.

5.1. *Escherichia coli*, *Salmonella enterica* and Heterotrophic Plate Count Monitoring

Escherichia coli was found only twice in all of the samples and none of the prospective

or non-typical isolates were determined to be *Salmonella enterica* on either Feb. 8th or

22nd. *E. coli* enumeration and *S. enterica* isolation results are therefore not included in

this document but can be found in Ontario Ministry of the Environment, (2010).

The Septic location showed consistantly higher HPCs than all of the other

samples, at all days with the exception of the final sample taken on Apr. 12th, 2010 (see

Figure 5.1). The Septic location, although not exposed to oxytetracycline (OTC),

displayed a slight half-log increase during exposure. The increase observed in the

'Septic' may be due to the absence of vacuuming the day prior, as tanks are vacuumed

only on Monday, Wednesday and Friday. The Feb. 24th sample was taken on a

Wednesday morning before vacuuming occurred (Allison Snow, personal

communication, Mar. 9th, 2010).

The 'Cattail', 'Sedge/Rice' and 'Final Pump/ Combined' are all depicted using

solid lines in Figure 5.1, as well as all subsequent time-course figures. This is because

these locations are all within the field-scale CW planted soil area, whereas the other
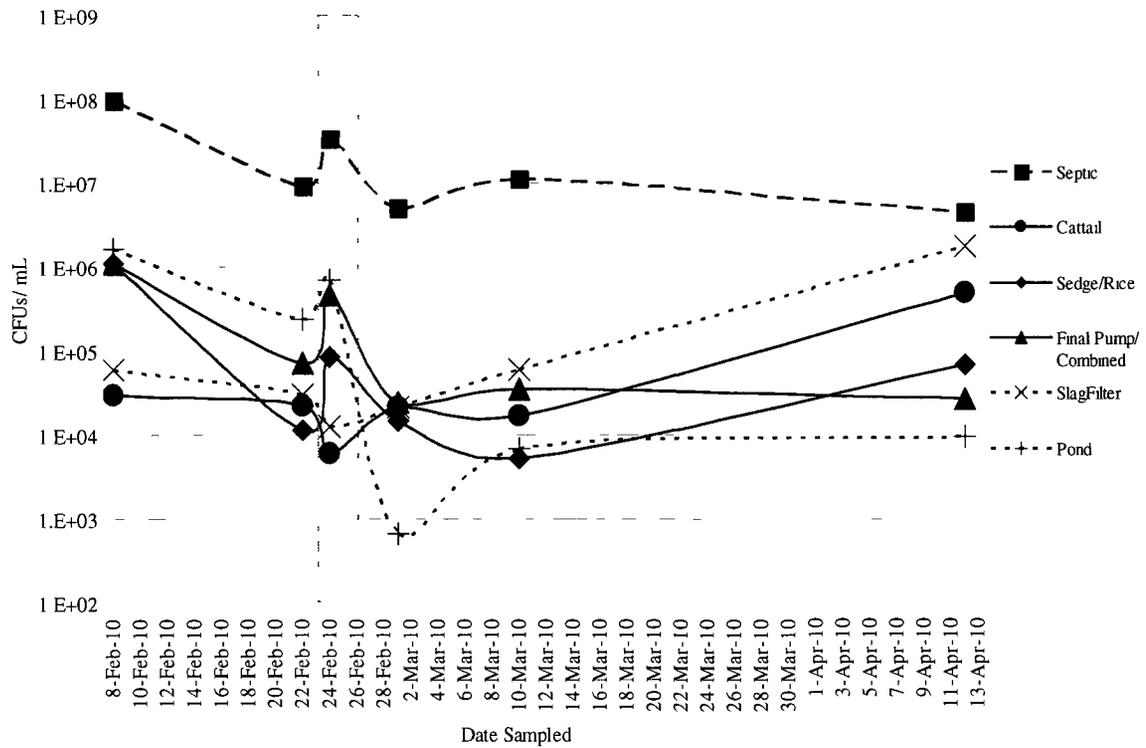
Figure 5.1. Graphical representation of heterotrophic plate counts from R2A plates after 7 days of incubation at 22°C. The grey box is added to assist visualization of the daily addition of oxytetracycline immediately following the collection of sample on Feb. 22nd, lasting until Feb. 26th.

locations monitored are not. Interestingly, following addition of OTC, the 'Cattail' and

'Sedge/Rice' sides showed distinctly different response patterns. The 'Cattail' displayed

an observable decrease in HPCs, while the 'Sedge/Rice' displayed an increase. These

results may indicate that there is a macrophyte-based difference in the amount of

culturable, heterotrophic communities that are able to survive within this wetland system.

It may also be indicative of the variable ability of the receiving communities to handle

OTC-related stress while maintaining the competitiveness of those environments, which

would ultimately act to remove the poorer competitors.

It appears that, upon acute exposure to OTC, the 'Cattail' community provided an

environment which was able to maintain its competitiveness despite the presence of a

deleterious chemical. Conversely, the 'Sedge/Rice' environment seemed to provide a less

competitive community with respect to HPC removal during OTC exposure. There was also an increase in the number of HPCs in the 'Final Pump/Combined' location during antibiotic exposure; increases that were surprisingly similar to the trend observed in the 'Septic' sample. There does not appear to be an acute effect of OTC on the HPCs in the 'Slag Filter', although a very slight decrease was observed.

OTC, a tetracycline-class antibiotic, is almost exclusively decomposed through photodegradation, although there is evidence of removal through hydrolysis and inactivation through binding to metal-ions or heating (Xuan et al., 2010). OTC is also known to persist for an extended period of time in sediment (Lunestad & Gokosyr, 1990), but adsorbs poorly to soil with low organic content (Jones et al., 2005), but moderately well (i.e., on average 25%) to soils with moderate to high organic content (Rabolle & Spliid, 2000). Due to the fact that the field-scale system is a subsurface flow CW operating during the winter with relatively spotty vegetation (see Figure 3.2), minimal removal of OTC can be expected prior to it entering the 'Pond' location. The majority of OTC would not be expected to become lodged within the wetland; instead it likely washed through until reaching the sediment of the 'Pond' location. Accordingly, there was a very slight increase in the 'Pond' HPCs during antibiotic exposure, followed by a three order of magnitude decrease by the sample following cessation of OTC addition. This observation may be due to build-up of antibiotic runoff in that endpoint location and may furthermore indicate extended environmental persistence of OTC through subsurface CWs.

Extensive monitoring of site characteristics was completed by various researchers on the this field-scale CW prior to, during and following the microbial community

sampling done here-in; including temperature, conductivity, flow rate, enumeration of the OTC within the system and a variety of other measurements effects and can be obtained from the Ontario Ministry of the Environment (2010). Ambient temperature was found to increase steadily over the sampling period, oscillating daily between minus -5 and -10°C during the Feb. 8th to Mar. 10th period, and increasing from -5 to 10°C between Mar. 10th and Apr. 12th (Ontario Ministry of the Environment, 2010). This steadily increasing temperature, and associated increase in free energy, may be responsible for the increasing trend of HPCs between Mar. 10th and Apr. 12th in the 'Cattail' and 'Slag Filter'; as on Apr. 12th these increased by an order of magnitude or more from both of their pre-OTC exposure samples.

Additionally, work done to enumerate the concentration of oxytetracycline in the field-scale as part of this collaborative project found that only the 'Cattail', 'Sedge/Rice' and, intermittently, the 'Combined', 'Slag Filter' and 'Pond' locations studied were exposed to the antibiotic at concentrations greater than $1\mu g/L$ (100 ppb; Ontario Ministry of the Environment, 2010). At some points, the 'Cattail' and 'Sedge/Rice' locations were exposed to nearly 200 mg/L (i.e., 200 ppm; Ontario Ministry of the Environment, 2010), well above the intended concentration of 5 mg/L (i.e., 5 ppm) and even further above what is considered an environmentally deleterious amount of 1 mg/L (i.e., 1 ppm; Kummerer, 2008). The actual amount of antibiotic entering the 'Pond' location may have been much larger than the amount detected considering the dilution that would have occurred (Ontario Ministry of the Environment, 2010), and the fact that antibiotic quantification was not completed after 120 hours following the first addition on Feb.

22nd. The decrease in the HPC from the 'Pond' location following OTC addition alludes to a possible impact of the OTC on the indigenous heterotrophic microbial community.

5.2. Functional Fingerprinting

CLPP was preformed on all 6 sample locations (see Figure 3.2) on 5 sampling dates, in triplicate, resulting in the generation of $n=90$ carbon source utilization patterns (CSUPs). CLPP was not completed for the first sample collected on Feb. 8th. The average blank-corrected colour development of all 93 Biolog™ ECOplate™ wells containing carbon sources (i.e., average well colour development; AWCD) was calculated for each location and presented in Figure 5.2. AWCD can be considered an overall estimate of the relative rate of community carbon metabolism (Garland *et al.*, 2007).
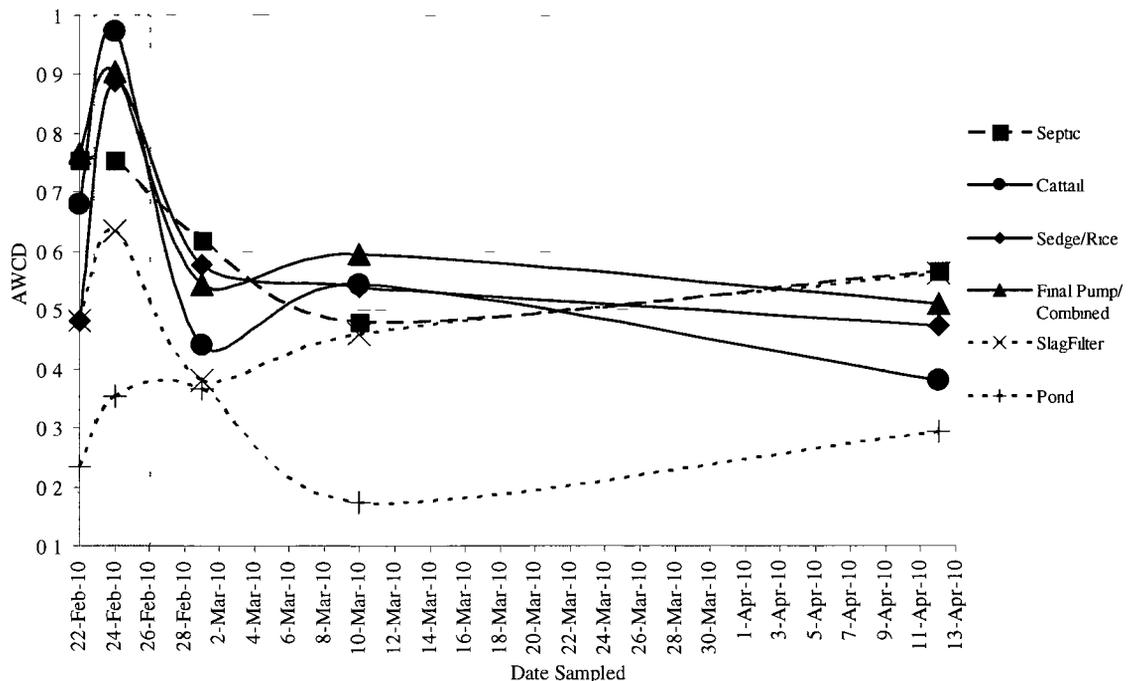
Figure 5.2. Average well colour development (AWCD) from field-scale CLPP after 48 hours of incubation at 22°C. The grey box is added to assist visualization of the daily addition of oxytetracycline immediately following the collection of sample on Feb. 22nd, lasting until Feb. 26th.

Of all the locations, the 'Pond' had the consistently lowest average rate of carbon metabolism. Carbon is not generally considered a limiting factor in the environment (Atlas & Bartha, 1998) and especially not within wetlands, which are generally considered to be carbon sinks (Lewis, 1995). Therefore, organisms efficient at sequestering carbon are not likely to have any competitive advantage compared to organisms that would be able to sequester biologically limiting essential elements (e.g., nitrogen, phosphorous, sulphur, etc.).

Generally, all locations downstream of the OTC addition (i.e., not the 'Septic') showed an increase in the rate of carbon metabolism during antibiotic exposure on Feb. 24th. This may indicate the presence of an acute effect of OTC that resulted in an increased capacity for carbon metabolism. It is likely that this increase in carbon metabolism is related to increased general physiological stress-response in the microorganisms (White, 2003). This increase in general carbon metabolism may also be due to selection of organisms that are OTC-resistant, able to metabolize carbon with increased rate and were under ecological stress from organisms now removed that had previously limited their ability to display this in the Biolog™ ECOplate™ wells. The subsequent decrease on Mar. 1st, the first sample following exposure, lends more likelihood to the stress-response related increase in the rate of carbon metabolism. Carbon metabolism should have remained elevated if the change was a result of the removal of susceptible and competitively or functionally dominant members. The 'Pond' location did not show a decrease in the Mar. 1st sample, likely due to the continued presence through leaching from the CW or persistence of the antibiotic (see Section 5.1).

The 'Cattail' location displayed a noticeable 50% decrease in carbon metabolism between the Feb. 22nd and Apr. 12th. This may allude to a long-term functional effect of the OTC treatment, as it is possible that the observed decreased capacity for carbon metabolism is a result of a loss of cooperative community relationships. It should be noted that, as mentioned in Section 3.1.2, the 'Septic' (on every event) and 'Slag Filter' (on Feb. 22nd) required dilution by an order of magnitude due to methodological concerns. The results displayed for these points should be interpreted with this in mind.

Richness, defined as the number of utilized carbon sources, is plotted over time in Figure 5.3. Changes in the number of carbon sources that a community can use should provide further clues regarding the type of effect OTC had on the functional characteristics of the community. The ability of a CW system to metabolize multiple different types of carbon sources is important for its applied functionality, as influent to these treatment systems may not always be entirely known, but require remediation nonetheless.

All samples exposed to OTC used more carbon sources upon addition of the antibiotic, followed by a return to near-normal usage following cessation of OTC administration; indicating an acute functional impact of the antibiotic perturbation. The 'Pond' location, however, displayed its highest number of carbon sources metabolized following OTC addition, which may be further evidence of the collection and persistence of OTC in this location. A total of 60 grams of OTC would have been added to the CW prior to this date and, based on the mechanisms of OTC breakdown and inactivation presented in Xuan et al. (2010), it is very likely that a large proportion of the OTC added would have made it to the 'Pond' location unabated. The drop observed in the other

Figure 5.3. Substrate richness from field-scale CLPP after 48 hours of incubation at 22°C. The grey box is added to assist visualization of the daily addition of oxytetracycline immediately following the collection of sample on Feb. 22nd, lasting until Feb. 26th.
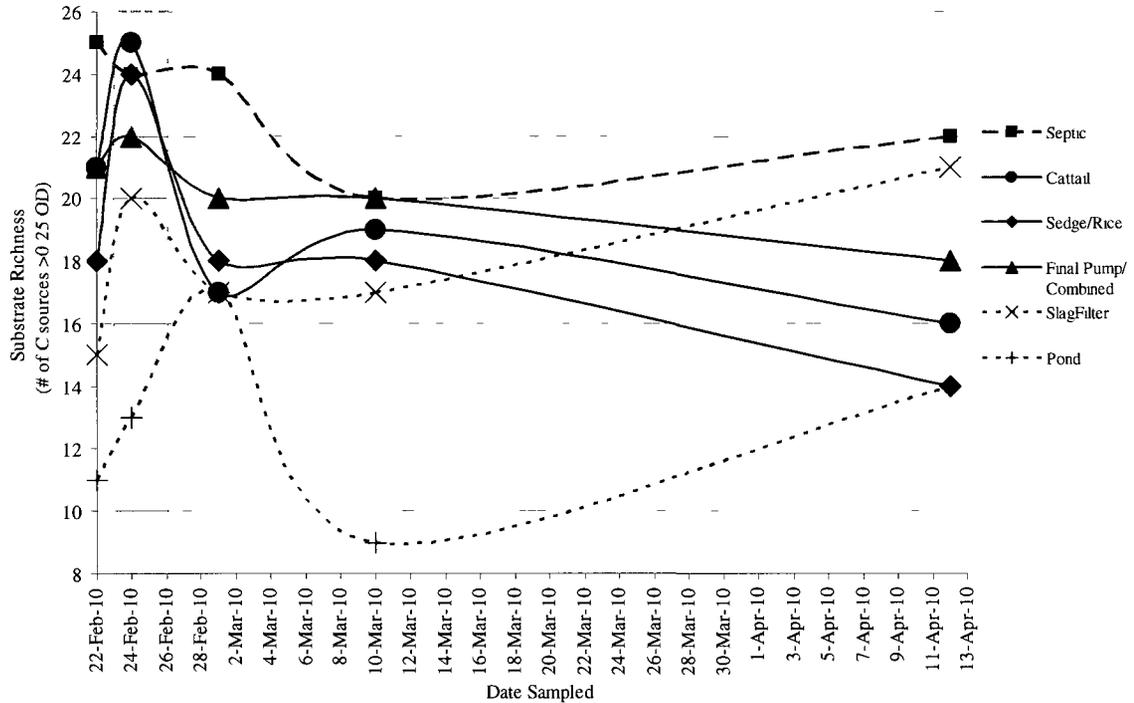
OTC-exposed locations was observed on March 10th, indicating that the OTC-derived stress was overcome or removed by this date.

The 'Slag Filter' displayed a noticeable increase in the number of carbon sources used between the Feb. 22nd and Apr. 12th samples, indicating a possible long-term effect of the antibiotic on this location. It may also be due to the dilution, done on Feb. 22nd and not on Apr. 12th. This increase may partially be attributed to increased temperatures observed between Mar. 10th and Apr. 12th, resulting in the increased metabolism of mesophilic populations and subsequently contributing to the modification of the ability to use previously unusable carbon on Apr. 12th. The 'Cattail', 'Sedge/Rice' and 'Final Pump/ Combined' locations, however, displayed a decreasing trend in the number of usable carbon sources over this same period despite increasing temperatures, although this change was only noticeably different from the Feb. 22nd samples for the 'Cattail'

location. Continued sampling would be required to determine if this decreased functionality continued or was overcome before concluding whether or not it may be long-term effect of antibiotic exposure or a seasonal effect.

It may be that the changes in carbon metabolism observed in the inter-CW sites are also due to the temperature influence, as the newly-awaking, previously-dormant mesophilic populations within the planted CW area may be causing increased competitive pressure and making carbon-metabolism a less of a priority for continued survival. It is clear, however, that there was an acute, likely physiological, effect of the OTC exposure on the functional richness of the field-scale CW community as the temperature recorded during this time period did not change in a considerable manner (Ontario Ministry of the Environment, 2010).

Temporal changes in H' (Shannon, 1948) as illustrated in Figure 5.4, provide further evidence of an acute effect of OTC exposure. Diversity generated from CLPP, an estimate of capacity and ability to utilize the supplied carbon sources, has been suggested as metric that provides an idea of the overall community response or function (Weber & Legge, 2008). Prior to OTC addition the ability and capacity for carbon metabolism, like general carbon metabolism and the number of carbon sources utilized, appeared to decrease with increasing retention time. This finding further indicates that carbon source utilization may not be an environmentally competitive trait within a prolonged treatment environment. Trends in CSUP H' also followed much of the same trends observed in the richness and AWCD figures. H' displayed an immediate increase following OTC exposure in all non-'Septic' locations, most of which returned to near-normal in the sample on Mar. 1st. The 'Pond' location exhibited an attenuated increase in H' from

Figure 5.4. Substrate diversity (Shannon H') calculated from CLPP after 48 hours of incubation at 22°C. The grey box is added to assist visualization of the daily addition of oxytetracycline immediately following the collection of sample on Feb. 22nd, lasting until Feb. 26th.
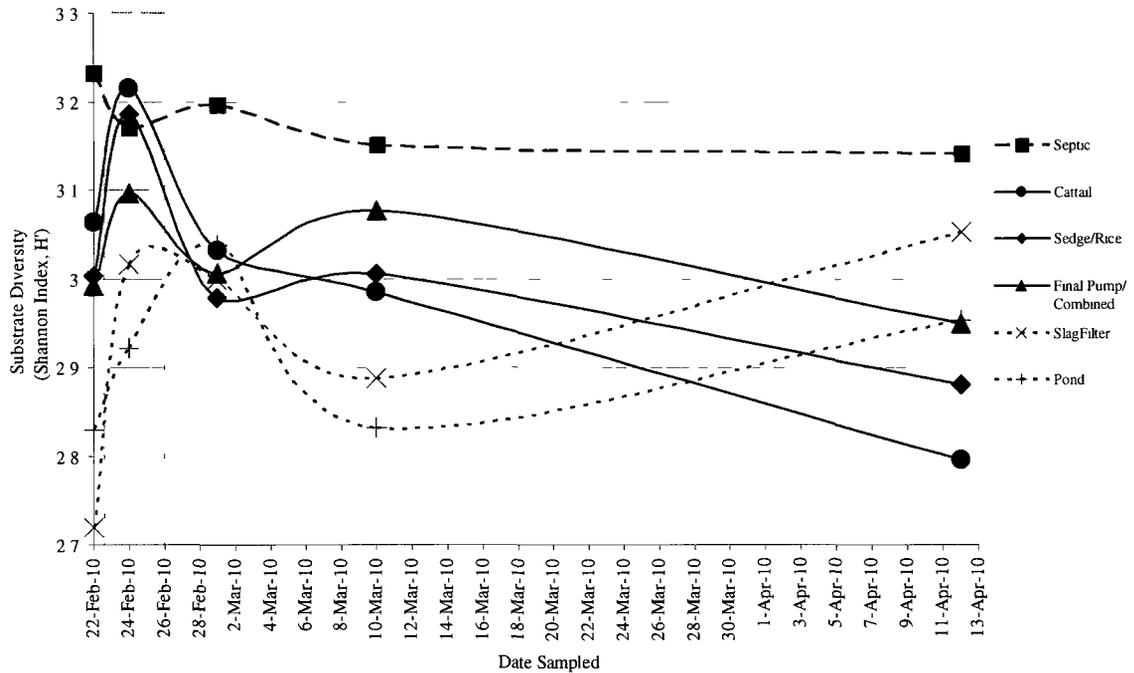
the pre-OTC sample. The trend observed between Mar. 10th and Apr. 12th was similar to the trends for the same period in the previous figures.

