

**Spatial and Temporal Biogeochemical Changes of  
Groundwater Associated with Managed Aquifer  
Recharge in Two Different Geographical Areas.**

This thesis is presented for the degree of Doctor of Philosophy at  
Microbiology and Immunology, School of Biomedical, Biomolecular  
and Chemical Sciences.

By

Deborah A. Reed B.Sc. (Hons)

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## Declaration

The work presented in this thesis is my own account of my own work. All contributions made by others to this work are explained in the Acknowledgements. The thesis contains no material which has been accepted for a degree at this or any other University. To the best of my knowledge, this thesis does not contain any material previously published or written by another person, except where due acknowledgement is made in the text.

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## ***ABSTRACT***

Managed Aquifer Recharge (MAR) is a technique that can be used to capture and store water in aquifers for later reuse. This method recycles water that would normally be lost or discarded to the environment. MAR has been observed to have the potential for improving the quality of recharged water through a combination of physical, chemical and biological processes.

The aim of this study was to investigate the changes in groundwater microbial population structure during MAR and the major influences that drive these population changes. Biogeochemical MAR studies have the potential to assist in the improved prediction of the removal of contaminants such as nutrients, pathogens and trace organics from the recharged water. Biological clogging during recharge also has the potential to overwhelm an aquifers ability to process wastewater thus reducing the hydraulic conductivity of the aquifer. Therefore further research into the spatial and temporal biogeochemical processes that occur during MAR is required.

The geochemical and microbial population dynamics of two contrasting MAR techniques were investigated at two different geographical locations (Perth, Western Australia and Adelaide, South Australia). These MAR sites contained aquifers of dissimilar properties that were recharged with wastewater that contrasted in water quality. The Perth MAR site received secondary treated effluent which continuously infiltrated the unsaturated zone into an unconfined aquifer aided by infiltration galleries. Reclaimed water was extracted from a well at distance from the infiltration gallery. In contrast the Adelaide, Aquifer Storage and Recovery (ASR) site involved the injection of tertiary treated wastewater into a confined aquifer for storage followed by recovery of that water from the same well.

Denaturing Gradient Gel Electrophoresis (DGGE) using DNA (from non-cultured and cultured bacteria) was extracted from groundwater. Changes in DNA banding patterns (e.g. genetic fingerprinting) were used to detect changes in microbial population dynamics. Multivariate statistics were used to evaluate geochemical and microbial changes in groundwater during MAR. Principal component analysis (PCA) was used to investigate spatial and temporal changes in the overall 'chemical signature' of the aquifer using an array of chemical analytes. Multivariate statistics such as

PERMANOVA, multi-dimensional scaling (MDS) and principal coordinate analysis (PCO) were used to evaluate changes in microbial population dynamics in response to the migrating nutrient plume.

Principal component analysis demonstrated a migrating chemical plume for both MAR sites. Microbial and chemical evidence suggested that groundwater from observation wells at greatest distance from the infiltration gallery were least affected by treated effluent recharge. The results suggested that groundwater microbial populations responded to the migrating chemical gradient and to the changes in aquifer geochemistry. Distinct microbial populations developed in a distance dependent successional manner concomitant with the geochemical plume migration. Evaluation of microbial and chemical interactions indicated that although the biogeochemistry of the aquifer was complex, discrete microbial populations could be explained by specific environmental parameters.

Notably the background and recovered water was most dissimilar in microbial and chemical population structure to that described for the infiltration gallery and injection well. Microbial and chemical evidence suggested that the background and extraction well groundwater were unaffected by plume migration. These results suggested that extraction well groundwater was similar in quality to that of ambient groundwater. Significant geochemical and microbial changes of secondary treated effluent during infiltration and lateral movement through aquifer were implicated in addition to the forced hydraulic gradient created from extracting five times the volume of infiltrating wastewater.