Functional divergence, or the one-dimensional difference between two CLPP-derived functional fingerprints, is displayed in Figure 5.5. The one dimensional difference between each functional profile and the functional profile of the sample taken immediately before the OTC addition (i.e., Feb. 22nd) is depicted in Figure 5.5.A. This is considered to be a good, simple measure for comparing the pattern of change of a microbial community in response to a perturbation (Weber and Legge, 2009). All of the OTC-exposed locations sampled displayed a minor amount of change from Feb. 22nd during and immediately following the exposure period. Following this, however, there was very little change from Feb. 22nd observed. Only the 'Slag Filter' continued to
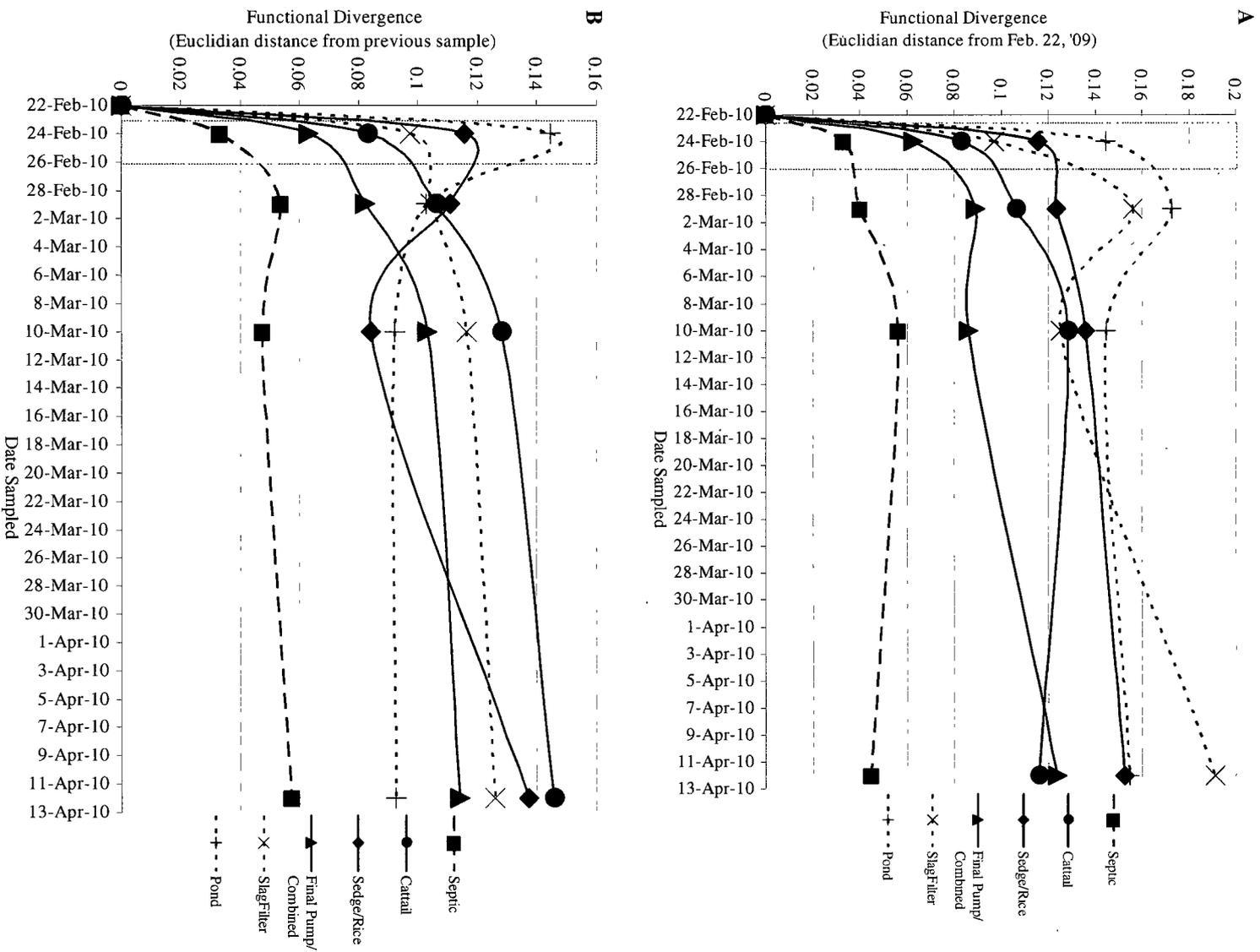
Figure 5.5. Functional divergence based on Euclidian distances of untransformed CLPP profiles from the field-scale CW. A) Divergence from the sample taken on Feb. 22nd and B) Divergence from the previous sample. The grey box is added to assist visualization of the daily addition of oxytetracycline immediately following the collection of sample on Feb. 22nd, lasting until Feb. 26th.

display functional change from the pre-OTC sample after Mar. 10th. This lack of functional divergence may be indicative of a long-term effect of OTC on the variability of community function, although it is unclear what the undisturbed amount of functional change was prior to OTC addition or during this seasonal period.

The one dimensional difference in carbon utilization between each previous sample's functional profile (i.e., Feb. 22nd from Feb. 24th, Feb. 24th from Mar. 1st, etc.) is presented in Figure 5.5.B. The rate at which change in the functional profile is occurring is given by the slope of Figure 5.5.B 'Cattail' and 'Sedge/Rice' locations displayed both the highest amounts of observed change from the pre-exposure sample, as well as the largest rates of change. The former was observed during antibiotic exposure on Feb. 24th, while the latter was seen following on Mar. 1st. These observations may be further evidence of a stress-response related increase in metabolism as the organisms attempt to deal physiologically with the OTC threat. The 'Pond' location, interestingly exhibited a prolonged divergence rate, which may be further evidence of the build-up of OTC in this location. All of the locations appeared to settle into a consistent rate of functional divergence following the acute OTC response and subsequent recovery, demonstrated by the relatively flat slope. Although there is a clear, acute, impact of OTC on the rate of functional divergence from the results presented in Figure 5.5, there does not appear to be evidence of a long term effect of OTC exposure on the rate of functional change in the field-scale CW.

Principal component analysis (PCA) is presented in Figure 5.6 and was done the supplied carbon sources as variables and the ability to utilize that carbon as the quantitative measure. It appears that the 'Septic' samples were very similar throughout
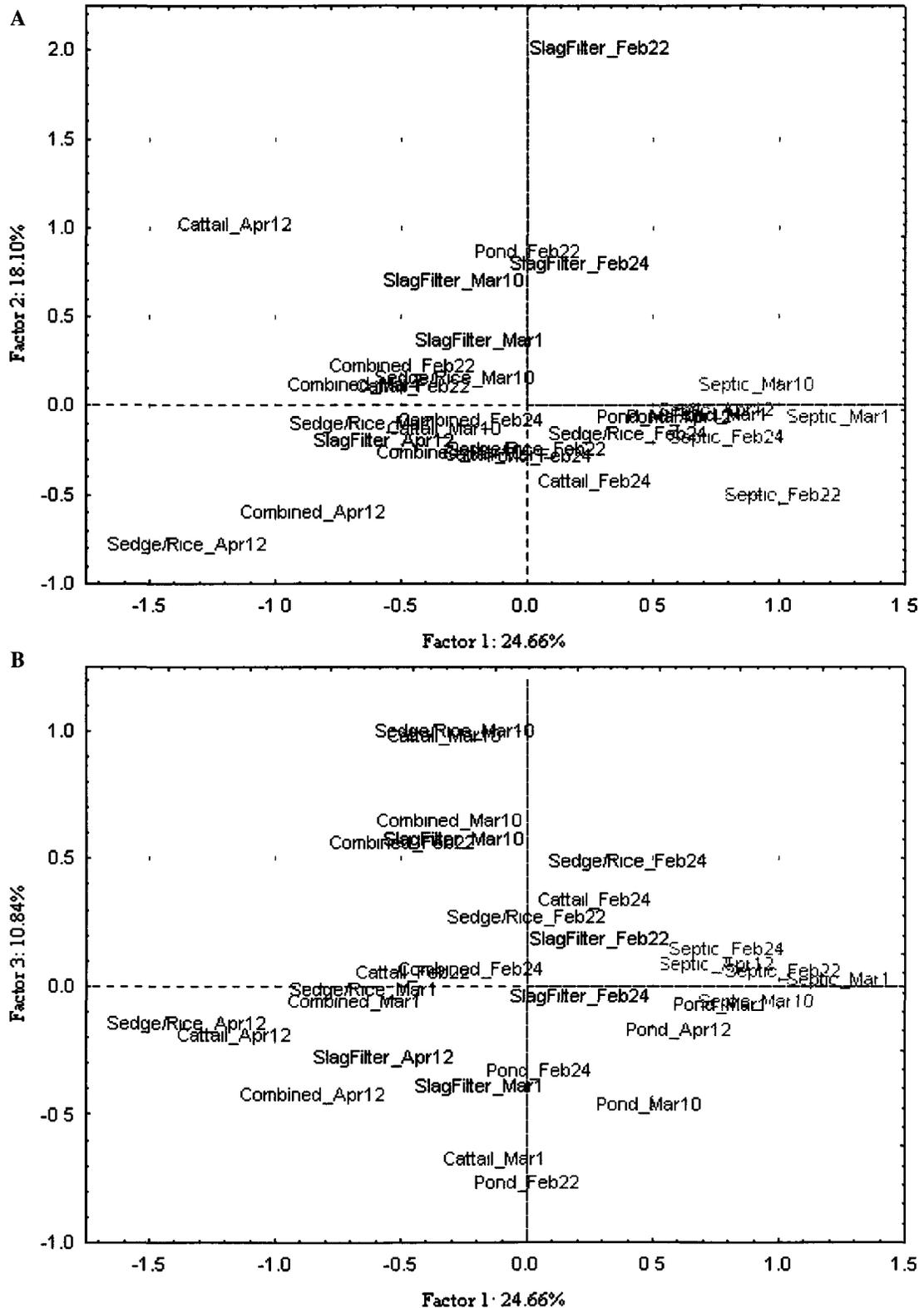
Figure 5.6. PCA using the covariance matrix from Taylor-transformed blank-corrected means from the field-scale CW CLPP. A) Plot of cases on the 1 x 2 factor plane and B) Plot of cases on the 1 x 3 factor plane. Oxytetracycline was added daily immediately following the collection of sample on Feb. 22nd, lasting until Feb 26th.

the investigation, providing evidence that the fish-supplied waste effluent is highly

similar in functional profile over time. However, only the 'Pond' samples taken following

OTC addition are highly similar to the 'Septic'; and this similarity increased over time.

This may allude to the persistence of the community primarily responsible for the

functional properties of the 'Septic' sample through the CW as a result of OTC addition.

It is unclear whether this functional similarity is due to the acute effect of the antibiotic;

which may have let a fraction of the 'Septic' through unabated during the exposure

period and it has persisted and is beginning to integrate into the 'Pond' community; or, if

there long-term and continual leaching of 'Septic' waste through the CW which is now

taking over the functional profile of the 'Pond' community. The Cattail', 'Sedge/Rice',

'Final Pump/Combined' and 'Slag Filter' locations also appeared to share a considerable

amount of carbon-based functional similarity. Of note, the 'Cattail' and 'Sedge/Rice' on

Apr. 12th appear to be distinct from each other and the other samples. This may indicate a

long-term effect of the OTC exposure and/or a continuing seasonal ecological shift.

Monitoring over multiple seasons would be required to elucidate which of these factors

were likely most responsible.

Tree cluster analysis is presented in Figure 5.7. Like PCA, a large degree of

functional similarity within the 'Pond' and 'Septic' locations is visible. Of note, the

'Pond' and 'Septic' carbon utilization was highly similar on Feb. 24th, although

maintained a consistent dissimilarity from then until April 12th, which may indicate that

is was an acute lapse in function that caused the addition of the functional 'Septic'

community responsible for carbon metabolism to the 'Pond'; not a persistent, long-term

decrease in functional removal as a result of OTC exposure.

The distance between the 'Cattail' and 'Sedge/Rice' locations on Feb. 22nd

compared to Feb. 24th suggest an acute impact of the antibiotic that has resulted in more

similar carbon metabolism in these areas. The 'Cattail' and Sedge/Rice' were highly

similar on Mar. 10th, which may indicate these locations were still in a stage of functional

recovery following OTC exposure by this date. The increasing dissimilarity of the

'Cattail' and 'Sedge/Rice' by Apr. 12th suggests that the functional stress caused by the

antibiotic and possibly also the recovery from exposure, were overcome by the end of the

monitoring period, but that the pattern of carbon metabolism had changed from pre-

exposure. This may also be due to mesophilic populations in these locations beginning to

add their metabolic functionality to functional fingerprint of the community.
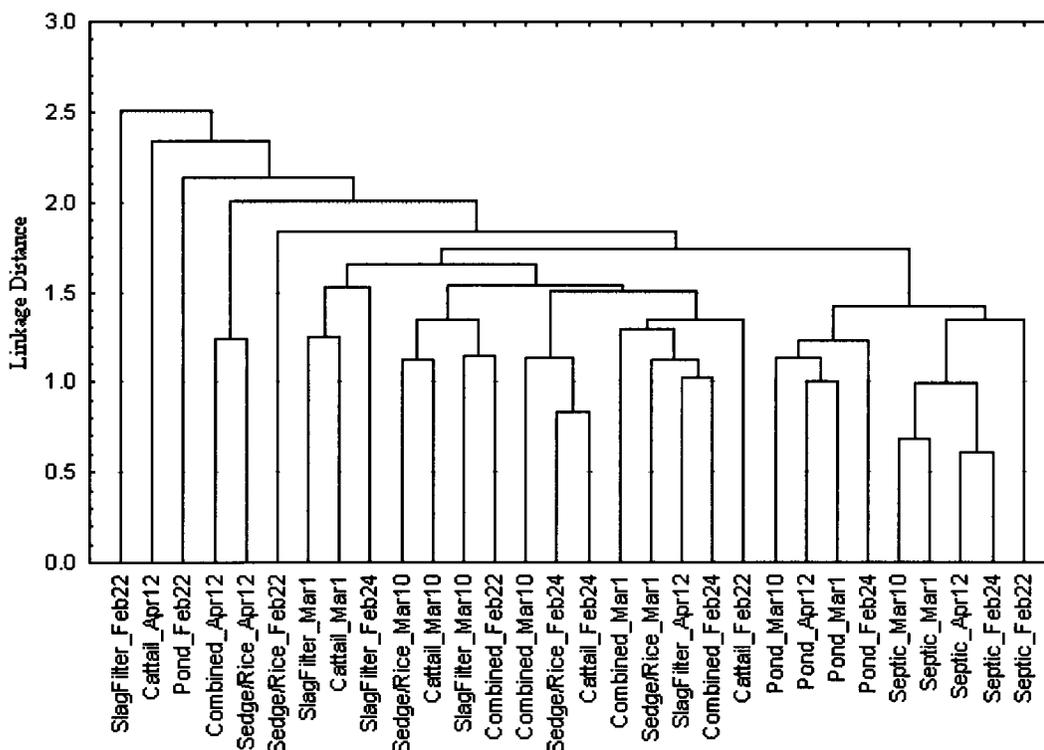
Figure 5.7. CLPP-based tree cluster analysis using the untransformed blank-corrected means from the field-scale CW. Linkage distance was calculated using UPGMA of Euclidian distances. Oxytetracycline was added daily immediately following the collection of sample on Feb. 22nd, lasting until Feb. 26th.

The persistent functional dissimilarity of almost every site from its respective pre-OTC site (i.e., loss of spatial dissimilarity), in combination with a large degree of similarity due to temporal factors (i.e., gain of temporal similarity), indicates that in response to OTC (i.e., a contaminant) the state of carbon metabolism for these communities may be the result of a standardized, general physiological stress-response.

5.3. Structural Fingerprinting

DGGE fingerprinting was completed on all samples taken, with PCR run on the duplicate template dilutions, resulting in a total $n= 64$. Genetic richness, defined as the number of bands above background observed in DGGE gels or operational taxonomic units (OTUs; Sneath, 2005; see Section 4.1. for discussion of this term's application within this thesis) was calculated and the time-course trends for this metric presented in Figure 5.8.
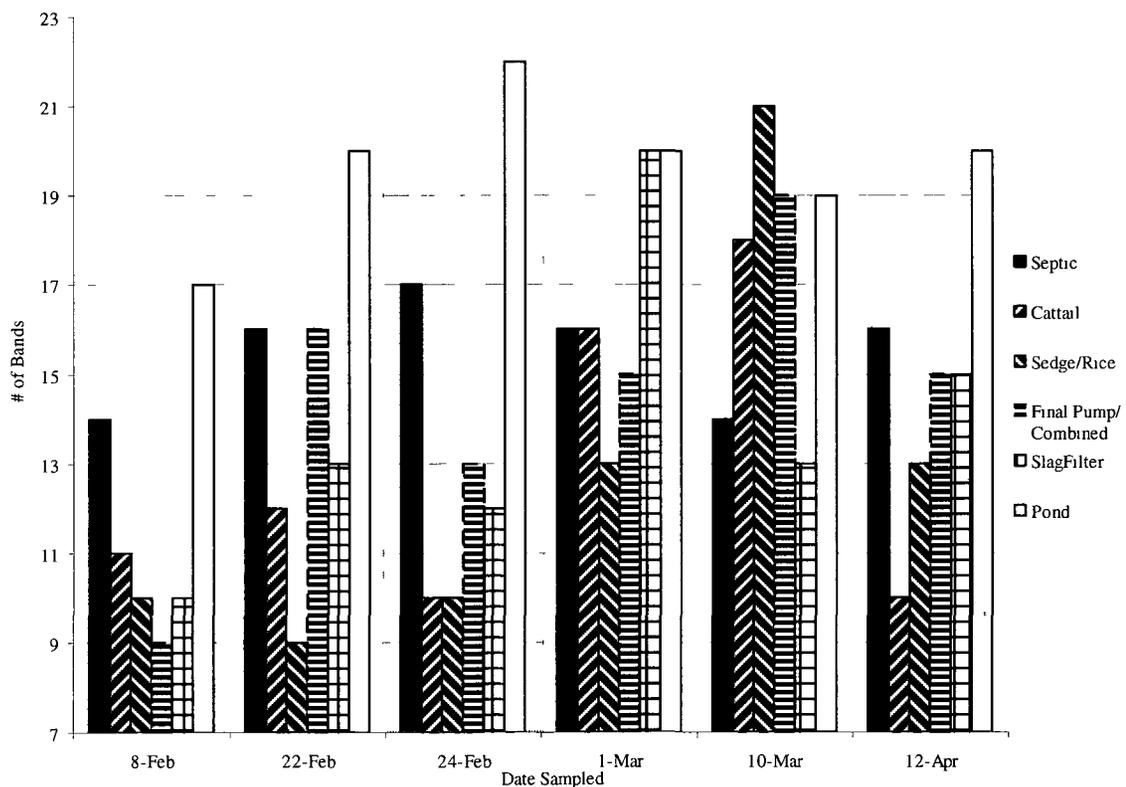
Figure 5.8. DGGE-based community richness (# of bands) from the field-scale CW. The grey line is added to assist visualization of addition of the oxytetracycline spike immediately following the collection of sample on Feb. 22nd, lasting until Feb. 26th.

There was generally only a small amount of change in the number of OTUs as a result of acute OTC exposure visible in Figure 5.8. The number of observed OTUs increased slightly in the 'Sedge/Rice' and 'Pond' locations and decreased slightly in the 'Cattail', 'Final Pump/Combined' and 'Slag Filter', although none of the observations during exposure differed noticeably from those taken prior to OTC addition. There is, however, clear and steep increases in the number of OTUs observed on Mar. 1st or Mar. 10th in most of locations. The 'Slag Filter' exhibited a 7 OTU increase on Mar. 1st compared to Feb. 22nd, followed by a 7 OTU decrease on Mar. 10th. The 'Cattail' and 'Final Pump/Combined' showed recovery of the slight Feb. 24th decrease in OTUs on Mar. 1st; with the Cattail displaying 4 more OTUs and the 'Final Pump/Combined' showing 1 OTU fewer than before OTC addition. This may indicate the acute antibiotic-related decrease of certain populations and subsequent recovery.

The 'Cattail' and 'Final Pump/Combined', as well as the 'Sedge/Rice', continued to display an increase in the number of observed OTUs on Mar. 10th; the point at which the largest number of OTUs for each of these three locations were observed. The 'Slag Filter' had already decreased back to the pre-OTC number of OTUs by Mar. 10th. This strongly suggests a delayed effect of OTC on the structural fingerprint of the communities, as the locations that were exposed to the highest concentration of OTC displayed the most delayed spikes in the number of OTUs observed; despite being the first exposed to the antibiotic. By Apr. 12th, the number of detectable OTUs present in all of the samples had decreased back to around the pre-OTC mark; although the 'Slag Filter' showed a slight increase. These changes in structural or genetic richness are juxtaposed to the changes in functional or substrate richness presented earlier (see Figure

5.3), which displayed little change after the antibiotic, but generally large increases during the exposure period.

To further explore the behaviour of the field-scale CWs, the Shannon index of diversity (H'; Shannon, 1948) and the probability of interspecific encounters (PIE; Hurlbert, 1971) were calculated, and can be seen in Figure 5.9. Diversity calculated from a genetic fingerprinting method can be interpreted as measure of both the number of distinct OTUs and the relative proportion with which they occur. H' is displayed in Figure 5.9.A. The only obvious acute change in the presence and abundance of the distinct phylogenetic groups in response to OTC exposure can be seen in the 'Slag Filter' location, which displayed a very low H'-based diversity during the exposure period. However, this decrease was also observed in the first sample taken on Feb. 8th, which may indicate large variability in the genetic diversity of this location.

A distinct spike in the 'Cattail', 'Sedge/Rice' and 'Slag Filter' H' was also observed on Mar. 1st. Interestingly, a decrease in the H' in the 'Cattail' and 'Sedge/Rice' on Mar. 10th was also observed, despite increased numbers of OTUs. This decreased genetic diversity in spite of the increasing number of observed OTUs was also seen in the PIE estimate for those locations on the same day (see Figure 5.9.B). PIE values displayed a slight increase in genetic diversity during and immediately following OTC exposure in the 'Cattail', 'Sedge/Rice' and 'Slag Filter' locations, similar to the H'-derived trends. Also, a drop in the 'Cattail' and 'Sedge/Rice' genetic diversity was observed on Mar. 10th and the diversity displayed continued decrease on Apr. 12th. The 'Final Pump/Combined' location displayed little to no change in genetic diversity over the monitoring period, along with the 'Pond'.

A

B

Figure 5.9. DGGE-based indices of community diversity from the field-scale CW A) Shannon index (H'),
B) Hurlbert index (PIE) The grey box is added to assist visualization of addition of the oxytetracycline
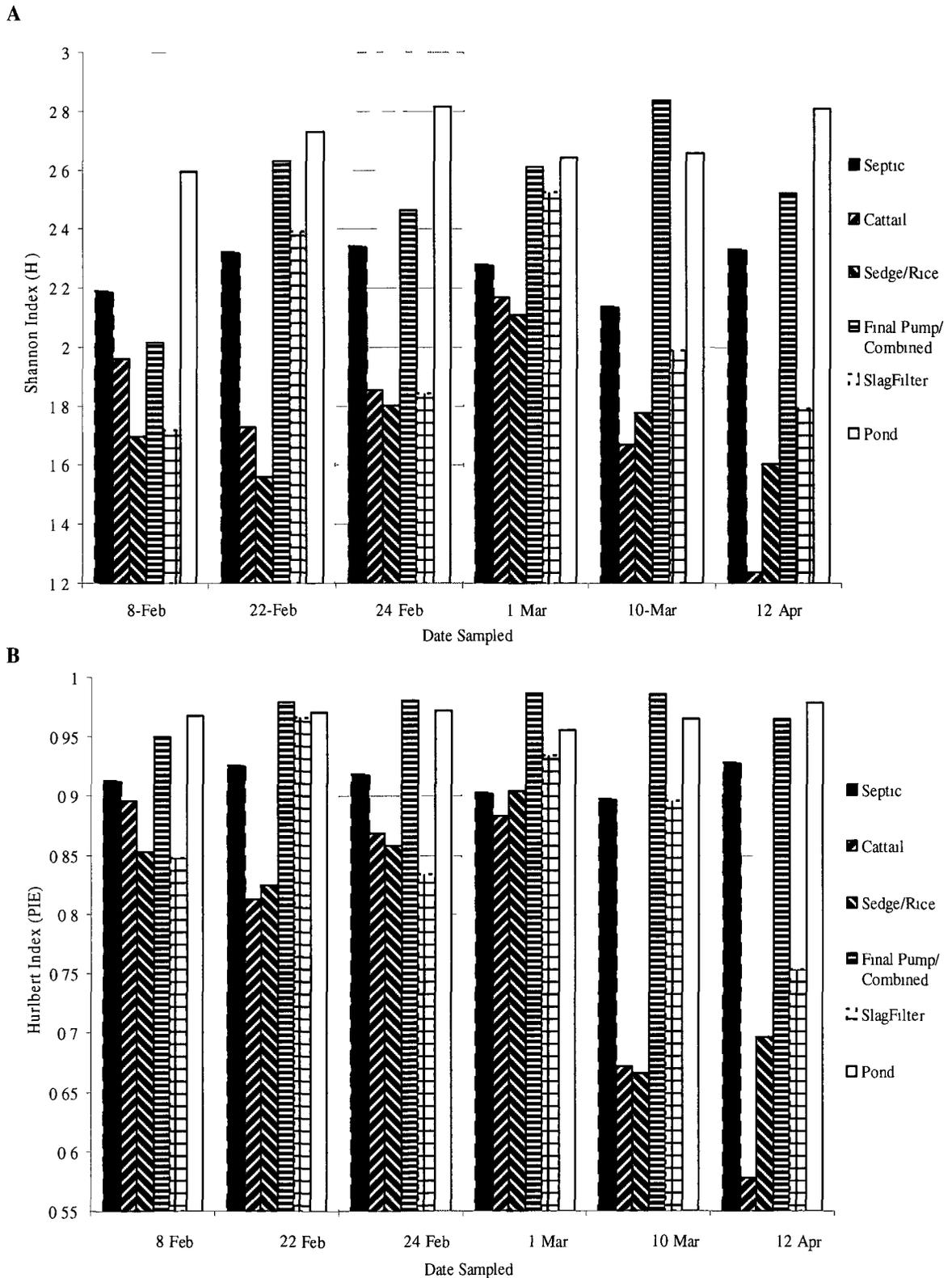spike immediately following the collection of sample on Feb 22nd, lasting until Feb 26th

The 'Final Pump/Combined' location displayed consistently high levels of genetic diversity in Figure 5.9 and a lack of response to the OTC, even though it had displayed a flux in the number of OTUs in Figure 5.10. Interestingly, water from this location is thought to be composed of the combined 'Cattail' and 'Sedge/Rice' flow-through; both of which displayed possible impacts of exposure. This suggests that there may be a distinct community residing prior to or within the 'Final Pump/Combined' location. It is also interesting that the genetic diversity indices displayed little, if any, change in the 'Pond' location, considering this location was intended to be the end point for the OTC. Further monitoring may be needed to observe the genetic changes in this location considering the apparent delay in the genetic responses in other the other field-scale locations.

Rank-abundance plots are displayed in Figure 5.10. The relative band intensity of the OTU movement (R_f) groups was used as the ranking value. Plots were included only for the 'Cattail' (Figure 5.10.A), 'Sedge/Rice' (Figure 5.10.B), 'Final Pump/Combined (Figure 5.10.C) and 'Slag Filter' (Figure 5.10.D) locations. The rank-abundance plots for the remaining two locations can be found in Appendix A, Figure A.6. Based on the appearance of 'Cattail' and 'Sedge/Rice' rank-abundance plots in Figure 5.10.A and B, it appears that the community structure in these locations underwent a change towards less even structure following exposure to OTC; culminating in both communities being dominated by a single OTU by Mar. 10th. The 'Final Pump/Combined' location (see Figure 5.10.C) showed little to no change in community structure over the OTC exposure and subsequent monitoring period, in contrast to the other of locations, further indicating that there may be a unique community in this area, separate from the communities inundating it from the 'Cattal' and 'Sedge/Rice' locations.
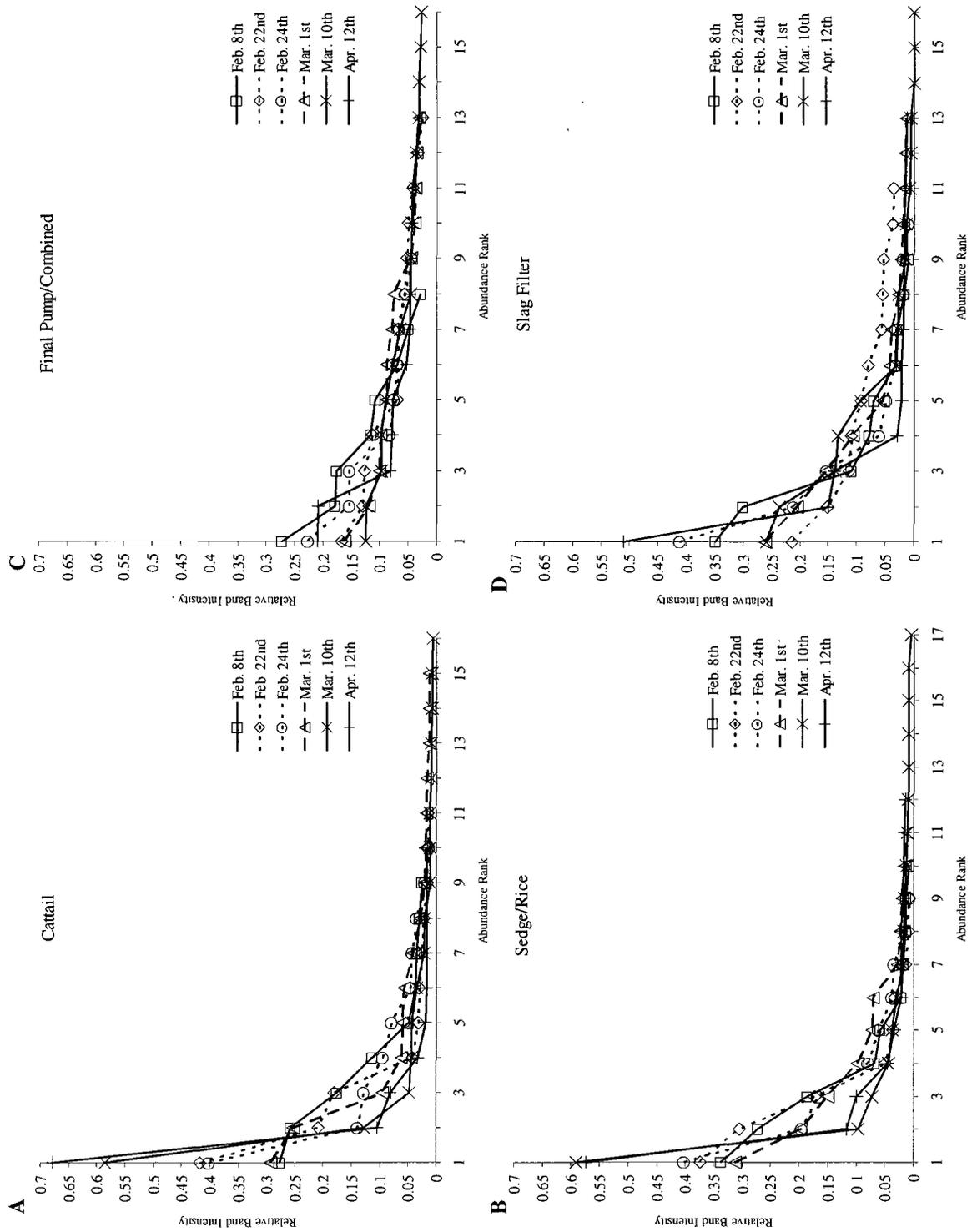
Figure 5.10. DGGE-based rank-abundance curves for selected locations in the field-scale CW. Addition of the oxytetracycline spike immediately following the collection of sample on Feb. 22nd, lasting until Feb. 26th. A) The'Cattail' location, B) The 'Sedge/Rice' location, C) The 'Final Pump/Combined' location and D) The 'Slag Filter' location.

The 'Slag Filter' location appeared to produce a similar pattern of change regarding the genetic community structure as did the 'Cattail' and 'Sedge/Rice' locations (see Figure 5.10.D); although there appeared to some maintenance of increased community evenness in the 'Slag Filter' compared to the 'Cattail' and 'Sedge/Rice' locations.

Important information may also be contained in not just changes in the ranked abundance of the relative population representation, but also in the G+C content possessed by the groups. To visualize this difference Figure 5.11. portrays the averaged relative abundances from the genetic profile, maintaining the movement of these locations in the DGGE gel. Plots were included only for the 'Cattail' (Figure 5.11.A), 'Sedge/Rice' (Figure 5.11.B), 'Final Pump/Combined (Figure 5.11.C) and 'Slag Filter' (Figure 5.11.D) as the remaining locations showed little to no visible changes over the sampling period. The 'Cattail' and 'Sedge/Rice' locations are largely dominated by 3 dominant OTUs with moderate G+C content. Following the addition of OTC, there appears to be a drop in the relative abundance of the 2 moderately dominant OTUs with slightly more and less G+C content, respectively, than the most dominant OTU. In samples following exposure, these 2 OTUs continued to decrease from their pre-exposure representation, replaced by the appearance of a number of scarce OTUs with much higher and much lower G+C content.

Persistence of the major OTU with moderate G+C content and comprising nearly 70% of the community representation remained throughout, and was noticeably increased in the Apr. 12th sample in both the 'Cattail' and 'Sedge/Rice' locations. The appearance of novel and scarce OTUs on Mar. 10th lends weight to the possible explanation of
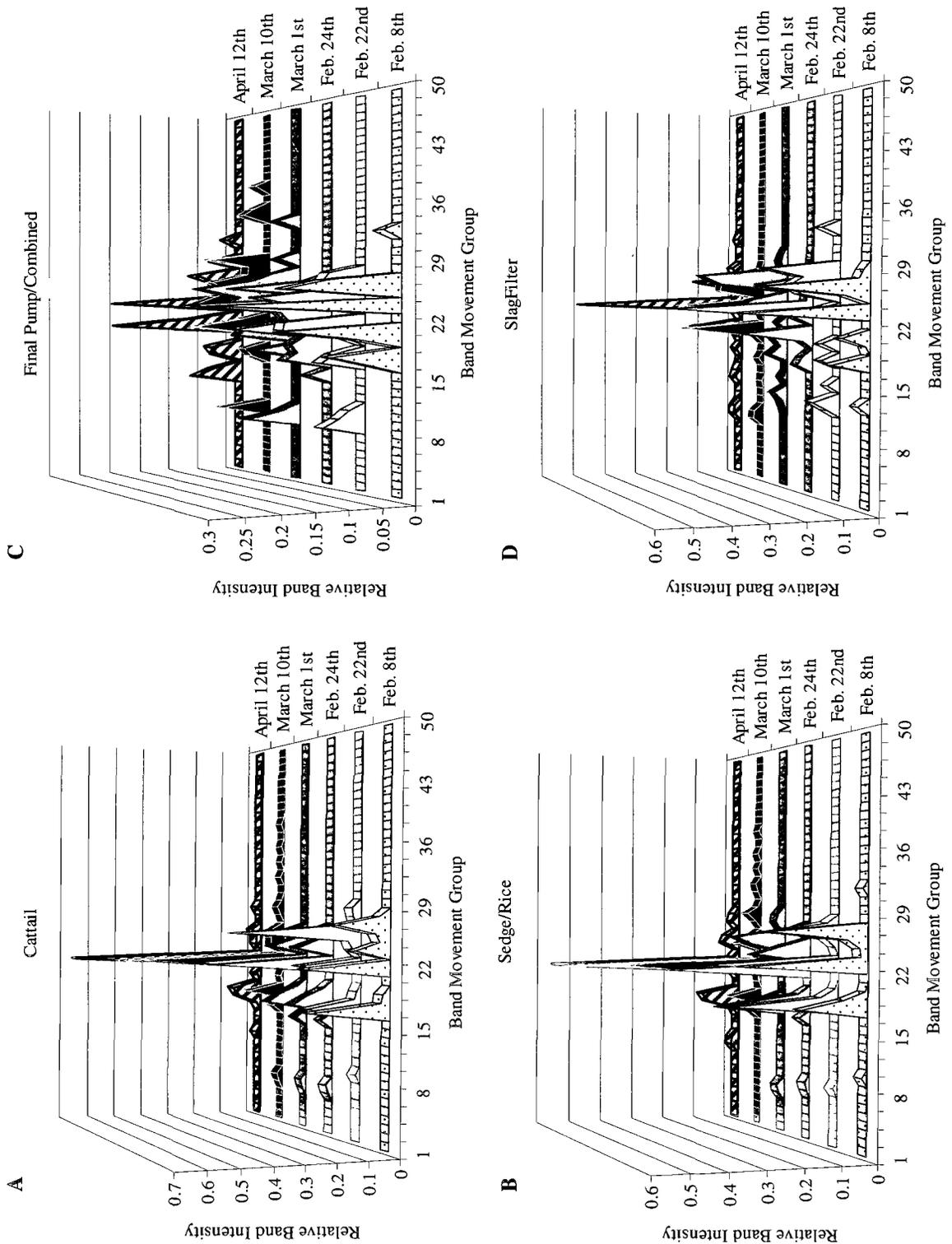
Figure 5.11. DGGE-based averaged banding pattern for selected locations in the field-scale CW. Addition of the oxytetracycline spike immediately following the collection of sample on Feb. 22nd, lasting until Feb. 26th. A) The'Cattail' location, B) The 'Sedge/Rice' location, C) The 'Final Pump/Combined' location and D) The 'Slag Filter' location.

decreased mesophilic dormancy for the changes in the functional fingerprints with the warmer temperatures from Mar. 10th to Apr. 12th. The changes in the 'Slag Filter' community structure also appeared to mirror those in the 'Cattail' and 'Sedge/Rice' locations, as all three locations had visually very similar appearance on Apr. 12th. These three locations also displayed a considerably different banding pattern on Apr. 12th than prior to OTC addition, providing clear evidence of a possible long-term effect of the OTC administration. The 'Final Pump/Combined' location displayed a unique community structure, confirming the indication from the genetic diversity curves (see Figure 5.9) that there may be a distinct community residing prior to or within the 'Final Pump/Combined' location despite the contributions from the earlier areas of the CW.

To quantify these visible population changes, the genetic divergence was calculated and is presented in Figure 5.11. Genetic divergence is a very simple one dimensional metric used to assess the amount of change between two communities. The divergence between the Feb. 8th and 22nd samples, not available in Figure 5.11, are displayed in Table 5.1. The values in Table 5.1 can be interpreted as an estimate of prior, undisturbed divergence. However as only 2 samples were taken prior to exposure, resulting in only a single pre-OTC divergence value, caution should be used in assuming that these numbers truly and fully describe the expected or average undisturbed divergence.

The 'Cattail', 'Sedge/Rice' and 'Slag Filter' locations showed the most genetic divergence, both during OTC exposure and afterward; although the rate

Table 5.1. Genetic divergence between Feb. 8th and Feb. 22nd.

Location	Divergence
Septic	0.106
Cattail	0.425
Sedge/Rice	0.170
Final Pump/ Combined	0.324
Slag Filter	0.391
Pond	0.167

of divergence in these locations declined steeply from Mar. 10th to Apr. 12th (see Figure

5.11.B). The pre-OTC genetic variation (see Table 5.1) was greatest in the 'Cattail',

indicating a moderate degree of variation. The 'Slag Filter' experienced a similarly

moderate divergence prior to OTC addition. Although the 'Slag Filter' appeared to return

a near pre-OTC divergence rate following exposure, the 'Cattail' continued to drop to

below half the rate of pre-OTC divergence on Apr. 12th. The 'Sedge/Rice' location

displayed a low pre-OTC divergence, which was increased to a rate nearly 6 times higher

during OTC exposure and remained relatively high by compared to pre-OTC divergence

until the Apr. 12th sample. This may be an indicator of both a possible short- term and

long-term effect of OTC administration on the genetic variability of these two sites, one

that has resulted in an increased divergence following acute exposure and a decreased

genetic variability in the long-term.

This could be potentially detrimental to the function and sustainability of CW

systems, as communities with decreased genetic variability and resultantly less plasticity

may be less likely to overcome further deleterious events. The 'Pond' and 'Septic'

samples showed very limited divergence from the initial sample, as well as a low natural

divergence between Feb. 8th and 22nd. These two locations also displayed a very similar

rate of genetic change over the entire investigation period (see Figure 5.11.B), providing

further evidence toward the increased persistence of the 'Septic' effluent through the CW

following OTC treatment, as the total genetic variability within this site appears as if it

may be linked. If this is the case it may indicate that the CW is ineffective in removing a

portion of the community indigenous to the waste effluent, and that this community is

subsequently finding a niche in the 'Pond'.

A

B
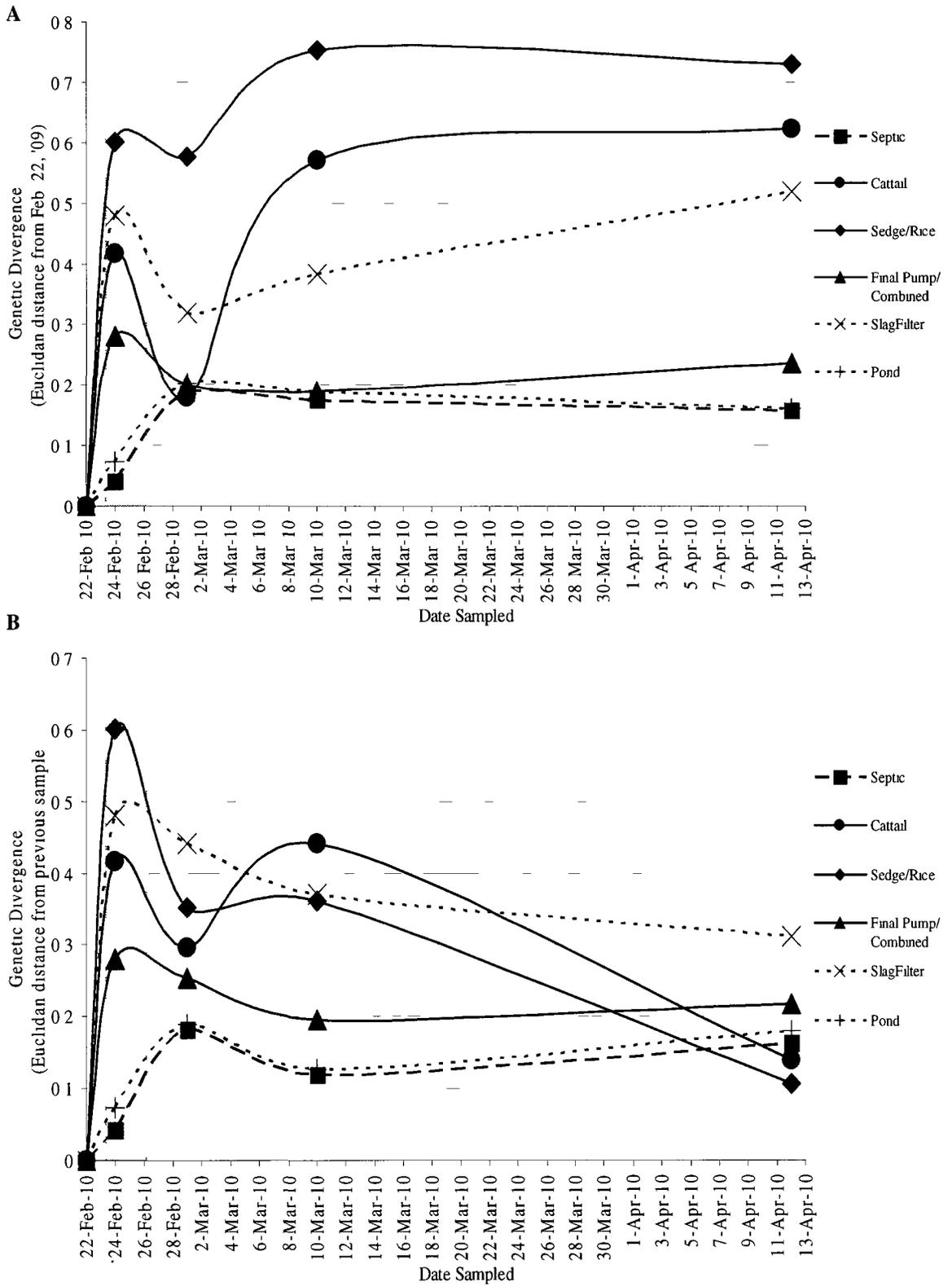
Figure 5.12. Genetic divergence based on Euclidian distances of untransformed DGGE profiles from the field-scale CW. A) Divergence from the Feb. 22nd sample, for each sample at each location and B) Divergence from the previous sample.

Investigation of the genetic fingerprints using PCA and tree cluster analysis should be able to distinguish if this amount of change is occurring in similar areas of the structural fingerprint, or if the similarity in the amount of structural divergence is due to changes in dissimilar areas. PCA is presented in Appendix A, Figure A.7. Evident from both plots is a high degree of spatial similarity in the genetic profiles, as all the sampled locations seem to be clustered near themselves. Additionally, the Feb. 22nd and Feb. 24th samples are all located in generally different areas in both Figures A.7.A and B. This may indicate that each of the locations sampled in this CW contain distinct microbial communities prior to addition of the antibiotic, and maintained these distinctions following acute OTC exposure.

By the Mar. 10th samples however, the 'Cattail' and 'Sedge/Rice' locations have appeared to lose much of the distance between them on the plots, indicating a possible convergence toward genetically-similar communities following OTC addition. This was also seen on Apr. 12th, which further indicates a delayed effect of the antibiotic on the community structure, and may also allude to long-term effects regarding the community structures in these two locations. The 'Cattail' and 'Sedge/Rice' locations also displayed a considerable dissimilarity from all the other locations sampled following OTC exposure, which may be a result of the much larger concentration of the antibiotic present in these locations. The 'Final Pump/Combined' location displayed a unique community structure, landing between the 'Slag Filter' and the 'Cattail' and 'Sedge/Rice' locations, confirming the indication from the rank-abundance and genetic diversity curves that there may be a distinct community residing prior to or within location despite the contributions from the earlier areas of the CW.