This study demonstrated that microbial populations and the geochemical processes associated with MAR can be studied and compared. Multivariate statistical methodology greatly simplified a vast array of dynamic biogeochemical information that could be dissected for meaningful interpretation over distance and time. The study evaluated the major biogeochemical influences which resulted in microbial and geochemical changes where it was noted that microbial populations were more dynamic than geochemical variation over time. Additionally biogeochemical comparative analysis indicated that microbial populations could change in population structure before a shift in aquifer geochemistry was detected. It is anticipated that the results from this study will benefit further research into the biogeochemical processes

involved in water quality changes (e.g. nutrient removal, pathogen decay and biodegradation of trace organics) as well as controlling biological clogging of MAR schemes.

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## GLOSSARY/ABBREVIATIONS

Allocthonous	Derived from outside a system: OM produced on land and then moved to a river or stream
AOC	Assimilable organic carbon
APHA	American public health association
ANOSIM	Analysis of similarity
ASR	Aquifer Storage and Recovery
Autochthonous	Derived from within a system: OM produced within a river or stream
BDOC	Biodegradable dissolved organic carbon
CCWA	Chemistry Centre, Western Australia
Centroid	The centroid of a triangle is the point of intersection of its median
DIC	Dissolved inorganic carbon
DGGE	Denaturing gradient gel electrophoresis
DOM	dissolved organic matter
Karstic	An area of irregular limestone in which erosion has produced fissures, sinkholes, underground streams and caverns.
MAR	Managed Aquifer Recharge
MDS	Multidimensional scaling
Oligotrophic	Refers to a body of water with very low nutrient levels that would offer little to sustain life
OM	Organic matter
PERMANOVA	Permutational multivariate analysis of variance
PERMDISP	Permutational analysis of multivariate dispersion
PCA	Principal component analysis
PIC	Particulate inorganic carbon
PCO	Principal coordinate analysis
PLFA	Phospholipid fatty acid analysis
POM	particulate organic matter
Pristine	A completely natural state—a water body remaining in a pure state
QAQC	Quality assurance and quality control
TEAPS	Terminal electron accepting processes
vMAX	Binding affinity at saturating substrate levels

## SECTION 1: INTRODUCTION

Managed Aquifer Recharge (MAR) is being extensively investigated in Perth for its potential to supplement freshwater supplies. Perth is heavily reliant on groundwater supplies for potable use (Davidson, 1995) and during the past ten years groundwater levels have substantially decreased within this region. MAR is being investigated as part of a portfolio of water management strategies to secure future demand for Perth's freshwater supplies. The MAR scheme being studied here is for water reuse to non-potable standards for irrigation use.

The Perth MAR scheme was commissioned in July 2005 for a three year trial to examine water quality improvements of treated effluent (Bekele *et al.*, 2006). The wastewater is pumped into subsurface infiltration galleries. The wastewater then undergoes treatment processes via filtration through the unsaturated zone to a shallow unconfined aquifer. It is expected that water quality improvements will continue to occur during lateral movement through the aquifer to an extraction well. The MAR site was designed so that infiltrating treated effluent had sufficient residence time within the aquifer for water quality improvements to occur via chemical, physical and biological processes. It is hoped that extracted water will meet irrigation guidelines thus demonstrating significant improvements in water quality. The trial site at CSIRO represents a worse case scenario for improvements in water quality as secondary treated effluent is used; no additional water treatment methods are implemented other than natural aquifer treatment processes.

In Adelaide, an Aquifer Storage and Recovery (ASR) scheme (1999-2004) injected water of higher quality (tertiary treated wastewater) into a confined, deep, brackish aquifer (Martin and Dillon, 2005). The scheme examined the feasibility of injecting the 'winter' excess of reclaimed water into the aquifers beneath the Northern Adelaide Plains for extraction in summer months. The ASR scheme resulted in significant geochemical changes and the quality of recovered water substantially improved (Vanderzalm *et al.*, 2006). The recovered water is diverted for irrigation of horticultural land in the North Adelaide Plains. Prior to the ASR scheme, irrigation of this area used approximately  $1.6 \times 10^{10}$ L per year of groundwater which resulted in aquifer depletion and increased groundwater salinity. Additionally the wastewater used for ASR was

previously discharged to the sea. Significant long-term environmental impacts along the South Australian coastline have been observed as a consequence of this discharge. The recycling project was thus developed to reduce the adverse environmental impacts on the groundwater and marine environments while sustaining expansion of the horticultural industry (Kracman *et al.*, 2001).

The subsurface is a unique environment where most of the energy is transferred solely through the microbial food web. Geochemical characterisation of the ASR site has been fully evaluated (Vanderzalm *et al.*, 2006) but no microbial population studies have been undertaken to date. In addition, the Perth infiltration site has neither been geochemically or microbiologically characterised in detail. The work reported here investigates the microbial population dynamics and aquifer geochemistry of groundwater infiltrated with treated effluent in Perth and injected tertiary effluent in Adelaide. Thus wastewater which is treated to different standards is introduced into two different aquifers at two different geographical areas using two different MAR strategies.

The overall aim of this study was to investigate aquifer geochemistry in conjunction with microbial population dynamics. It was a priority of this study to develop techniques to analyse genetic fingerprinting data obtained from both culture and non-culture samples in association with environmental parameters. Only two groundwater studies have been published on combined genetic population studies and environmental parameter analyses in conjunction with multivariate statistics. These studies were for groundwater from non-MAR contaminated aquifers (Fahy *et al.*, 2005; Haack *et al.*, 2004). Currently there are no biogeochemical characterisation studies of MAR sites using treated wastewater which extensively investigates their microbial populations.

The research strategy for the project was as follows:

Firstly DNA was extracted directly from groundwater samples to obtain DNA from as many microbes as possible within the groundwater environment. Additionally DNA was obtained from sulphate-reducing, iron-reducing, nitrate-reducing and fermentative cultures grown in specific media inoculated from groundwater samples to evaluate microbes associated with groundwater biogeochemical cycles. The DNA was amplified using bacterial universal primers and the subsequent fragments were separated by



electrophoresis on a denaturing gradient gel (DGGE). The resulting DNA banding pattern for each sample, which represented the bacterial genetic diversity for a given sample based on the specificity of the primers, was then used to compare spatial and temporal microbial population profiles. Variations in microbial populations in response to the nutrient gradient were evaluated by analysing samples at different distances and depths from the origin of wastewater penetration to groundwater over time. Therefore changes in microbial populations at each specified sampling station could be tracked. Chemical analyses were also undertaken at identical sampling stations for identical sampling periods for a vast array of environmental parameters. The latest ecological multivariate statistical techniques (Anderson, 2001) were then used to individually evaluate microbial and chemical changes over time. Lastly microbial and chemical changes were analysed in unison to fully evaluate aquifer biogeochemical changes in response to MAR. The Adelaide study also additionally evaluated the spatial and temporal differences between free-living and attached microbial community structures.

## SECTION 2: LITERATURE REVIEW

### 2.1 Aquifers and Groundwater

Aquifers are water-bearing geological formations below ground which are able to receive, store or transmit water. Aquifers are typically saturated regions of the subsurface which may be confined or semi-confined in nature. Confined aquifers exist between an upper and lower layer of relatively impermeable material and are deeper than the unconfined aquifer. Unconfined aquifers have their upper boundary at the level known as the water table and are often referred to as superficial aquifers (Back, 1986). Numerous aquifers can occur at different depths separated by impervious rock (Back, 1986). These sandy areas or porous rocks contain significant amounts of underground water. The water contained in these aquifers is known as groundwater.