Tree cluster analysis is depicted in Figure 5.13. Based on these results there is further evidence for the existence of spatially distinct community structures within this CW system, as the 'Cattail' and 'Sedge/Rice' samples show the largest amount of dissimilarity from the other samples taken. There is also considerable dissimilarity visible between 'Cattail' and 'Sedge/Rice' samples, although this is really only visible prior to OTC addition. This dissimilarity is almost completely lost by Apr. 12th, as these locations displayed convergence to a considerable genetic similarity on this date. This is likely the long-term effect of both the acute OTC loss and the fallout of modified ecological relationships, which may have caused the communities on Apr. 12th in these locations to be comprised of only members, likely similar considering the amount of OTC used, which were resistant or unaffected.
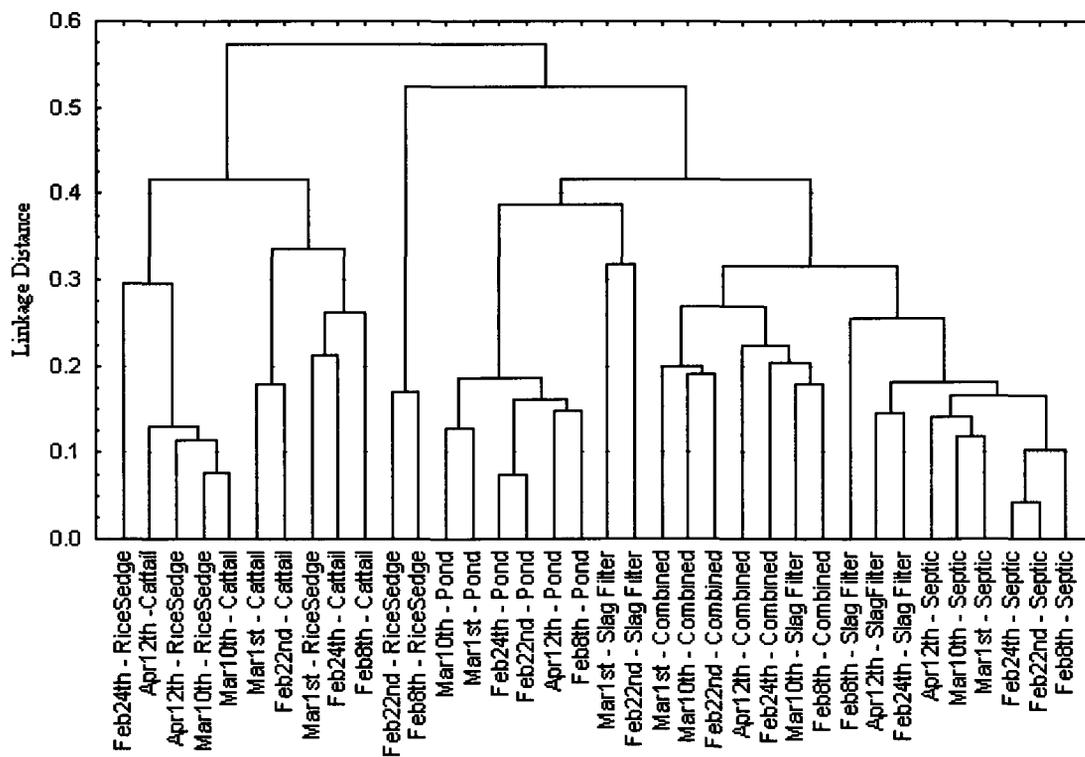
Figure 5.13. DGGE-based tree cluster analysis using the untransformed means from the field-scale CW.

Additionally, the similarity of the 'Final Pump/Combined' and 'Slag Filter' locations through the investigation period indicate that the communities coming from the 'Final Pump/Combined' may largely comprise the community present in the 'Slag Filter', although these two locations also share a large degree of structural similarity with the 'Septic'. Spatial genetic dissimilarity appears prominent in the 'Pond' location, as there is little change with time. The 'Pond' also appears closely related to the 'Slag Filter'; which may indicate that a fraction of the community is able to persist through the CW and also able to survive in the 'Pond'.

Both PC and cluster analysis were able to produce additional visualization of the spatial dissimilarity within the various locations of the field-scale CW. Changes observed through the functional fingerprinting and associated metrics have indicated an acute effect of OTC exposure, while changes observed through the structural fingerprinting metrics have made it clear that there is some effect of antibiotic perturbation on the microbial community, though this effect appeared either depressed or delayed.

Due to the stringent monitoring of the field-scale system during the week of antibiotic exposure, it is very likely the trends observed during this time are largely due to the antibiotic itself. However, trends following antibiotic exposure are more difficult to attribute to the OTC alone, due to the possible and pertinent influence of environmental factors like increasing temperature and other factors that may not have been assessed.

5.4. Conclusions from Field-scale Characterization

Based on the results previously discussed, the following conclusions from the field-scale

CW can be made:

➢ An acute effect of OTC exposure was observed in the functional fingerprints of

CW locations, in which nearly all metrics used to analyze the changes or patterns

in carbon metabolism showed clear increases during the exposure period. A

subsequent drop in function was observed following exposure, indicating a

possible recovery period.

➢ There was no clear evidence of a long-term effect of OTC exposure on the

observed functional fingerprint of the locations studied. However, continual

decline in the number of carbon sources utilized and the diversity of this

utilization tended to decrease from Mar. 1st through to Apr. 12th, despite

increasing temperatures. Continued monitoring is suggested before concluding

whether or not a long-term effect of OTC exposure was observed.

➢ There was no clear evidence of an acute structural impact of OTC exposure,

although there were slight changes observed during the exposure period. The best

indication of a possible acute effect of OTC was visualized in the rate of genetic

divergence; through which a moderate increase in sample-to-sample structural

community change was observed during the exposure period.

➢ A delayed and possibly long-term structural effect of the OTC exposure was

clearly observed in the field-scale CW following OTC administration was

completed. It appeared that structural communities changes tended to occur

during the recovery stage. Rank-abundance plots displayed a clear change in

community structure and PIE-based genetic diversity estimates showed a decrease in community diversity in the planted soil areas of the CW one week following cessation of OTC exposure.

➢ The functional behaviour of the 'Pond' location suggests that the ability of this field-scale CW to remove OTC was marginal, and that the majority of OTC was able to persist into this location.

➢ The difference in H' trends compared to those from the PIE genetic diversity indices suggests that PIE may be a more appropriate diversity metric than H' when considering techniques providing genetic or OTU-related data.

6.   Industrial-scale Treatment Lagoon Characterization

In order to address part of the overall goal of this study, which was to profile the structural and functional characteristics of predominant microbial communities in CWs at multiple system scales, an industrial-scale treatment lagoon was monitored using CLPP and DGGE, along with culture-based methods to monitor pathogens present. Other associated site characteristics monitored are presented in Appendix B, Table B.2 and include pH, ambient and water temperature and dissolved oxygen.

Although lagoons and wetlands differ in a number of important characteristics (see Section 2.1), there are also a number of very similar characteristics (i.e., wet, submersed soil, vegetation distinct from surrounding areas, use for treatment of wastewater) that make the microbial communities present within these treatment environments suitable for comparison to each other.

6.1. *Escherichia coli, Salmonella enterica* and Heterotrophic Plate Count Monitoring

HPCs were only completed on June 24th (2009) as part of the preliminary investigation of the site. Results from these HPCs are presented in Table 6.1. Three different heterotrophic growth media types were used to assess whether or not HPC were dependent on different types of concentrations of nutrients supplied. All media types promoted the growth of similar sized heterotrophic communities, indicating little difference, if any, in

Table 6.1. HPCs (CFUs/mL) from the industrial-scale treatment lagoon on June 24th, 2009 using LB, NB, and R2A after 7 days of incubation at 25°C.

Location	R2A	1/5th LB	LB
PostFloc	2.1×10^6	1.9×10^6	1.4×10^6
Lagoon 1	4.0×10^5	7.1×10^5	6.2×10^5
$1 \rightarrow 2$	1.1×10^5	2.2×10^5	3.4×10^5
Lagoon 2	8.8×10^4	6.6×10^4	3.9×10^4
$2 \rightarrow 3$	6.2×10^5	2.8×10^5	4.7×10^5
Lagoon 3	3.1×10^4	5.5×10^4	2.9×10^4

the fastidiousness of this group of microorganisms within the lagoon system. Septic

HPCs were at least an order of magnitude greater than any other site, and very little

difference, if any was noticed between HPCs within the lagoon system. HPCs were

decreased two fold by the third lagoon. Faecal coliforms (FCs) and *Escherichia coli*

enumerations, as well as *Salmonella enterica* isolation, were completed to provide a

measure of wetland function with regard to biological contaminant removal. *S. enterica*

presence and absence is presented in Table 6.2. FC and *E. coli* counts are displayed

graphically in Figure 6.1.

'Lagoon 2' was the only location that displayed consistent, decreasing trends with

respect to pathogen removal. It was also the only location where *S. enterica* was not ever

detected. Pathogens were decreased by 99.9% from 'Lagoon 1' to 'Lagoon 3' on Sept.

21st, Nov. 17th (2009) and July 5th (2010). The June 24th, 2009 sample displayed a 99%

removal of *E. coli*. None of these samples dates were positive for *S. enterica* in the final

lagoon. It appears from this that the unknown amount of untreated kill floor effluent

leaking into 'Lagoon 3' on Sept. 28th, 2010, as this location experienced both increased

FCs and *E. coli*, but also the emergence of *S. enterica* in the third lagoon; providing

Table 6.2. *S. enterica* presence and absence in the industrial-scale treatment lagoon based on culture isolation and PCR confirmation. '+' signs indicate the presence of PCR confirmed *S. enterica* in a sample, while '-' signs indicate it's absence.

| Location	24-Jun-09	21-Sep-09	17-Nov-09	5-Jul-10	28-Sep-10
PostFloc	+	N/A	+	+	+
Lagoon 1	-	+	-	+	+
1 → 2	+	+	+	+	+
Lagoon 2	-	-	-	-	-
2 → 3	+	-	+	-	+
Lagoon 3	-	-	-	-	+
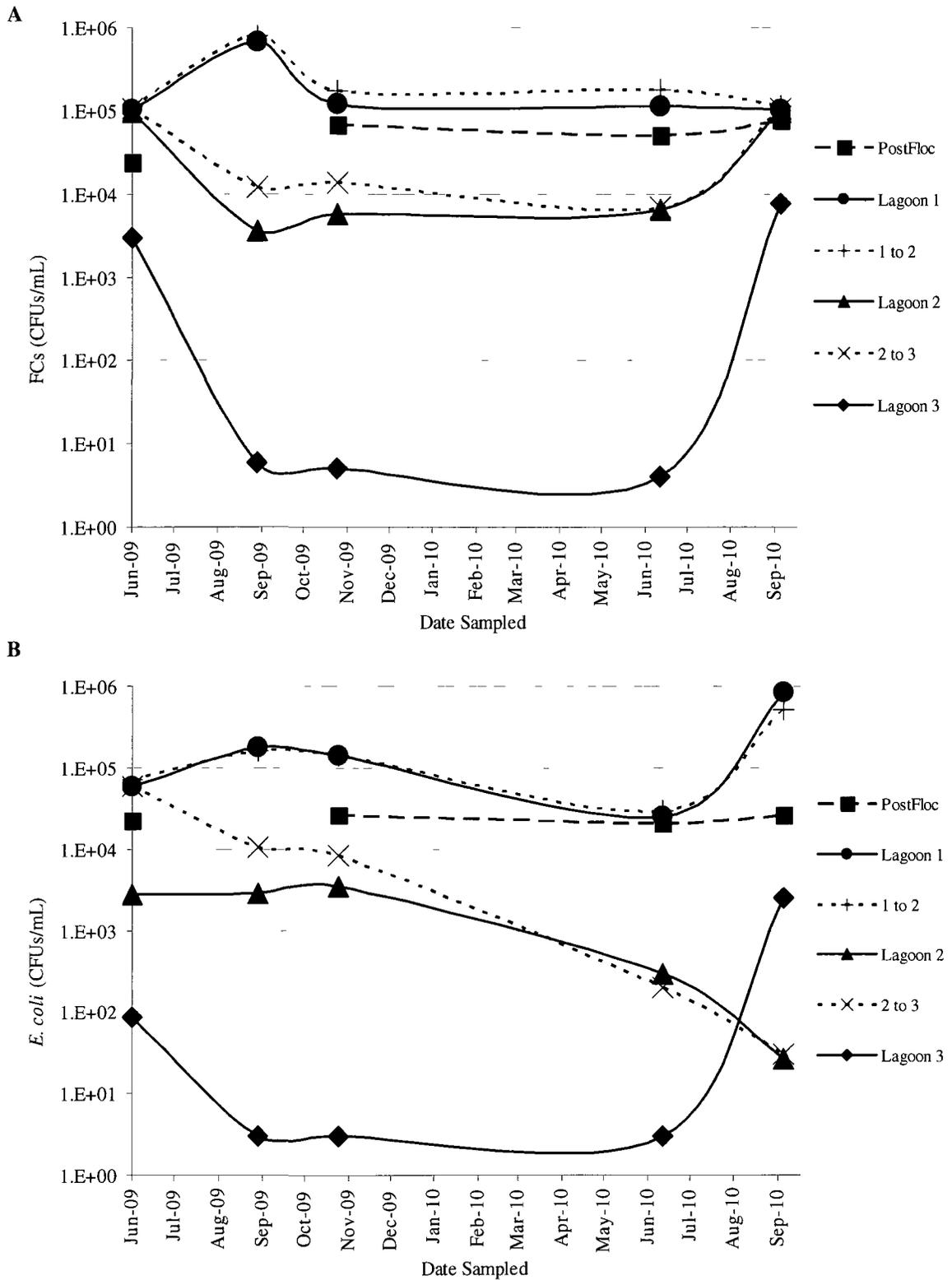
Figure 6.1. Graphical representation of FCs and *E. coli* enumerated in the industrial-scale treatment lagoon. A) Faecal coliform and B) *E. coli* enumeration on mFC + 1% Rosolic acid and mFC + 1% BCIG, respectively. Counts were completed between 24 and 48 hours of incubation in a water bath @ 44.5°C.

evidence to confirm the concerns of Krishnan & Smith, (1987) regarding the use of

untreated wastewater in alternative environmental treatment systems. As mentioned in

Section 3.1.3, the raw-effluent contamination event continued for an estimated three days

until the flocculator pipes could be cleared.

Another important site note is the impact of ferric chloride done on Sept. 21st

(2009) samples, added to counteract the addition of untreated effluent to the first lagoon.

The fact that there was little to no change as a result of this untreated wastewater

contamination may indicate that the ferric chloride treatment was an effective measure of

decontamination.

6.2. Functional Fingerprinting

CLPP was preformed on all 6 sample locations (see Figure 3.1.3) on 4 sampling dates in

triplicate, excluding the Sept. 21st (2009) 'PostFloc' sample. This resulted in the

generation of $n=$ 69 carbon source utilization pattern (CSUPs) for this industrial-scale

site. The first sample taken on Jun. 24th (2009) was not used in creation of CSUPs. The

average blank-corrected average well colour development (AWCD) of the 93 Biolog™

ECOplate™ wells containing carbon sources was calculated for each location and

presented in Figure 6.2. AWCD can be considered an overall estimate of the relative rate

of community carbon metabolism (Garland et al., 2007). The general rate of carbon

metabolism portrayed by each lagoon community stayed considerably consistent over the

monitoring period; displaying a slight general increase in metabolism in the hot, middle

of summer (July 5th) and decrease in the colder late fall (Nov. 17th). Notably, the rate of

carbon metabolism for the third lagoon was considerably increased from the fall to

summer, over which the remaining samples displayed a slight increase as well.
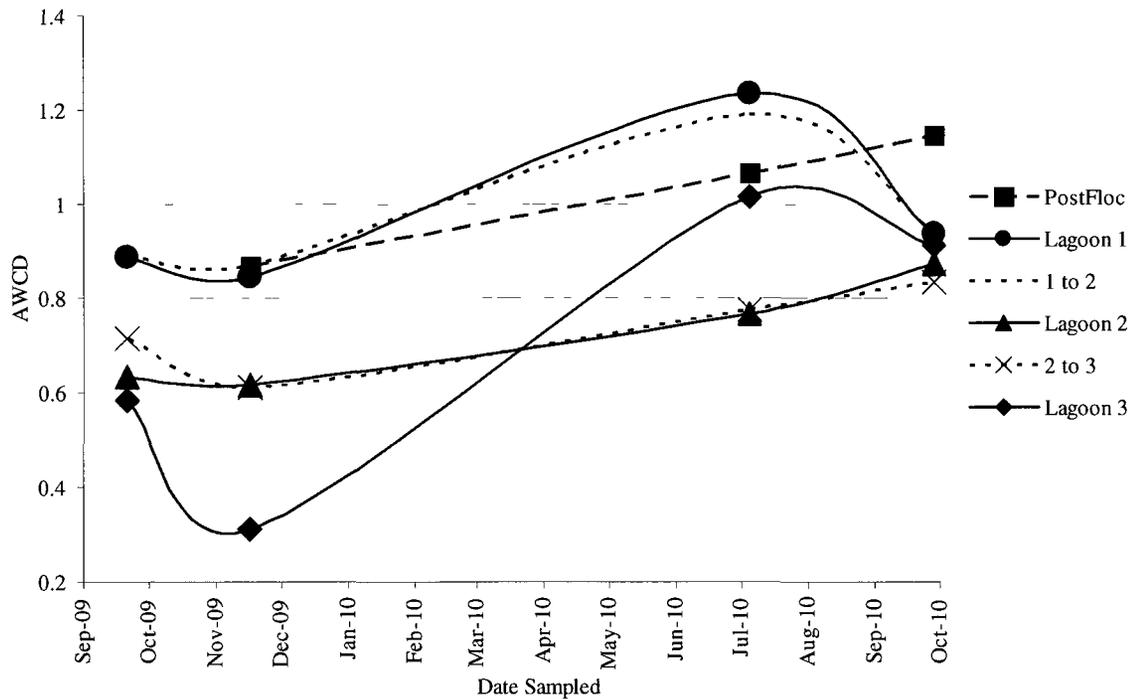
Figure 6.2. Average well colour development (AWCD) from the industrial-scale treatment lagoon CLPP after 72 hours of incubation at 22°C.

This may indicate that the rate of carbon metabolism is largely influenced by temperature or season.

Substrate richness, presented in Figure 6.3, is a measure of the number of carbon sources utilized by the community. Although most of the locations displayed a similar trends to those seen in the average amount of carbon metabolism (see Figure 6.2), the third lagoon showed a trend of decreasing carbon utilization with increasing temperature; displaying a decrease in the number of carbon sources utilized from Sept. 21st (2009) until its lowest point on July 5th (2010). This discrepancy between the increased rate of carbon metabolism and the decrease in total number of usable carbon sources in the third lagoon may be due to the influence of microbial populations that supply varying degrees of environmental competition depending on the ambient temperature or season in question.
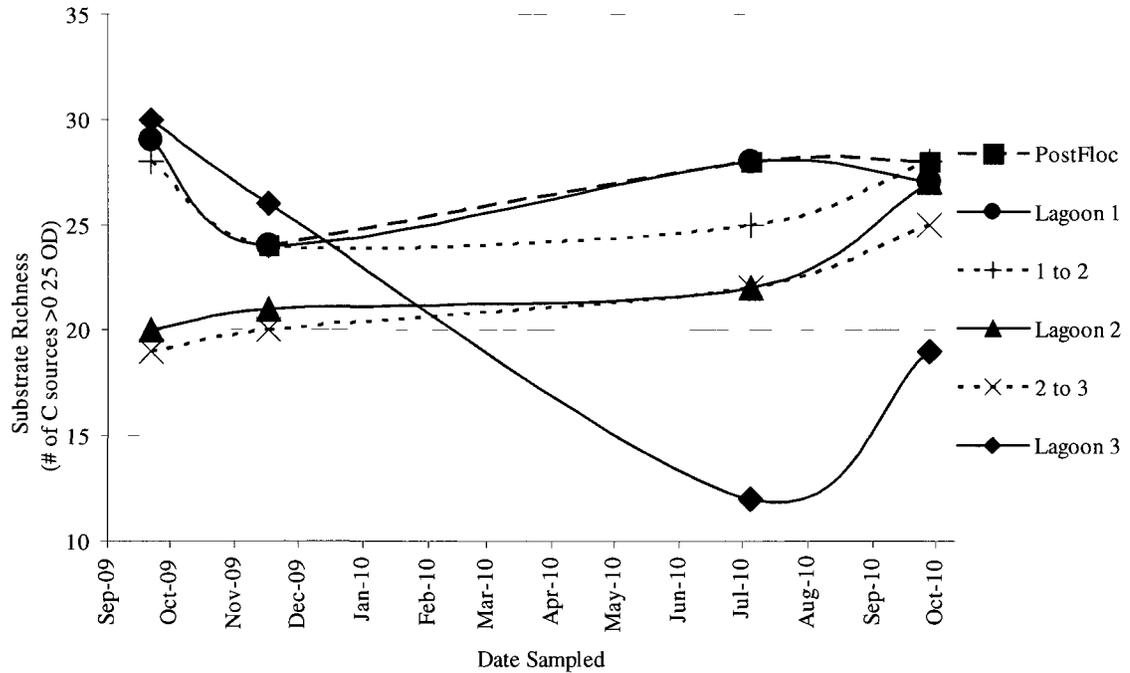
Figure 6.3. Substrate richness calculated from the industrial-scale treatment lagoon CLPP after 72 hours of incubation at 22°C

It may be the case that as the temperature increases (see Appendix B, Table B.2),

mesophilic organisms supply more competition for environmental niches or required

elements. As competition for scarce or limiting nutrients increases, the ability of a

population or community member to use a certain carbon source would become

dependent on its ability to sequester the required, environmentally limiting elements (i.e.,

phosphorous, nitrogen. etc). Those organisms able to obtain these limiting factors would

be able to metabolize their preferred carbon source with relative efficiency; resulting in

the increase rate of carbon utilization.

Conversely, those microorganisms that would not be able to obtain the same

limiting factors in the face of increasing competition would not be able to metabolize

their preferred carbon source, or any carbon source at all for that matter; resulting in a

decrease in the number of usable carbon sources. The lack of seasonally-attributed

change observed in the other lagoon locations may be a result of the continued supply of

waste effluent, which may contain enough biologically-usable limiting elements to

maintain the carbon metabolizing populations regardless of the amount of competition

present from other microorganisms.

Changes in H' (Shannon, 1948) over the monitoring period are presented in

Figure 6.4. Diversity generated from CLPP, an estimate of capacity and ability to utilize

the supplied carbon sources, has been suggested as a metric that provides an insight into

the overall community response or function (Weber & Legge, 2008). Based on these

trends, it appears that the seasonal effects in the number of carbon sources utilized and

the overall rate of carbon metabolism seem to effectively cancel each other out with

respect to diversity estimates; as H' takes into account both the number of carbon sources

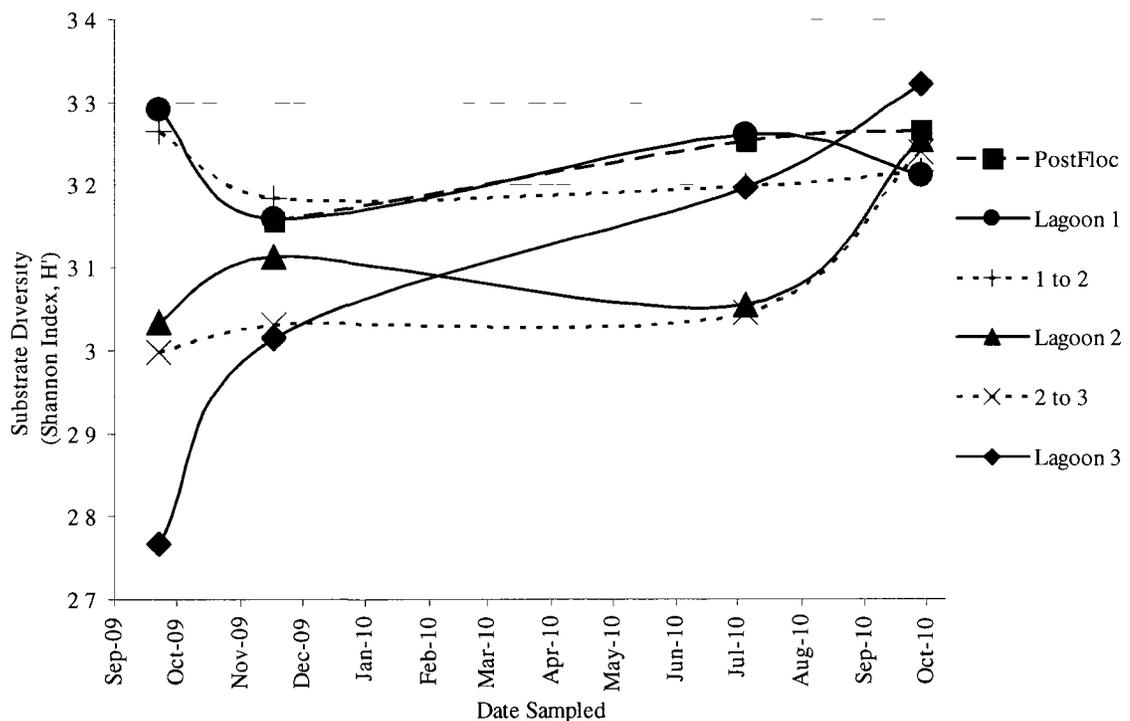utilized and the pattern of their usage. There, however, appears to be a major change in


Figure 6.4. Substrate diversity (Shannon H') calculated from the industrial-scale treatment lagoon CLPP after 72 hours of incubation at 22°C

the pattern of diversity regarding carbon metabolism coinciding with the two
contamination events.

The addition of ferric chloride to the third lagoon on Sept 21st (2009) seems to
have visually decreased the diversity of carbon metabolism; despite the observation that
nearly all of the supplied carbon sources were utilized (i.e., 30 of 31; see Figure 6.3) and
the rate of carbon metabolism was slightly increased on this same day (see Figure 6.2).
The first lagoon on this day, which received the untreated wastewater, showed a carbon
utilization diversity near the maximum observable (i.e., 3.434; see Section 2.3.1.2). A
diversity value very near this maximum was also observed in 'Lagoon 3' after it received
untreated waste on Sept. 28th (2010). This provides further evidence that these alternative
treatment systems may be primarily effective remediating waste effluent when receiving
pre-treated wastewater.

Figure 6.5 displays the functional divergence observed from the first sample taken
on Sept. 21st (see Figure 6.5.A) and the divergence from the previous sample (see Figure
6.5.B). Genetic divergence is a one dimensional metric considered to be a good, simple
measure for comparing the pattern of change of a microbial community; especially in
response to a perturbation (Weber and Legge, 2009). Unfortunately, due to the
unexpected nature of the perturbations in the form of untreated wastewater, there is no
sample to use as reference prior to the perturbation. As such, the curves depicted in
Figure 6.5.A should be interpreted as the amount of functional change following recovery
from a perturbation. All of the locations displayed a consistent amount of functional
divergence from the Sept. 21st (2009) sample on both Nov. 17th (2009) and July 5th
(2010). It is unclear if this lack of functional divergence from Sept. 21st (2009), despite

A

B
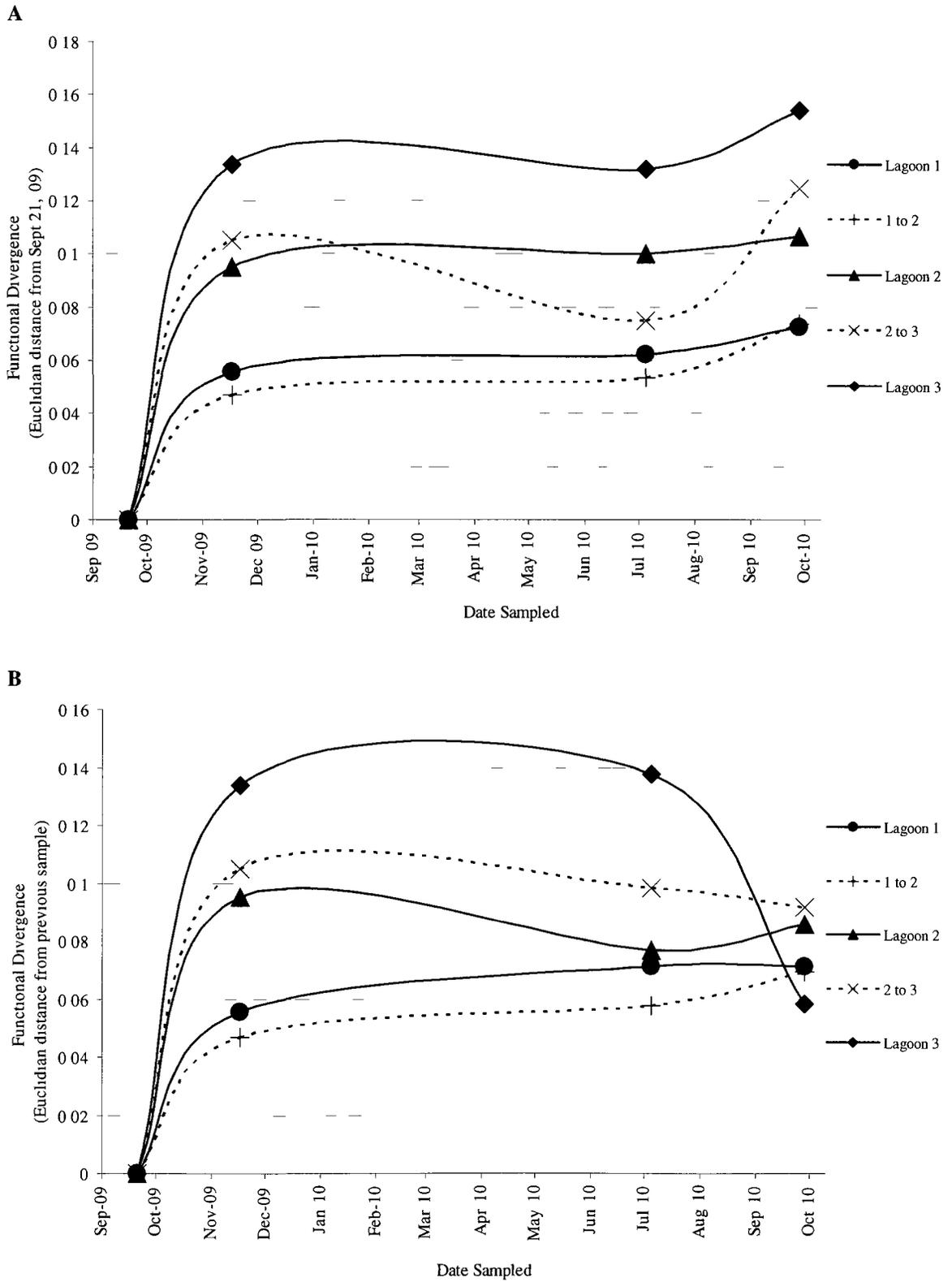
Figure 6.5. Functional divergence based on Euclidian distances of untransformed CLPP profiles from the industrial-scale treatment lagoon A) Divergence from the sample taken on Sept 21st and B) Divergence from the previous sample The PostFloc location was not included in this calculation

changes in temperature (see Appendix B, Table B.2) and other observed changes in metrics used to assess the functional profile, should be expected; or is resulting from a long-term effect of ferric chloride addition and untreated waste contamination.

Figure 6.5.B displayed very similar trends in sample to sample divergence, indicating that during normal operation there is a consistent rate of functional divergence, or variation in carbon metabolism. Following the contamination event on Sept. 28th (2010), however, there appears to be a considerable drop in the rate of functional divergence in 'Lagoon 3'; indicating a loss of variability in the functional profile. This loss of functional variability seems to indicate that the community responsible for carbon metabolism in the third lagoon may have suffered substantial duress following the addition of untreated wastewater. Further monitoring is required to elucidate the resiliency of the 'Lagoon 3' and if it is able to overcome this decrease in functional variability.

Principal component analysis (PCA) is presented in Figure 6.6 and treated the supplied carbon sources as variables along with the ability to utilize that carbon as the quantitative measures. It appears from both Figure 6.6.A and B that, in 2009, the third lagoon contained a community functionally distinct from the earlier lagoons, as well as the 'PostFloc', 'Lagoon 2' and 'Lagoon 3' generally displayed considerable temporal variation, which may be due to changes in temperature or other seasonal characteristics not monitored. Both 'Lagoon 1' and the 'PostFoc' samples were relatively invariable despite seasonal changes, and generally shared a large degree of similarity despite the addition of a commercial wastewater treatment agent (Active8, see Section 3.1.3) between them.
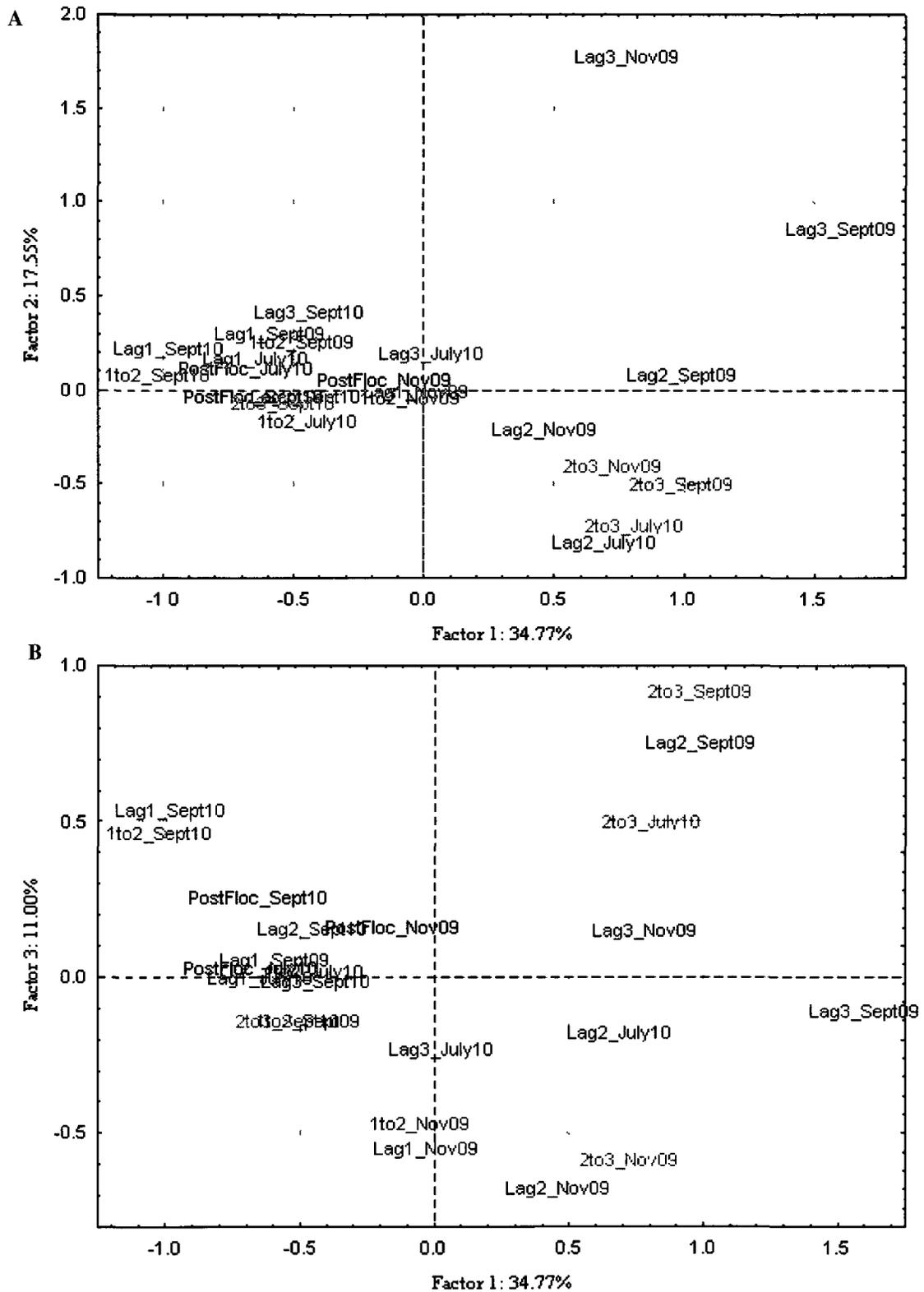
Figure 6.6. PCA using the covariance matrix from Taylor-transformed blank-corrected means from the industrial-scale treatment lagoon CLPP. A) Plot of cases on the 1 x 2 factor plane and B) Plot of cases on the 1 x 3 factor plane.

Following the contamination of 'Lagoon 3' on Sept. 28th (2010), this location became functionally very similar to the 'PostFloc' and 'Lagoon 1'; indicating that the untreated wastewater community responsible for carbon metabolism was able to persist in this location. As the July 5th (2010) community representation in 'Lagoon 3' appeared to share a high degree of functional similarity with the 'PostFloc' and first lagoon, there may also be an impact of the ferric chloride decontamination from the previous Sept. which may also be responsible for changes seen in the functional profile of the third lagoon.

This decontamination event may have left the surviving community in the third lagoon weakened and unable to compete with the 'PostFloc' or 'Lagoon 1' community that continued to influence it from Sept., until the end of operation in mid-Dec., when the apparent temperature-influenced number of carbon sources utilized would have been at its lowest. This may have left the mesophilic communities responsible for carbon metabolism in the third lagoon less metabolically fit due to both the decontaminant- and temperature-related stress; opening the door for any of the metabolically well equipped 'PostFloc' populations to compete for niches; resultantly changing the functional profile to one closer to that of the 'PostFloc'.

Tree cluster analysis is presented in Figure 6.7. Like PCA, a large degree of functional similarity within the 'PostFloc' and 'Lagoon 1' locations is visible. Of note, the 'PostFloc', 'Lagoon 1' and '1 to 2' locations were generally very similar to each other over the monitoring period, indicating the likely persistence of the environmentally
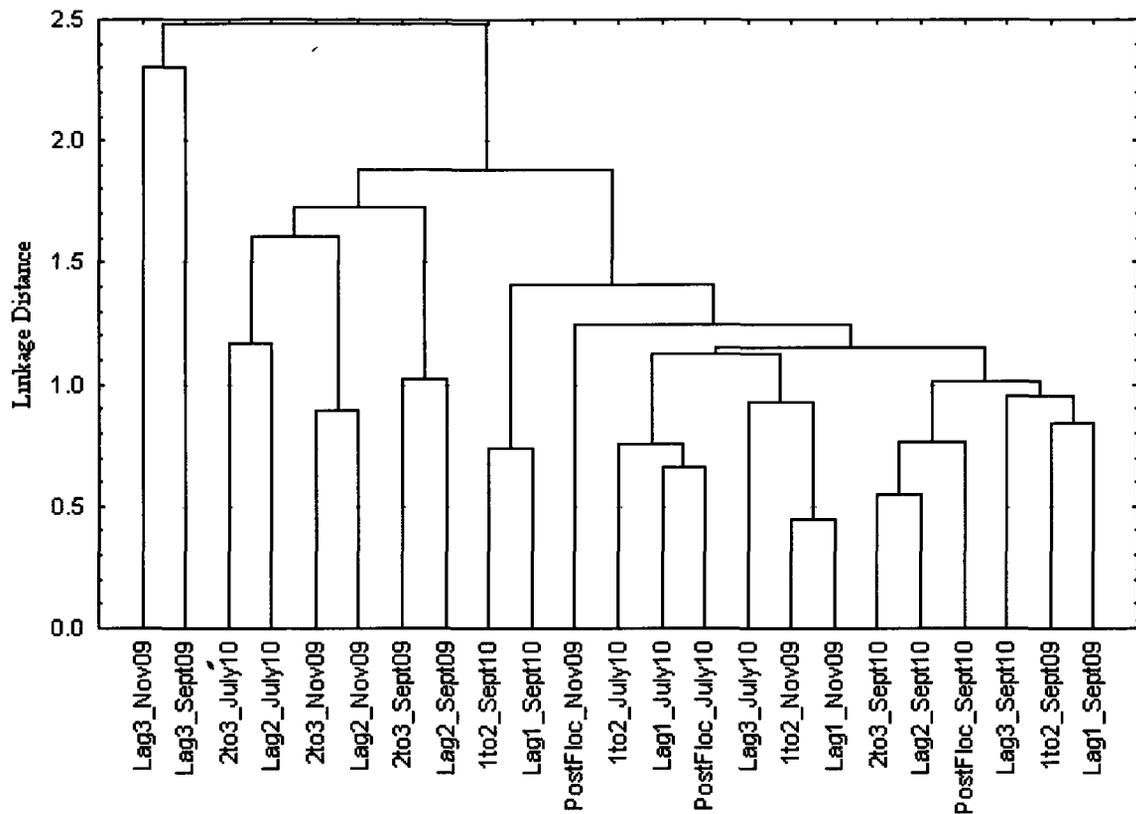
Figure 6.7. CLPP-based tree cluster analysis using the untransformed blank-corrected means from the industrial-scale treatment lagoon. Linkage distance was calculated using UPGMA of Euclidian distances

competitive functional community present in the 'PostFloc'. The 'Lagoon 2' samples

appeared distinct with considerable dissimilarity in functional profile when compared to

the earlier locations. This may indicate the presence of a functionally and spatially

distinct community in this location that is subject to considerable seasonal variation.

When comparing across the year of sampling, the linkage distance of the samples

taken from 'Lagoon 3' in 2009 indicates that this community was likely distinct and

variable under normal functioning. The samples taken in 2010 show a considerable

similarity to 'PostFloc' and 'Lagoon 1' locations; which is interesting, as these samples

did not show any apparent similarity to the second lagoon, despite the fact that 'Lagoon

3' receives a sizable load from 'Lagoon 2'. This is further complicated by the fact that

there was a noted dissimilarity between 'PostFloc' and 'Lagoon 1' locations in 2009.

6.3. DGGE Results and Discussion

DGGE was originally completed on all samples taken, including the 2010 samples using

both the centrifuge ('spin') method and filter method. To compare the two methods,

samples from both DNA extraction methods processed on the July 5th and Sept. 28th dates

were subjected to preliminary DGGE. The resulting gels were analyzed for the number of

bands visible and this is displayed in Figure 6.8. Both DNA extraction methods displayed

similarly shaped trend lines, but the filter extraction method resulted in consistently more

numerous bands than the spin method. The 'Lagoon 3' location on Sept. 28th displayed

the largest difference between extraction protocols, with a difference of 14 bands. Due to

the over-estimation of richness produced by the filtering method prior to DNA extraction
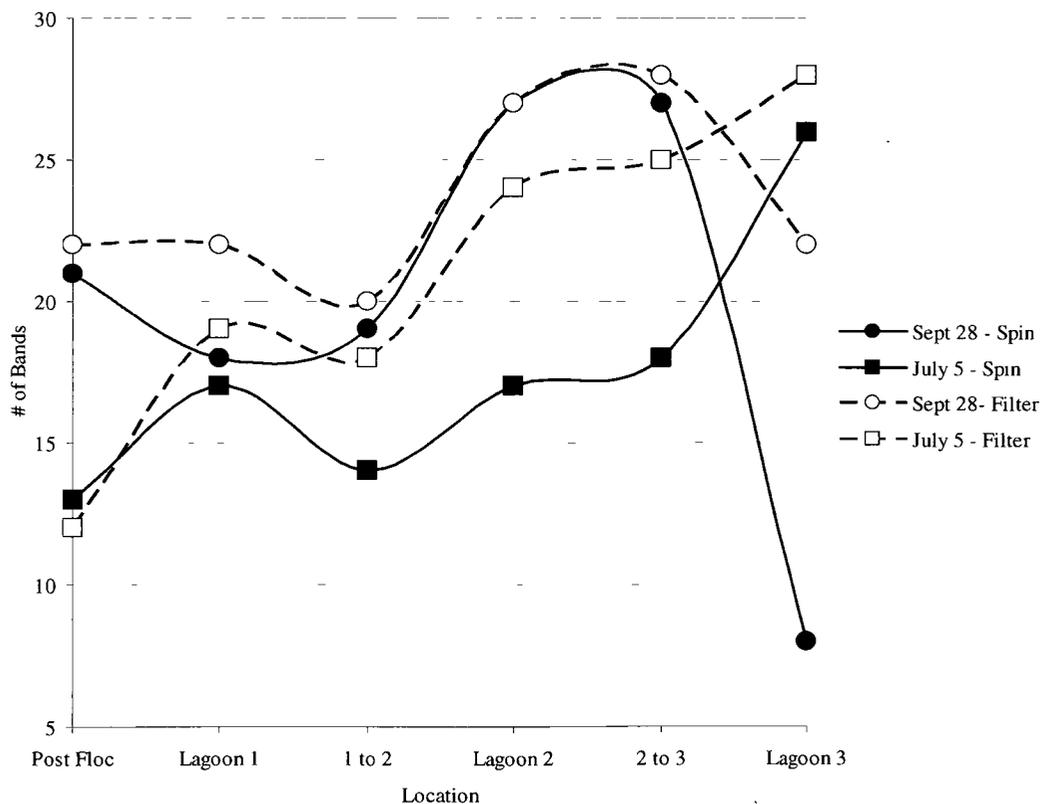
Figure 6.8. Comparison of richness from centrifugation ('spin') and filtration ('filter') of sampled lagoon water prior to DNA extraction.

produced by the filtering method prior to DNA extraction, along with the fact that only

the centrifuge method was completed on the 2009 samples, only DGGE on the samples

extracted using the centrifuge method was included for analysis in this document. This

resulted in the inclusion of a total n= 58 genetic fingerprints for analysis in this section;

given by the 6 locations monitored at 5 time points performed in duplicate and excluding

the unavailable Sept. 21st, 2009 'PostFloc' sample.