The hydrological cycle is a continuous and complex process driven by solar energy. Water from the land surface flows through the soil of the vadose (unsaturated) zone through the subsoil and rock via infiltration and percolation processes. The process of water soaking into the ground to become groundwater is known as groundwater recharge. The water within the aquifer tends to move slowly along a flow path the rate of which is determined by the permeability of the rock (Freeze and Witherspoon, 1967). Subsurface constituents are predominantly heterogeneous thus affecting aquifer transmissivity within the aquifer. For example the particle sizes of: sand (2.0-0.06mm), silt (0.06-0.02mm) and clay (<0.002mm) vary. Therefore such parameters need to be spatially characterised within the aquifer for an estimation of aquifer transmissivity. The hydraulic gradient of the aquifer influences the direction and rate of groundwater flow due to changes in the depth of the water table (Freeze and Cherry, 1979). Approximately 4% of the water contained in the hydrologic cycle is groundwater. Although groundwater is only a small fraction of total water resources it can be of significant importance in some destinations. It has been estimated that over 95% of the freshwater available in the US is groundwater (Asano 1985) and 60% of the drinking water supply in Perth, Western Australia comes from groundwater (Davidson, 1995).

## 2.2 Groundwater Geochemistry

The chemistry of groundwater is affected by the subsoils and rocks through which the water infiltrates and percolates during its passage from the surface. Groundwater has higher dissolved mineral concentrations than surface waters. This is because groundwater has a greater contact time with soils and rocks for dissolution to occur. Consequently there is a generally a relationship between the composition of groundwater and the soil and rocks through which the water has migrated. Therefore the geology of subsurface environments is of great importance as geological factors can affect inert changes in groundwater chemistry (Freeze and Cheery, 1979). The primary goal of groundwater geochemistry is to understand the processes governing the distribution of chemical species in groundwater. Geochemical changes can be measured from the fundamental principals of mineral equilibria, chemical kinetics and mass transport (Garrels and MacKenzie, 1967).

### 2.2.1 Groundwater Characteristics

Groundwater is a mix of waters from many sources of different ages. The general age of groundwater increases with depth. The age of a particular sample is the average age of all the constituent components. Groundwater dating can be determined by Carbon-14 analysis (Pearson and White, 1967). The main constituents of groundwater include: calcium, magnesium, sodium, bicarbonate, sulphate and chloride. They occur in the form of electrically charged ions (Matthess, 1982). The concentration of dissolved constituents tends to increase with the length of the flow path.

Many different subsurface environments enclose groundwater with a broad range of salinities. Thus groundwater can vary in quality from fresh, brackish to saline. The rate of groundwater flow can be extremely slow in deep aquifers which can be saline, with concentrations ranging up to ten times the salinity of seawater. Groundwater temperature can also vary with depth where an increase in temperature is associated with greater depths. Groundwater temperature commonly varies between 20 – 30°C. In contrast groundwater generally maintains a relatively constant pH. This is due to the contact time with the rock formations and subsequent buffering from the carbonate or silicate minerals that make-up the aquifers (Matthess, 1982).

Infiltrating surface waters contain varying amounts of organic matter but like many aquatic environments that are unpolluted, groundwater is typically oligotrophic in nature. In contrast with surface waters, pristine groundwater typically does not contain suspended solids known as particulate organic matter (POM). Suspended solids are removed due to the infiltration and percolation processes which act as a natural filter (Freeze and Cherry, 1979). Additionally biotic transformation of POM may occur within the highly productive aerobic unsaturated zone during its migration to the saturated zone (Konopka, and Turco, 1991; Fuller *et al.*, 1995). Thus the quality of infiltrating surface water is significantly improved as the water penetrates to the subsurface aquifer and characteristically contains considerably lower amounts of organic matter than surface waters (Asano and Levine 1996). Although groundwater is characteristically oligotrophic, dissolved organic matter (DOM) is ubiquitous in all water environments (Wotton, 1994).