Genetic richness, defined as the number of bands above background observed in

DGGE gels or operational taxonomic units (OTUs; Sneath, 2005) was calculated and the

time-course trends for this metric is displayed in Figure 6.9. In the initial samples taken

on June 24th (2009), the third lagoon displayed the largest number of observed OTUs,
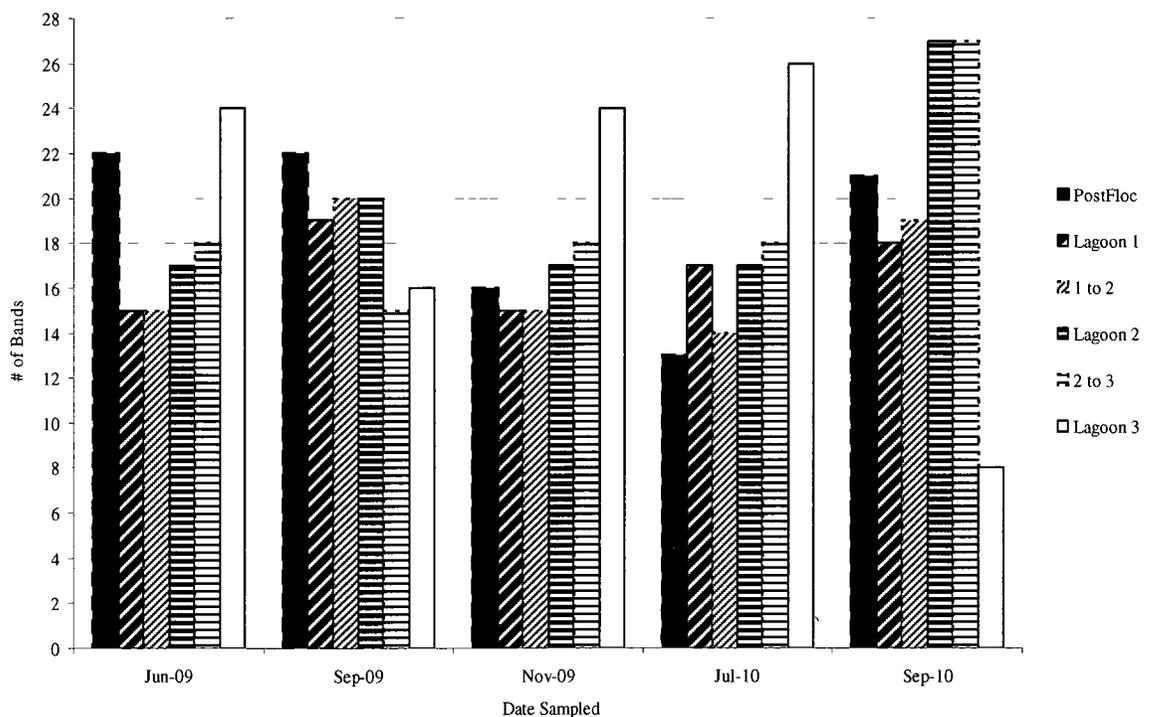
Figure 6.9. DGGE-based community richness (# of bands) from the industrial-scale treatment lagoon. PCR-DGGE was done using the DNA extracted from the 10 mL centrifuged pellet.

which subsequently showed a decline following ferric chloride addition on Sept. 21st, 2009.

On that same day, the first and second lagoons showed slight increases in the OTUs present, indicating both an acute effect of disinfection and a slight effect from the addition of raw wastewater. OTUs returned to almost completely identical amounts on Nov. 17th (2009) compared to June 24th (2009). This return may indicate that the number of OTUs within each location was not affected beyond an acute effect by the ferric chloride and initial wastewater contamination event. The samples taken on July 5th (2010) showed, again, nearly an identical number of OTUs in their respective sites, with slight increases visible. However, following the second contamination event the number of OTUs decreased drastically, with a drop of an average 14 OTUs, while all of the other locations displayed an increase in the number of observed OTUs.

To further explore the behaviour of the industrial-scale treatment lagoons, H' (Shannon, 1948) and PIE (Hurlbert, 1971) were calculated and are displayed in Figure 6.10. Both H' and PIE provide a simultaneous indicator of the number of OTUs present, and the proportion with which they occur. Both metrics showed decreases in the structural diversity of the community following the 2009 and 2010 contamination events. Both indices also depicted an apparent recovery of diversity back to June 24th levels into the Nov. 17th and July 5th samples. There was no apparent change in structural diversity between the late fall and summer samples, which is surprising considering the differences in temperature between Nov. and July.

A

B
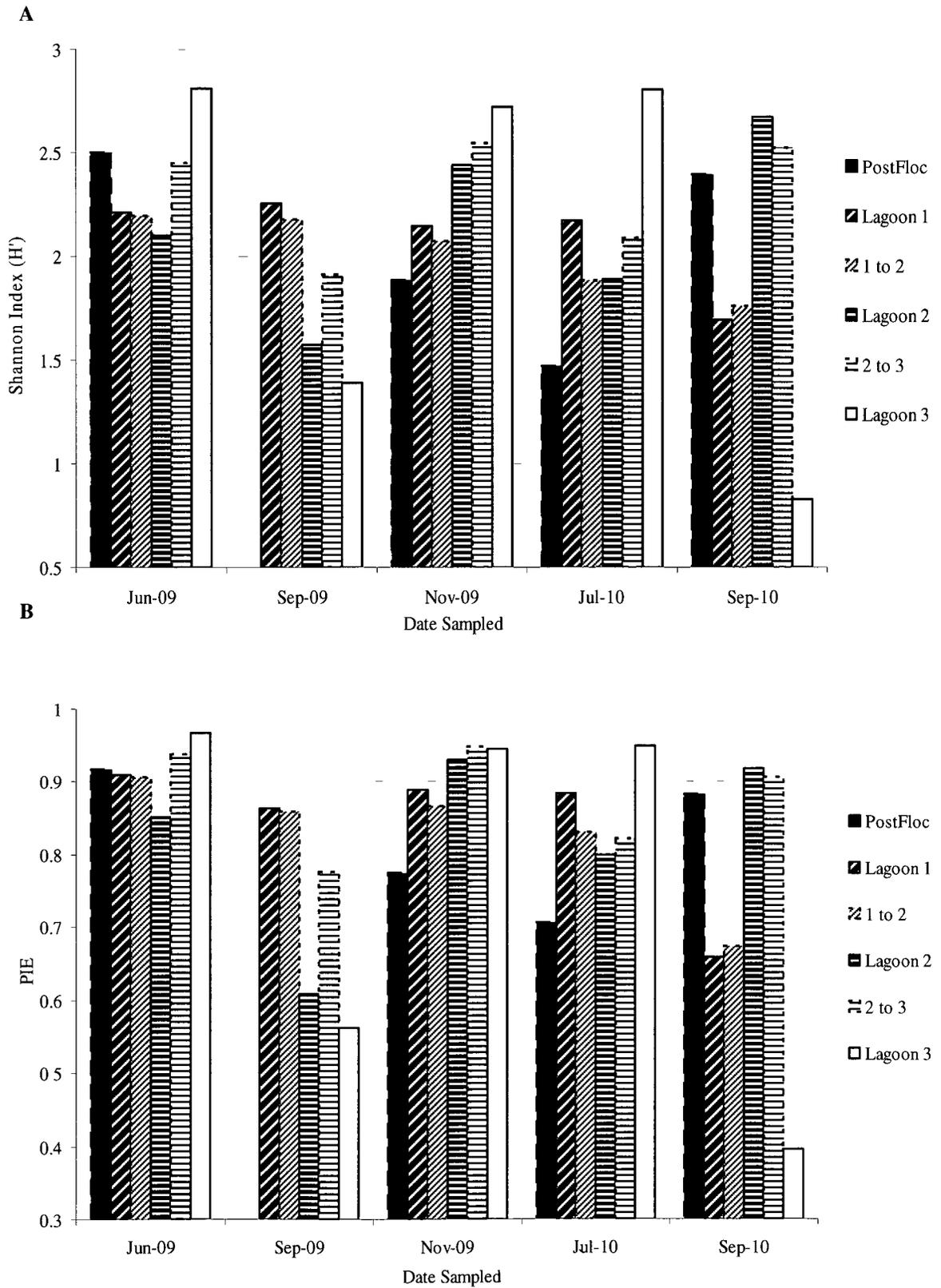
Figure 6.10. DGGE-based indices of community diversity from the industrial-scale treatment lagoon. A) Shannon index (H') and B) Hurlbert index (PIE).

Rank-abundance plots were created for the three different lagoons and can be seen in Figure 6.11. Plots for the two flow-through locations were not included as these were not noticeably different from the rank-abundance plots for the lagoon providing the flow-through. The first lagoon (see Figure 6.11.A) appeared to maintain a very similar genetic profile throughout the monitoring of the system, although this was not conserved on the final monitoring day (Sept. 28th, 2010). Both 'Lagoon 1' and 'Lagoon 3' (see Figure 6.11.C) displayed similar shifts in the rank-abundance curve toward communities largely dominated by a single OTU on the final day of monitoring; coinciding with a contamination event in which raw, untreated waste effluent was added to both the 1st and 3rd lagoons. There was also a visible shift in rank-abundance curves in all three lagoons following the contamination event on Sept. 21st, 2010. This may indicate that the addition of untreated waste water to the treatment system any step in retention causes a change in the relative genetic structure of the community. It is unclear whether this structural change is related to long term changes in community function, although it should be noted that the only time S. enterica was detected in the 3rd lagoon was following the contamination event on Sept. 28th, 2010; indicating that this change may be detrimental to the function of the treatment system. 'Lagoon 2', the only location consistently devoid of S. enterica, showed little to no change in the community structure over the monitoring period, with the exception of Sept. 21st, 2010 sampling date. The lack of S. enterica despite a community profile dominated by a single OTU may be due to the addition of the ferric chloride decontaminant prior to sampling.

To visualize this difference Figure 6.12.illustrates the averaged banding pattern for each of the three lagoons, and were created using the averaged relative abundances
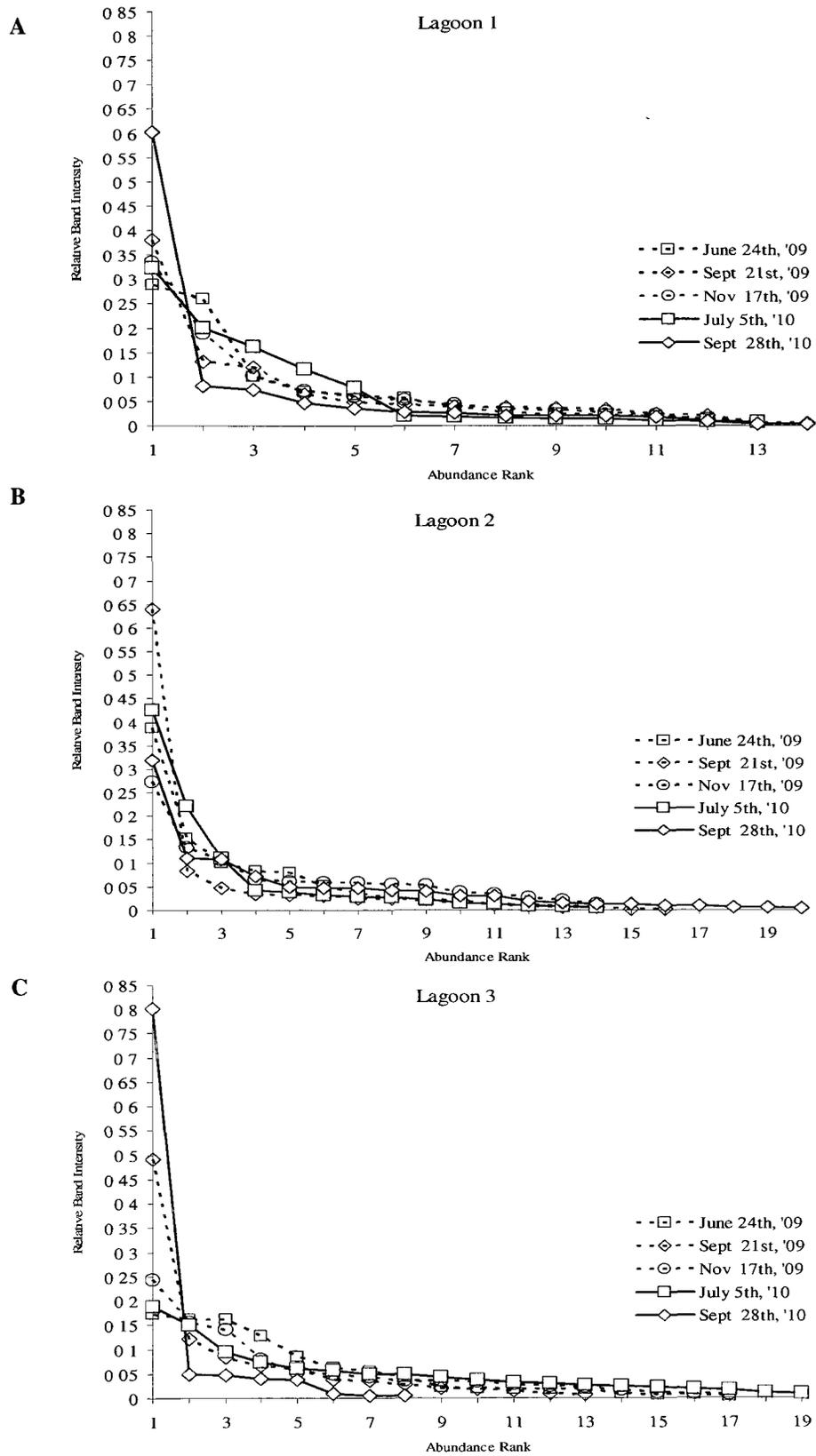
Figure 6.11. DGGE-based rank-abundance curves for the lagoon locations in the industrial-scale treatment lagoon. A) 'Lagoon 1', B) 'Lagoon 2' and C) 'Lagoon 3'.

from the genetic profile while maintaining the movement of these locations in the DGGE gel.

In Figure 6.12, there appears to be only minor changes in 'Lagoon 2' and 'Lagoon 3' visible between the Nov. 17th and July 5th sample despite the seasonal changes. 'Lagoon 3' appeared dominated by a number of moderate G+C OTUs, all contributing to relatively equal proportions of the community. However, following the Sept. 21st (2009) contamination event, a prominent OTU that was only present in minor representation previously increased substantially, contributing less than 5% of the community to representing over 40%. As the temperature decreased, the appearance of high G+C OTUs were visible, and the previously dominant phylogenetic groups appeared decreased to normal levels. July 5th produced little change in the population structure beyond the appearance of a novel low G+C OTU and the shuffling of community structure representation amongst those previously observed. This may indicate that the ferric chloride disinfection may have affected the ability of this community to undergo normal, seasonal successions.

The genetic structure on Sept. 28th (2010), however, showed the presence of only one excessively dominant phylogenetic group; accounting for nearly 80% of the total community. This community structure was similar to that of 'Lagoon 2' following the first contamination event, as well as the first lagoon on following the second contamination event; with both these locations displaying a community dominated by a single OTU accounting for over 50% of the total community representation with moderate G+C content. This may indicate that, following the addition of untreated
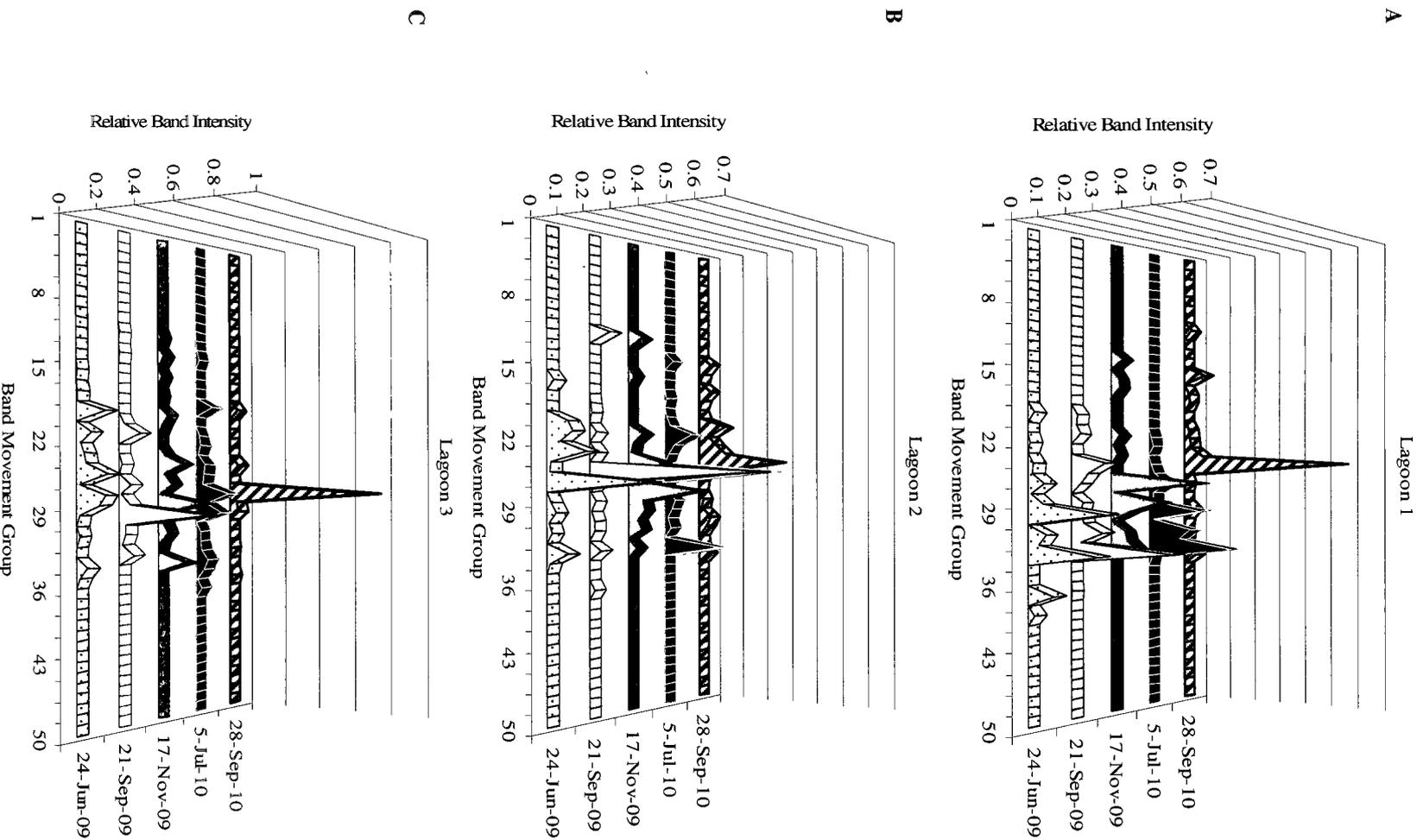
Figure 6.12. DGGE-based averaged banding pattern for the lagoon locations in the industrial-scale treatment lagoon. A) 'Lagoon 1', B) 'Lagoon 2' and C) 'Lagoon 3'.

wastewater, the structural profile of the undisturbed community dynamics are modified in such a way that only OTUs with moderate G+C content are able to flourish.

Genetic divergence is illustrated in Figure 6.13. Figure 6.13.A, shows the total amount of divergence in a given community structure from the initial sample taken on June 24th, while Figure 6.13.B displays the sample to sample change in community structure. On Sept. 21st 'Lagoon 2' displayed the largest amount of change from its respective sample on June 24th, while 'Lagoon 3' displayed the least. This was followed by increased structural change in the third lagoon and decreased structural change in the second lagoon on Nov.17th. Upon sampling on July 5th, the second lagoon maintained a consistent divergence from the initial sample, while both the first and third lagoon displayed somewhat of a reversion back to the structure observed on the initial sampling day one year earlier. This may indicate the presence of seasonal changes in the microbial community, as the June 2009 and July 2010 samples may be considered to represent the same season. Considerable structural change was observed in the first lagoon in the final sample, while 'Lagoon 3' continued to display very moderate structural divergence from June 24th, as did 'Lagoon 2'.

The sample to-sample divergence presented in Figure 6.13.B shows very similar trends to those observed in Figure 6.13.A. The flatter slope between Nov. 17th (2009) and July 5th (2010) in Figure 6.13.B compared to Figure 6.13.A may indicate that seasonally dependent populations, abundant in summer and not the late fall, were responsible for approximately half of the structural change observed. The increased rate of change in the third lagoon in Nov. 17th and July 5th compared to both September samples may indicate that decreases in the rate of structural change of a community may provide evidence
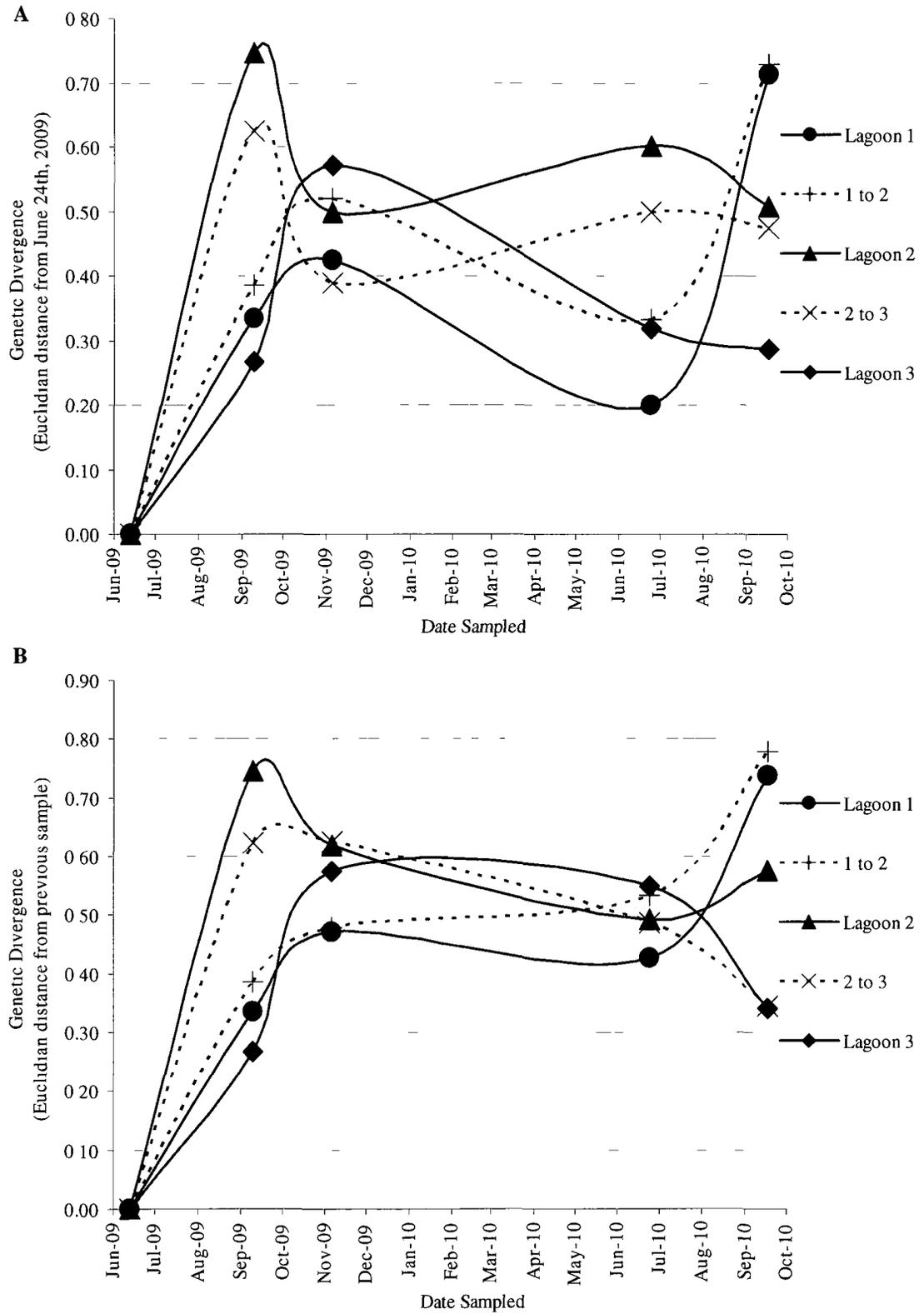
Figure 6.13. Genetic divergence based on Euclidian distances of untransformed DGGE profiles from the industrial-scale treatment lagoon. A) Divergence from the sample taken on June 24st and B) Divergence from the previous sample.

regarding the deleterious events, as both of these decreases coincided with raw waste contamination or chemical decontamination events.

It is clear from Figure 6.13 that both the genetic or structural divergence change from June 24th and the rate of structural change was highly variable over the over the monitoring period. Additionally, despite some small differences in rate or amount of change between locations, there was generally similar amount and rate of structural divergence on the Nov. 17th and July 5th sampling dates. However, due to the variable amount of genetic divergence observed and the similarity of the two divergence curves it is important to understand if this divergence is occurring in similar or dissimilar areas of the structural profile. Principal component analysis (PCA) or tree cluster analysis may be able to shed light on the current pictures of the behaviour of the community structures in these lagoons.

PCA plots are illustrated in Appendix A, Figure A.8. Unlike the functional fingerprints, PCA based on the structural fingerprints indicates that the 'PostFloc' and 'Lagoon 1' communities are considerably distinct. Both the second and third lagoons appear to be somewhat structurally similar to the each other, although are more temporally variable than the 'PostFloc' or 'Lagoon 1' communities. Of note, samples taken in June and September of 2009 appear to be located in distinct areas of both PCA plots, while both samples taken in 2010, as well as the Nov. 2009 sample appear to be converging on the area of the PCA plot containing the 'PostFloc' samples. This may indicate that, even after the ferric chloride decontamination event, some of the community from the 'PostFloc' was able to persist within the third lagoon.

Tree cluster analysis is presented in Figure 6.14. From this it is clear that the

'PostFloc' contained a relatively distinct community structure. Although the Sept. 28th

'Lagoon 3' sample appeared to be highly similar with in community structure to the

'PostFloc' based on the PCA plots, the third lagoon displayed a considerable degree of

structural dissimilarity to the 'PostFloc' based on tree clustering. Additionally, the Sept.

28th (2010) samples showed considerable similarity to every other location. All of the

samples taken from 'Lagoon 2' displayed moderate dissimilarity to the 'Septic' and

'Lagoon 1' locations.
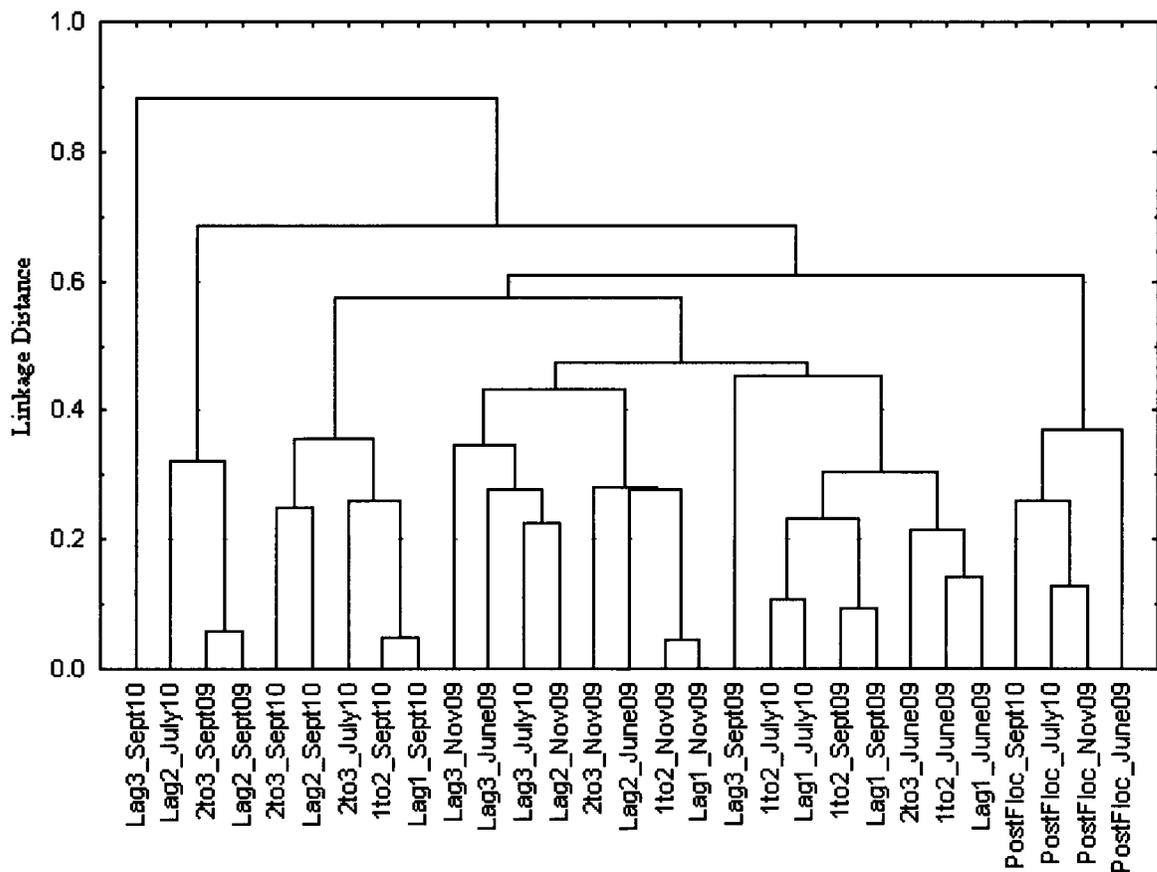
Figure 6.14. DGGE-based tree cluster analysis using the untransformed means from the industrial-scale treatment lagoon Linkage distance was calculated using UPGMA of Euclidian distances.

6.4. Conclusions from Industrial-scale Treatment Lagoon Characterization

Based on the results previously discussed, the following conclusions from the industrial-scale treatment lagoon can be made:

> Pathogen monitoring indicated the persistence of *S. enterica* in the third lagoon following contamination with untreated wastewater, providing evidence to confirm the concerns of Krishnan & Smith, (1987) regarding the use of untreated wastewater in alternative environmental treatment systems.

> Structural fingerprinting provided clear visualization of an acute effect of ferric chloride decontamination, as well as the raw wastewater contamination event in 'Lagoon 3'. This was very clearly visualized through both rank-abundance plots, averaged banding pattern plots and the use of diversity indices. The structural fingerprint was not able to provide clear evidence regarding seasonal changes between the late fall (Nov. 2009) and early summer (July 2010) in the microbial communities, although this may be a result of the stress placed on the community by the addition of ferric chloride and untreated wastewater during the monitoring period.

> Functional fingerprinting was not able to show much evidence of the first contamination event on the microbial community in the second or third lagoon. It was, however, able to produce clear evidence of the second event through multivariate statistical analysis and changes in functional diversity. The functional fingerprint was also able to provide information regarding changes in the capacity and ability for carbon metabolism between fall and summer samples.

➢   This industrial lagoon treatment system displayed very good (sometimes 99.99%)

removal of the *E.coli* and faecal coliforms, as well as consistent and complete

removal of *S. enterica*. However, this was not maintained following two

contamination events. This reiterates the need for understanding the capacity and

community dynamics within environmental or alternative wastewater treatment

systems, as well as proper management and usage of these systems.

7.   General Discussion

7.1. Discussion of Community Characterizations with Respect to System Scale Under Normal Operation

The first overall goal of this thesis was to investigate functional and structural characteristics of microbial communities within bench-, field-, and industrial-scale constructed wetlands (CWs) or alternative wastewater treatment systems under normal, undisturbed condition in the hope of gaining a better understanding of the ability of controlled studies to predict microbial communities found in functioning field-scale environmental sites. The following two subsections will discuss how this goal was accomplished.

7.1.1. Commonalities from the Functional Fingerprints and Other Culture-based Methods

The ability of a CW to produce an environment harsh enough to provide sufficient selection against populations of environmentally unfit or less-fit microorganisms is the major reason why these systems are able to remove unwanted microorganisms. The majority of unwanted microorganisms in wastewater are the result of faecal contamination, either human or animal (Kadlec & Knight, 1996). Most human pathogens of concern in wastewater treatment, therefore, are also important members of human or animal gastrointestinal tracts (i.e., members of the Enterobacteriaceae); as they possess an ability to rapidly break down a diverse number of carbon sources, proteins and lipids which are the usual sources of energy for higher order eukaryotes.

The external environment is, however, a very different place than a gastrointestinal tract to survive in; the temperature can change drastically; chemically-

bound energy and required elements are not supplied at regular intervals; there are no homeostatic mechanisms maintaining pH; among other differences. Although CWs, and wetlands in general, are considered carbon sinks (Lewis, 1995) and there is a considerable amount of usable energy stored within them, the fact that unwanted pathogenic microorganisms need to compete for other required elements is of prime importance. Because of this competition, organisms that have evolved within a human or animal host may not be equipped to sequester the biologically-important elements like nitrogen, phosphorous or magnesium which are often limiting in many environments. Organisms with a supply of free, useable CW carbon, but without the ability to compete for the building blocks of DNA, proteins and membranes will quickly realize the true severity of the famous quote "everything is everywhere... the environment selects" (Baas Becking, 1934, p. 15; translated by De Wit & Bouvier, 2006; as cited by O'Malley, 2008). This fundamental principle of microbial ecology was clearly observed during the normal functioning of the treatment system communities investigated.

Heterotrophic plate counts (HPCs) from field- and industrial-scale systems indicated that these system scales were able to support similarly sized heterotrophic populations (approx. 10^4, see Figure 5.1 and Table 6.1), and that both displayed the ability to decrease the size of these heterotrophic communities from their respective supplied waste effluents by at least two orders of magnitude. Results from the functional fingerprints of the field- and industrial-scale systems indicated that both systems provided competition against carbon metabolism; as both the Nov. 17th (2009) and July 5th (2010) samples from the industrial-scale treatment lagoon and the Feb. 22nd samples from the field-scale CW displayed a pattern of decreasing carbon metabolism following

increased retention time of the 'to-be-treated' effluent. Additionally, the monitoring of

enteric pathogens in the industrial-scale site showed an incredible capacity for removal of

the unwanted contaminants under normal functioning. These findings indicate that, under

normal operation, both the field-scale and industrial-scale treatment systems were able to

provide sufficient selective pressure against the survival of unwanted microorganisms.

This indicates that smaller-scale treatment systems may be able to provide similar

functional environments to those in larger-scale treatment systems.

7.1.2. Commonalities from the Structural Fingerprints

From the results of the three separate structural investigations it appears that the

bench-scale CWs were able to produce a community with a similar number of observed

OTUs and similar genetic diversity as the industrial-scale treatment system. However,

these estimates from the bench-scale CW consistently overestimated what was observed

in the field-scale CW, although the differences in operating temperature may be

responsible for this discrepancy. Genetic divergence showed an opposite trend between

the three scales, as the bench-scale CW underestimated the amount of structural

community change when compared to the field-scale CW; while overestimating the

amount of genetic variation in the industrial-scale system. Rank-abundance plots from

these three varying scales, however, produced similar pictures for all of the locations

under normal functioning. Even more importantly the shift toward a community

dominated by a single OTU was observed in all three scales following a perturbation.

Multivariate statistical analyses performed on the structural fingerprints of the

microbial communities in the field- and industrial-scale treatment system also indicated

that each system was able to maintain spatially distinct communities which maintained a

reasonable degree of similarity despite temporal or seasonal factors. Unfortunately, the

bench-scale system was not monitored following the same time periods as the other two

larger systems. Because of this, factors that may become apparent following prolonged

development of the bench-scale CW may influence the ability of this model system to

approximate larger scale systems operating for long-term periods that are outside the

scope of this study. One important factor is microbial dispersal. Smaller-scale systems

provide less area for dispersal, which may result in altered population dynamics as

populations reach the carrying capacity of the system more rapidly. Because of the

frequency of emptying and refilling the bench-scale CW column, carrying capacity

effects may have been avoided during start-up, but may become prevalent following

extended operation.

However, based on the observations here-in, it seems that the bench-scale CW is a

viable indicator for the genetic or structural characteristics of larger systems. It may be

appropriate following the trends in the chosen metrics, that bench-scale CWs be

subjected to similar temperature shifts observed in the systems they are modeled after.

**7.2. Constructed Wetland Community Dynamics in Response to an Antibiotic
Stressor or Deleterious Perturbation**

The characteristics of CW communities in response to deleterious contaminants appear

complex, and dependent on the nature of the contaminant. This was illustrated by

increases in community function after acute exposure of an antibiotic, followed by

recovery. During recovery, communities displayed a clear structural response, shown

through both changes in the patterns in relative abundance of contributing populations

and divergence from the rate of this structural change observed under normal operating conditions.

The acute change in community function may be due to an increase in physiological stress-response mechanisms. Stress-response mechanisms are known to respond to various types of stressors through quorum-secretion, modification in cellular charge, increased enzyme production and general metabolism, sporulation and activation of a number of reparative or defensive pathways, among others (White, 2003). Some of these responses may serve to increase the capacity of carbon metabolism. In the same instance, increased stress-response may produce altered enzyme kinetics or cellular function; increasing the presence of chemically reduced molecules (i.e., metabolized substrates or waste products); subsequently lowering pH and increasing the amount of absorbance by the tetrazolium dye pH indicator; whether or not the reduction observed is from the supplied carbon sources. Of note, the functional fingerprinting performed on the bench-scale system produced the same acute response and subsequent recovery as observed in the field-scale system (Dr. Kela Weber, personal communication, Sept. 27th, 2010).

The delayed change in structural community fingerprint may be a result stemming from the modification in functional behaviour. Microbial populations within the CWs could be removed immediately or, if able to survive, have reproductive growth compromised by increased metabolic devotion to stress-response; while resistant organisms that may be normally poorer competitors would be allowed to flourish over the exposure period; filling in newly available niches left by susceptible organisms that may

have otherwise outcompeted the resistant organisms and consequently dominating the community.

These results indicate that the use of multiple metrics for community analysis is able to provide a comprehensive picture of the structural changes that occur within a developing CW community.

7.3. Integrative Nature of this Study

A mentioned in Section 2.4, the integrative biologist will have an incredibly important role in building an understanding of CWs. Integration should be evident throughout this document, visible first and foremost in the use of multiple techniques that measure different hierarchies of biological organization, through both evolutionary (i.e., genetic or structural) and physiological (i.e., metabolic or functional) changes. Additionally, not only were CWs monitored in their natural states to add to basic knowledge of these systems, but also in response to a deleterious stressor; which may help build a body of applied knowledge of CWs. Furthermore, the conclusions from a comparison of lab-based research mesocosms to currently applied industrial systems have supplied novel, integrative information regarding varying scales of environmental systems not previously investigated.

In addition, this work was done as part of a number of different collaborations, specifically mentioned in the bench- and field-scale CW systems. This novel information may ultimately assist with the future detection of weakened communities within environmental treatment systems and allow preventative behaviour to be taken prior to contamination events like those discussed in this thesis. To be truly and thoroughly integrative in the short period of time provided to complete a masters program is not

possible. However, building the skills, relationships and thought-processes required to create and accomplish an integrative research project is and was achieved over the course of this graduate research. As a result, although there is much more to learn regarding the integrative nature of CWs especially with regard to the sustainability and capacity of these systems, the steps taken in this study to emphasize the importance of integrative research in the field of alternative wastewater treatment are likely more valuable than the research conducted over the program duration.

7.4. Overall Conclusions and Recommendations

Based on the results from all three community characterizations the following overall conclusions and recommendations are summarized:

> ➤ Of primary importance from this study is the requirement of using these alternative treatment systems to remediate pre-treated wastewater.

> ➤ Under normal operation, bench-, field- and industrial-scale treatment systems were able to produce structural profiles that were comparable over a number of metrics. Functional profiles from field- and industrial-scale systems displayed relatively similar trends across the chosen metrics. This indicates that smaller-scale treatment systems may be able to provide similar environments to those in larger-scale treatment systems.

> ➤ Continued monitoring of all system scales is required to determine if the trends or similarities observed herein are able to be reproduced in systems with differing properties or treating different waste. It is also important to understand the seasonal dynamics of the community, as communities may be more susceptible to perturbation during times of increased natural variation or biological succession.

➤ Further research is required into the applied functionality of bench-scale systems with regard to their capacity for pathogen removal; as the systems are easier to overload and manipulate to get an idea of capacity per unit size.

➤ Increased retention time resulted in communities with consistently low performance in terms of functional capacity and consistently high performance on structural metrics and observed variability compared to areas initially receiving the wastewater for treatment. This indirect relationship was not maintained during antibiotic treatment and was reversed following exposure to untreated wastewater.

➤ These findings indicate the importance of continued application of both functional and structural profiling when monitoring microbial community characteristics within alternative treatment systems.

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Appendix A - Supplementary Figures
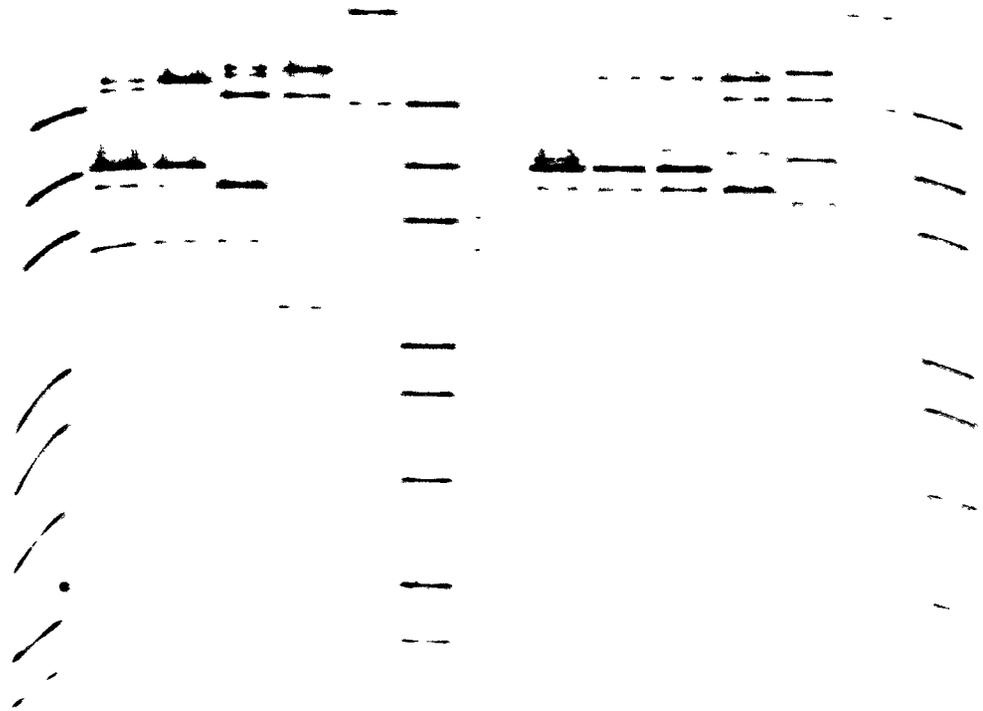
Figure A.1. Example DGGE from the bench-scale CW. Ladders are in lanes 1, 7 and 15. All remaining lanes are loaded with sample.
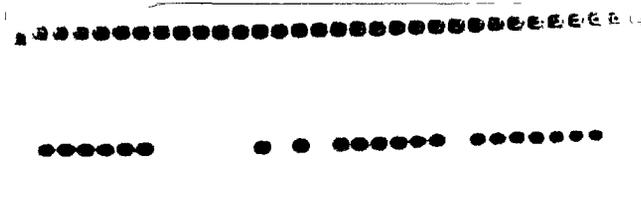
Figure A.2. Example of an agarose gel containing *S. enterica* colony PCR products. Lanes (from left to right) contain: 100 bp ladder, 6 'PostFloc' isolates, 3 'Lagoon 3' isolates, *E. coli* (negative control), empty lane, *S. enterica* (positive control), PCR blank, *S. enterica* (positive control from extraction protocol), empty lane, 6 '1 to 2' isolates, and 6 '2 to 3' isolates.
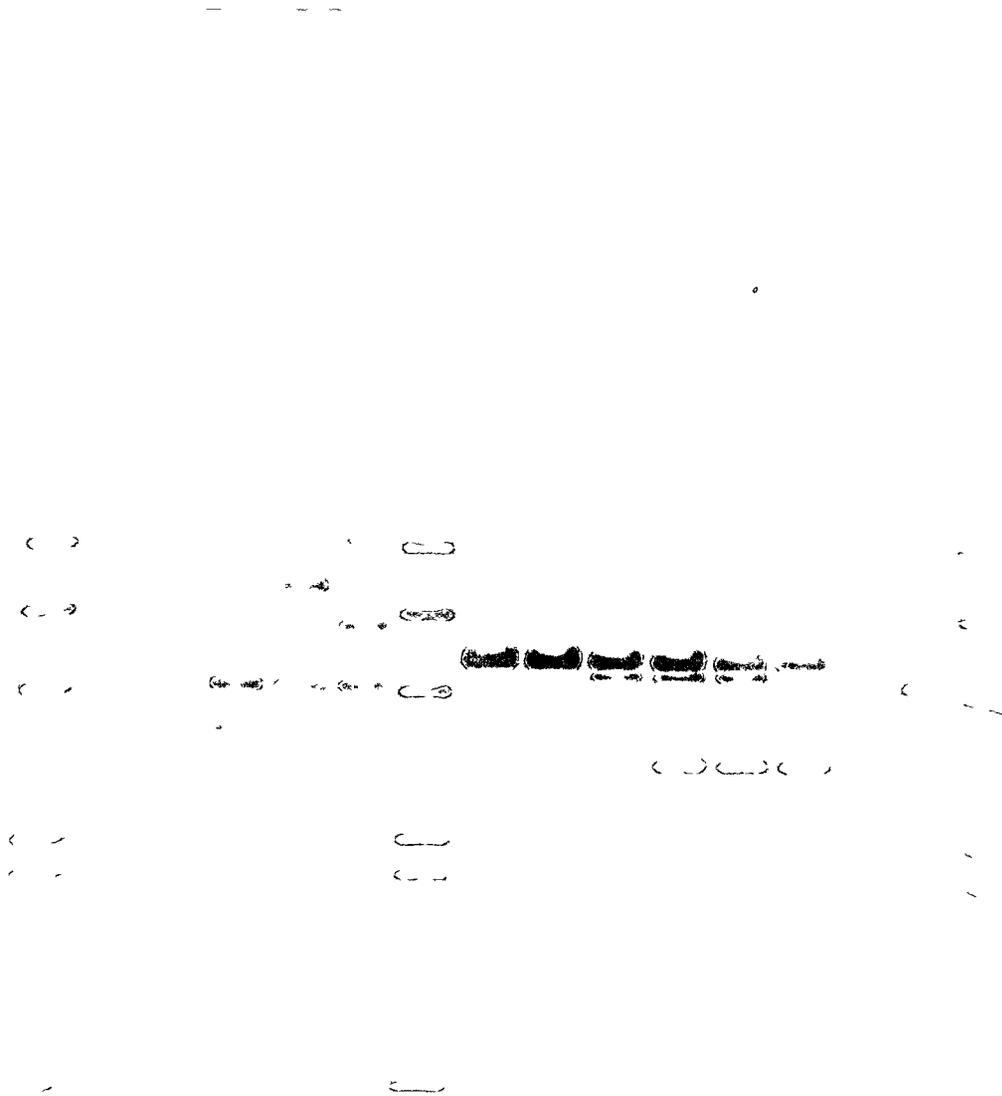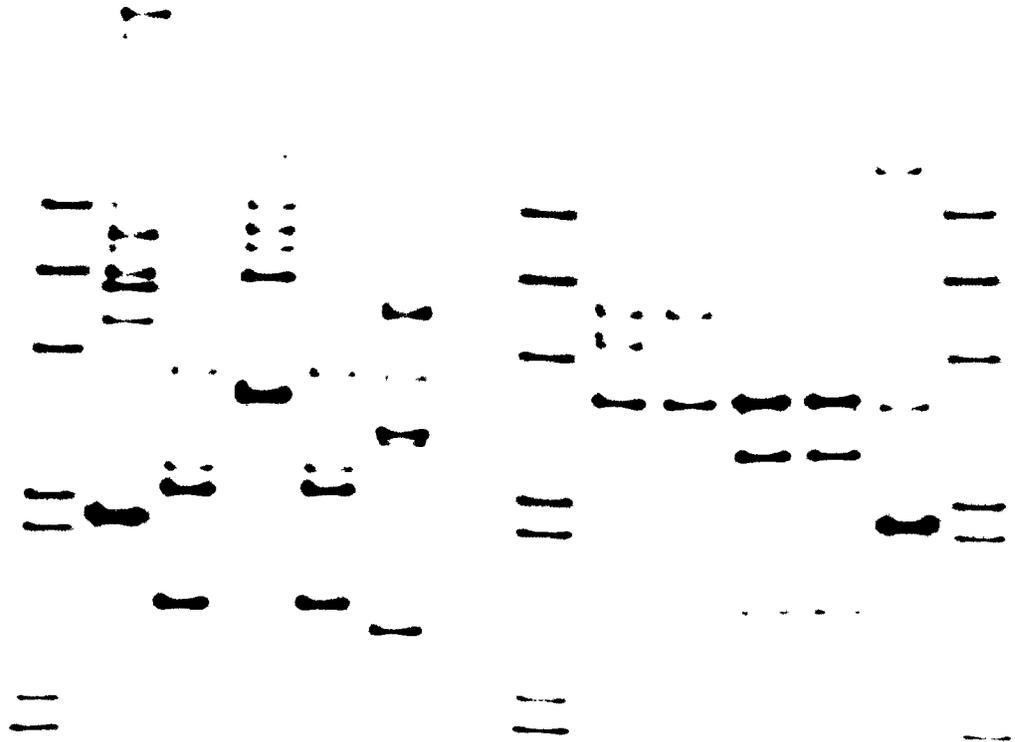
Figure A.3. Example DGGE from the field-scale CW Ladders are in lanes 1, 7 and 16, and Lane 14 contains the blank DNA extraction None of the bands visualized in this lane were above background All remaining lanes are loaded with sample