### 2.3 Superkingdom Classification of Life

The Three Domain System, proposed by Woese *et al.*, 1990 is an evolutionary model of classification based on differences in the sequences of nucleotides in the cell's ribosomal RNAs (rRNA), as well as the cell's membrane lipid structure and its sensitivity to antibiotics. The evolutionary conserved nature of rRNA nucleotide sequences are a good indication of how related or unrelated different cells and organisms are.

This system proposes that a common ancestor cell gave rise to three different cell types, each representing a domain. The three domains are the *Archaea* (archaeobacteria), the *Bacteria* (eubacteria), and the *Eukarya* (eukaryotes). The *Eukarya* are then divided into 4 kingdoms: Protists, Fungi, Animalia, and Plantae. A description of the three domains follows:

#### **The *Archaea* (archaeobacteria)**

The *Archaea* possess the following characteristics: *Archaea* are prokaryotic cells however unlike the *Bacteria* and the *Eukarya*, the *Archaea* have membranes composed of branched hydrocarbon chains attached to glycerol by ether linkages. The cell walls of *Archaea* contain no peptidoglycan. *Archaea* are not sensitive to some antibiotics that

affect the *Bacteria*, but are sensitive to some antibiotics that affect the *Eukarya*. *Archaea* contain rRNA that is unique to the *Archaea* as indicated by the presence molecular regions distinctly different from the rRNA of *Bacteria* and *Eukarya*. *Archaea* often live in extreme environments and include methanogens, extreme halophiles, thermophiles and hyperthermophiles. A recent study (Konneke *et al.*, 2006) has shown that one group of marine thermophiles are capable of nitrification, a trait previously unknown among the archae. Evolutionary-wise Archae are believed to have evolved very early in earth history when the atmosphere did not contain oxygen (Woese, 1981).

### **The *Bacteria* (eubacteria)**

The *Bacteria* possess the following characteristics: *Bacteria* are prokaryotic cells. Like the *Eukarya*, they have membranes composed of unbranched fatty acid chains attached to glycerol by ester linkages. The cell walls of *Bacteria*, unlike the *Archaea* and the *Eukarya*, contain peptidoglycan. *Bacteria* are sensitive to traditional antibacterial antibiotics but are resistant to most antibiotics that affect *Eukarya*. *Bacteria* contain rRNA that is unique to the *Bacteria* as indicated by the presence molecular regions distinctly different from the rRNA of *Archaea* and *Eukarya*. *Bacteria* include mycoplasmas, cyanobacteria, Gram-positive bacteria, and Gram-negative bacteria. *Bacteria* display a wide range of metabolisms and extensively colonise the subsurface (Balkwill *et al.*, 1989).

### **The *Eukarya* (eukaryotes)**

The *Eukarya* (also spelled *Eucarya*) possess the following characteristics: *Eukarya* have eukaryotic cells. Like the *Bacteria*, they have membranes composed of unbranched fatty acid chains attached to glycerol by ester linkages. Not all *Eukarya* possess cells with a cell wall, but for those *Eukarya* having a cell wall, that wall contains no peptidoglycan. *Eukarya* are resistant to traditional antibacterial antibiotics but are sensitive to most antibiotics that affect eukaryotic cells. *Eukarya* contain rRNA that is unique to the *Eukarya* as indicated by the presence molecular regions distinctly different from the rRNA of *Archaea* and *Bacteria*.

The *Eukarya* are subdivided into the following kingdoms:

**Protista Kingdom:** Protista are simple, predominately unicellular eukaryotic organisms. Examples includes slime moulds, euglenoids, algae, and protozoans.

**Fungi Kingdom:** Fungi are unicellular or multicellular organisms with eukaryotic cell types. The cells have cell walls but are not organized into tissues. They do not carry out photosynthesis and obtain nutrients through absorption. Examples include sac fungi, club fungi, yeasts, and moulds.

**Plantae Kingdom:** Plants are multicellular organisms composed of eukaryotic cells. The cells are organized into tissues and have cell walls. They obtain nutrients by photosynthesis and absorption. Examples include mosses, ferns, conifers, and flowering plants.