Figure A.4. Example DGGE image from the industrial-scale treatment lagoons. Ladders are in lanes 1, 8 and 14. Lane 7 contains the PCR-blank, of which only one of the bands visible in that lane was found to be above background, even though PCR-product check was negative. All remaining lanes are loaded with sample.
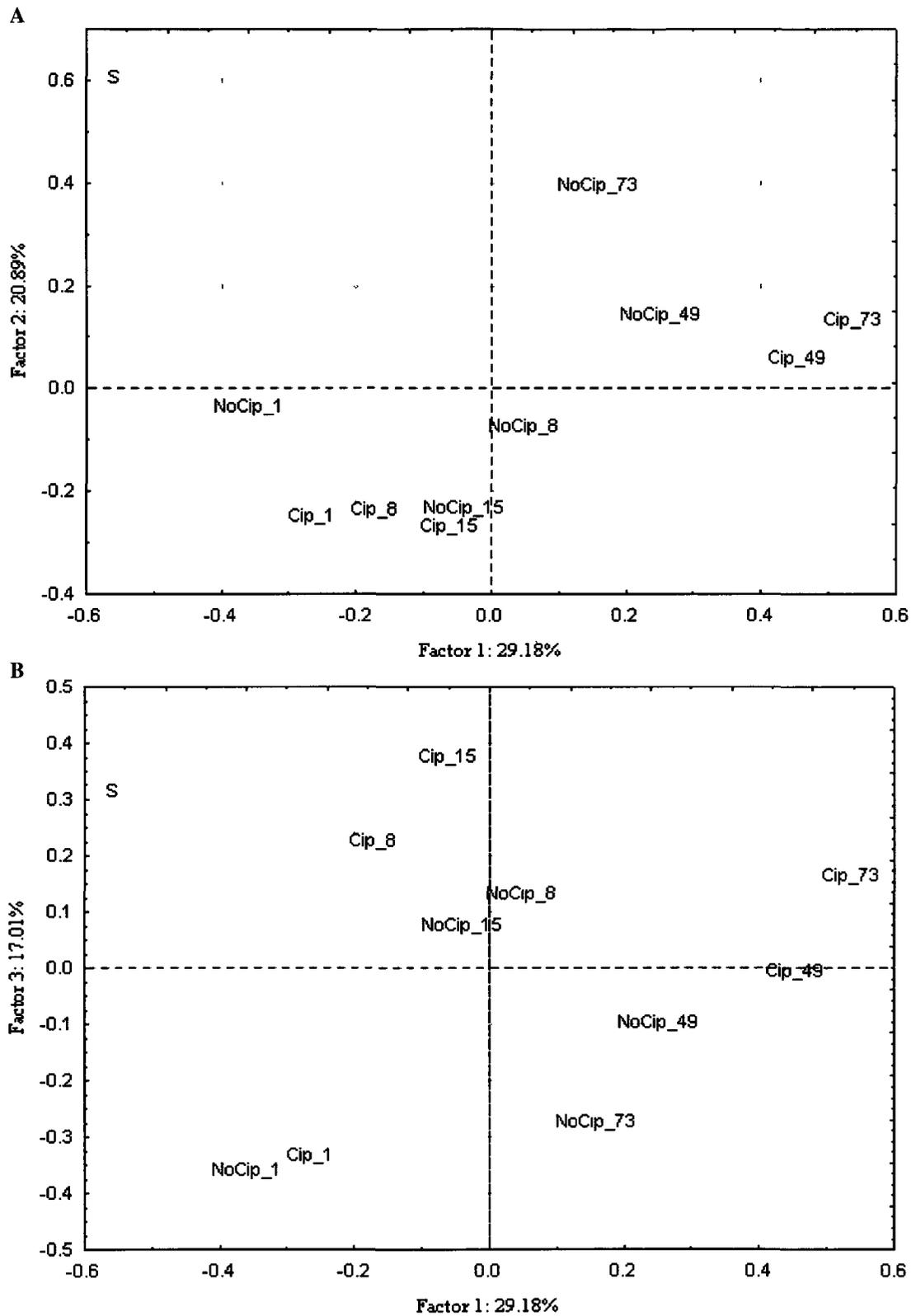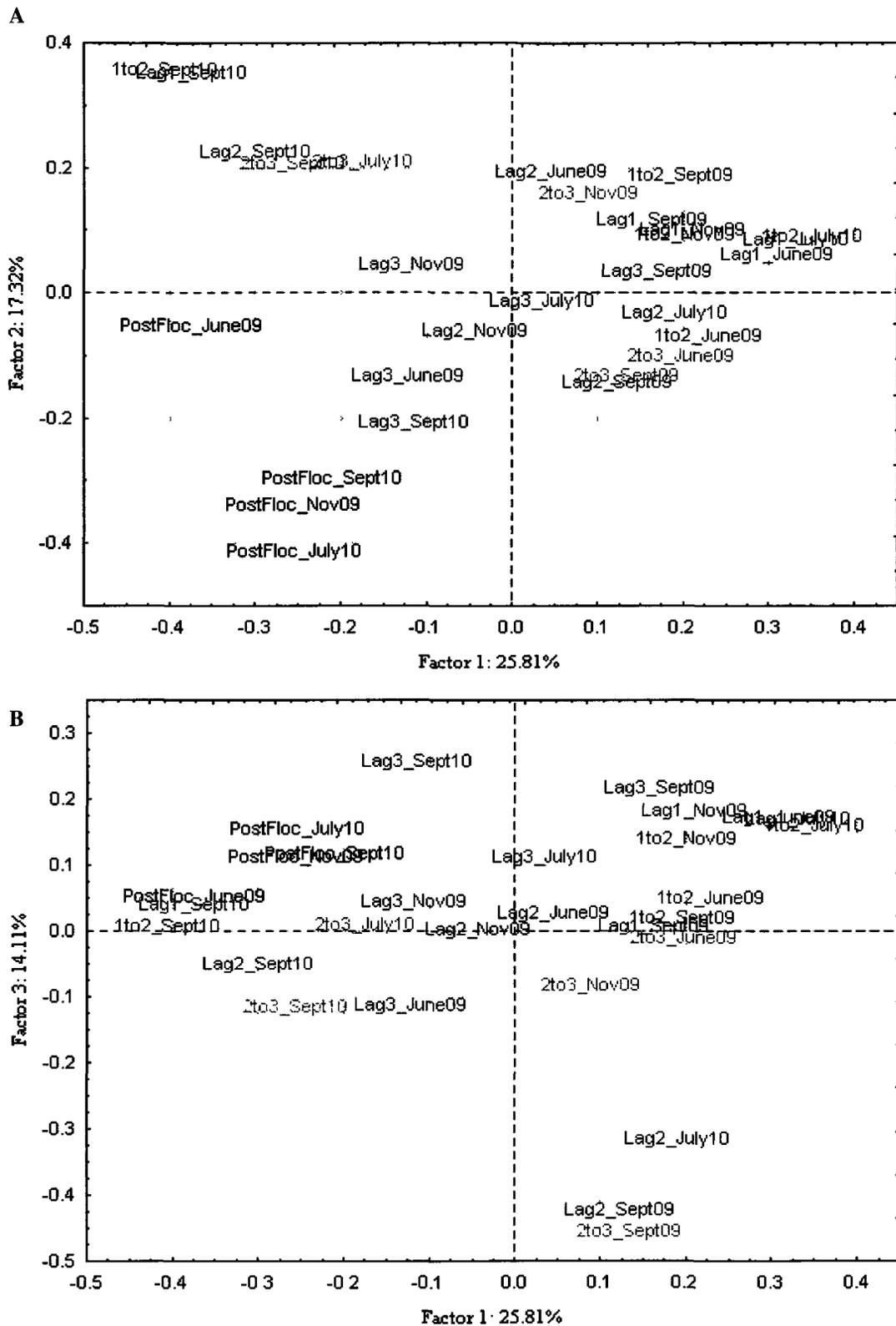
Figure A.5. PCA using the covariance matrix from Taylor-transformed means from bench-scale CW DGGE. A) Plot of cases on the 1 x 2 factor plane and B) Plot of cases on the 1 x 3 factor plane. Ciprofloxacin was administered between day 8 and 15 from start-up.
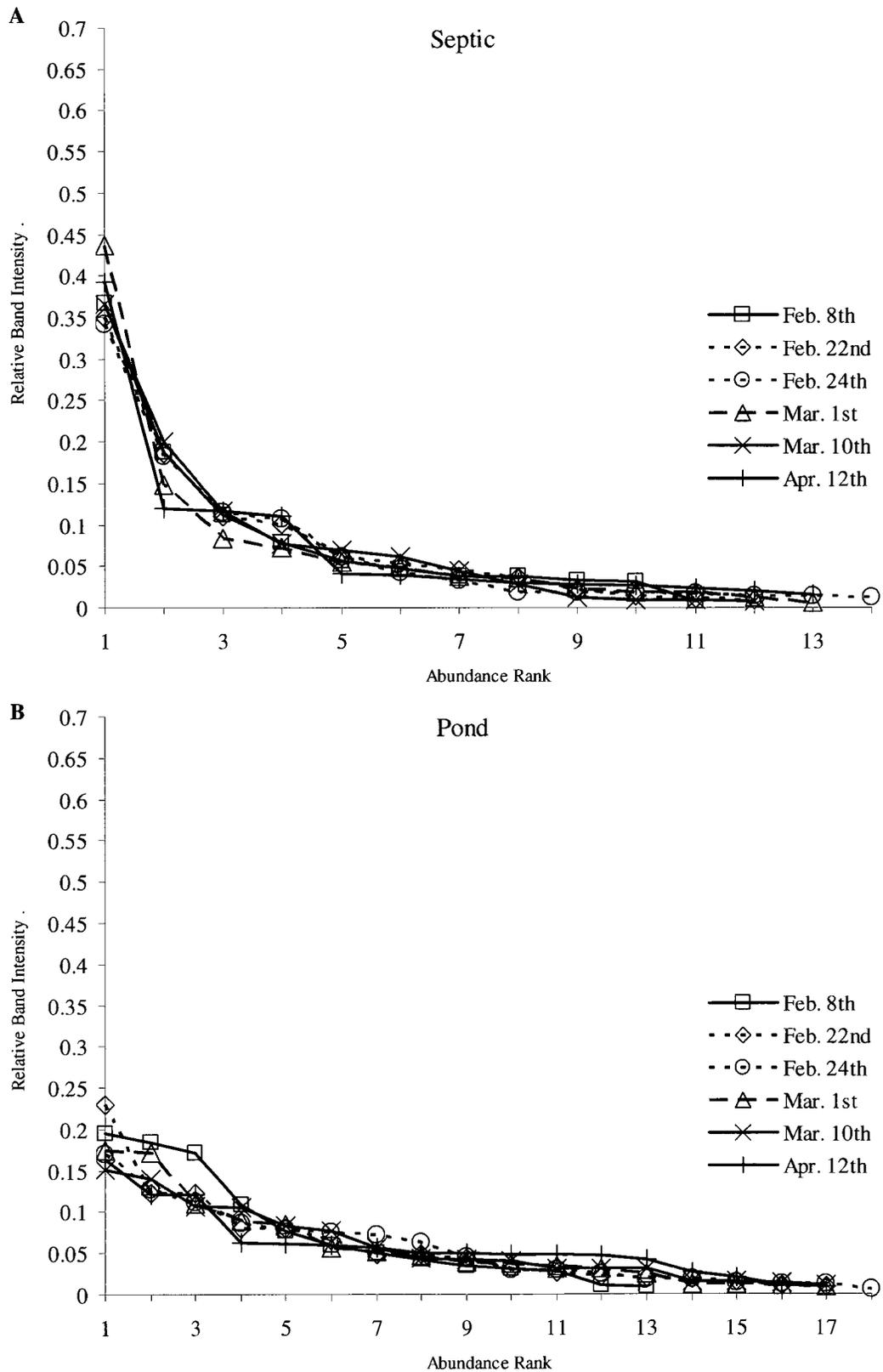
Figure A.6. DGGE-based rank-abundance curves for selected locations in the field-scale CW. Addition of the oxytetracycline spike immediately following the collection of sample on Feb. 22nd, lasting until Feb. 26th. A) The 'Septic' location, B) The 'Pond' location.

A

B
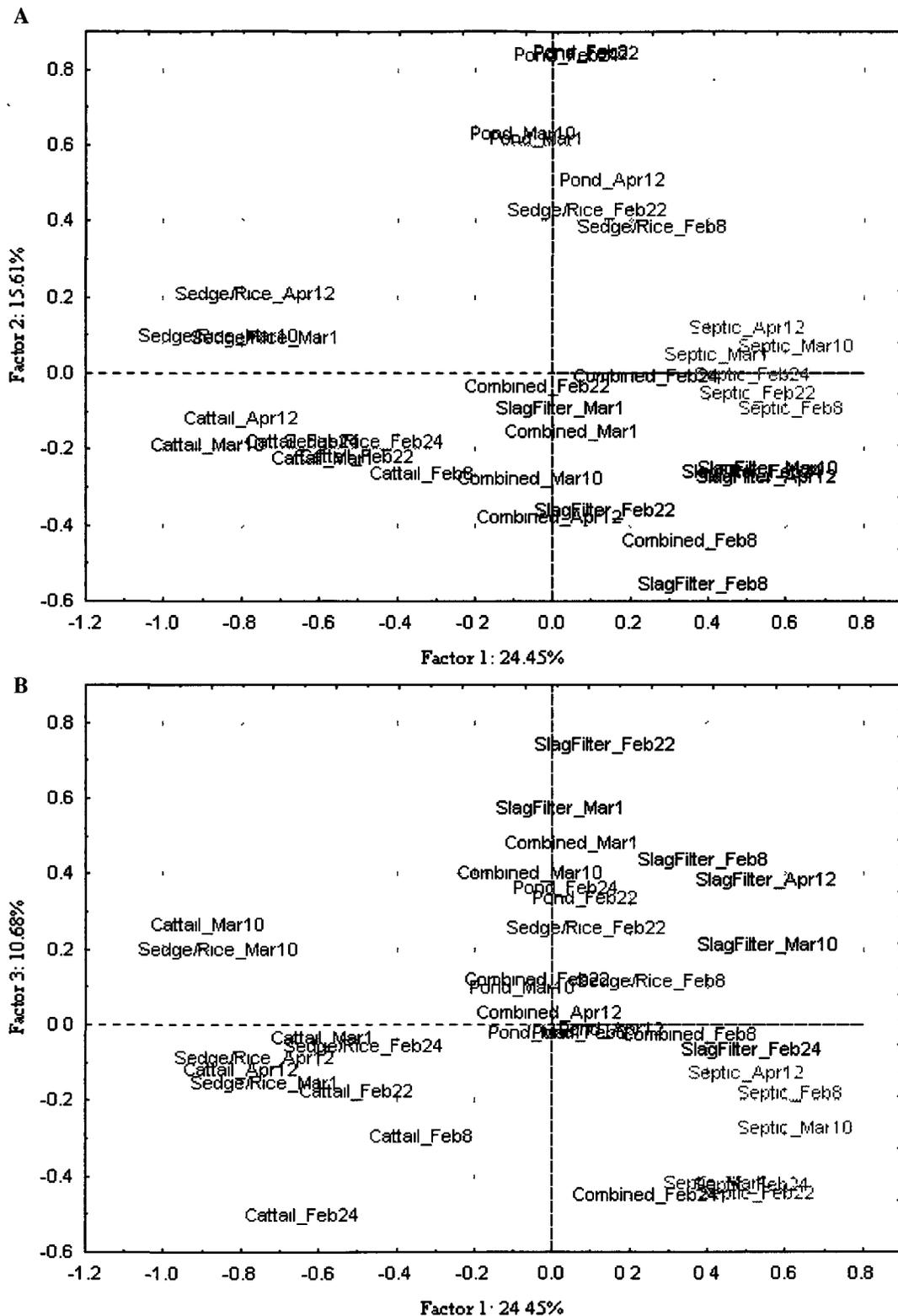
Figure A.7. PCA using the covariance matrix from Taylor-transformed means from the field-scale CW DGGE. A) Plot of cases on the 1 x 2 factor plane and B) Plot of cases on the 1 x 3 factor plane. Oxytetracycline was added daily immediately following the collection of sample on Feb. 22nd, lasting until Feb. 26th.

A

B

Figure A.8. PCA using the covariance matrix from Ln+1-transformed means from industrial-scale treatment lagoon DGGE. A) plot of cases on the 1 x 2 factor plane and B) plot of cases on the 1 x 3 factor plane.

Appendix B –Supplementary Tables

Table B.1. Characteristic *S. enterica* presentation on media used for biochemical confirmation.

		Media Type		
		Triple Sugar Iron (TSI)	Lysine Iron Agar	Urea Broth
Expected Visualized Result*; Biochemical Interpretation	Slant	Red; negative for lactose utilization	Purple; positive for lysine decarboxylase	
	Butt	Yellow & Black; positive for dextrose utilization, H₂S production visible	Purple & Black; negative for lysine deaminase, H₂S production visible	Yellow; negative for urease
	Gas	Produced	Absent	

* Not all results may be visible or displayed by a positive isolate (APHA, 1998). Gas production may be very weak, and acid produced by dextrose fermentation may hide H_2S production. Insufficient inoculating load in LIA may result in very weak or absent H_2S production.

Table B.2. pH, temperature and dissolved oxygen from the industrial-scale treatment lagoons.

Location	Date	pH	Ambient Temp. (Max. - Min., °C)	Water Temp. (°C)	Dissolved Oxygen (mg/L)
Lagoon 1	June 24th, '09	7.57	30.7 - 18.1	27°C	5.86
	Sept. 21st, '09	7.18	24 - 13	21°C	3.24
	Nov 17th, '09	7.61	7.9 - 2.7	11°C	9.16
	July 5th, '10	N/A	33.9 - 23	N/A	N/A
	Sept. 28th, '10	N/A	18.4 - 13.8	N/A	N/A
Lagoon 2	June 24th, '09	6.65	30.7 - 18.1	25°C	7.14
	Sept. 21st, '09	7.15	24 - 13	20°C	7.8
	Nov 17th, '09	7.05	7.9 - 2.7	9°C	10.69
	July 5th, '10	N/A	33.9 - 23	N/A	N/A
	Sept. 28th, '10	N/A	18.4 - 13.8	N/A	N/A
Lagoon 3	June 24th, '09	9.23	30.7 - 18.1	27°C	6.44
	Sept. 21st, '09	8.46	24 - 13	20°C	7.62
	Nov 17th, '09	9.50	7.9 - 2.7	8°C	11.56
	July 5th, '10	N/A	33.9 - 23	N/A	N/A
	Sept. 28th, '10	N/A	18.4 - 13.8	N/A	N/A

* Areas marked as unavailable have not been yet provided or released by site administrators. The request for this information is pending.