**Animalia Kingdom:** Animals are multicellular organisms composed of eukaryotic cells. The cells are organized into tissues and lack cell walls. They do not carry out photosynthesis and obtain nutrients primarily by ingestion. Examples include sponges, worms, insects and vertebrates.

**Viruses:** Viruses are microscopic particles that can infect the cells of a biological organism. Viruses can only replicate by infecting the host cells and therefore cannot reproduce on their own. They can infect a wide variety of organisms: both eukaryotes and prokaryotes. A virus that infects bacteria is known as a bacteriophage (Weinbauer and Rassoulzadegan, 2004) and they are abundant in aquatic systems (Wilhelm and Suttle, 1999).

Microbial communities inhabit many deep subsurface environments showing metabolic diversity (Krumholz, 2000).

## 2.4 Microbial Metabolism

In order to obtain energy from a substrate microbes remove electrons (electron donor) and transfer them to other chemicals that serve as electron acceptors. These electron donating compounds are found in groundwater in the form of mineral constituents, particulate organic carbon (POC), dissolved organic carbon (DOC) and inorganic carbon (PIC and DIC) (Wotton, 1994). Redox conditions are important in these electron transfer systems. Redox is the process of electron transfer which results in a

reduction/oxidation (redox) reaction. Electrons flowing from electron donors to electron acceptors results in kinetic energy. Microbes are able to transform this kinetic energy into chemical energy by chemiosmosis and store this energy as adenosine 5'-triphosphate (ATP) that can be used for cellular growth (Madigan and Martinko, 2005). The ATP provides the microbe with energy to undertake cellular work. Examples of energy utilising processes include the transport of chemicals such as sodium and chloride across membranes to maintain intracellular osmotic equilibrium and nutrient uptake (Oren, 1999; Neijssel and Teixeira de Mattos, 1994). Subsurface microbes sustain their life functions such as metabolism, growth and reproduction with electron transfer via reduction/oxidation (redox) processes.

Microbes can be classified based on nutrition or the type of substance used for carbon or energy source, namely heterotrophy, lithotrophy and chemolithotrophy. Heterotrophs are able to use organic carbon as an electron donor. These microbes are important in the global biogeochemical cycling of carbon, oxygen, nitrogen, phosphorous, iron and sulphur (Meganigal *et al.*, 2004). Lithotrophs in contrast generate energy from reduced inorganic molecules. These molecules have high potential electrons that can be used to drive the electron transport system. Lithotrophs frequently utilise carbon in the form of carbon dioxide using the calvin cycle. Energy yields from lithotrophy are low per substrate oxidized and thus a large amount of substrate has to be metabolised per cell. Lithotrophs which are at the base of the food chain in many groundwater systems link life to geology and can have a major impact on biogeochemical cycles. Chemolithotrophs obtain energy by oxidising reduced inorganic chemicals such as hydrogen sulphide, hydrogen or ferrous iron (Madigan and Martinko, 2005).

#### 2.4.1 Anaerobic Respiration

Microbes can also be classified based on tolerance to oxygen. Obligate anaerobes die in the presence of oxygen whereas facultative anaerobes make ATP by aerobic respiration if oxygen is present, but are also capable of switching to fermentation in anaerobic conditions. Groundwater is often anaerobic because the flux of oxygen is typically less than oxygen consumption via microbial respiration resulting in anaerobic conditions.

Anaerobic respiration is a process which couples the oxidation of organic chemicals with the reduction of inorganic chemicals as electron acceptors. Organic electron donors (POM/DOM) are transferred to a mineral terminal electron acceptor (oxidised compounds) such as nitrate, ferric iron, sulphate and carbon dioxide. Anaerobic respiration therefore incorporates the reduction of carbon to methane, sulphate to hydrogen sulphide and ferric iron to ferrous iron. Denitrification of nitrate results in nitrogen and nitric oxide as end products and dissimilatory nitrate reduction produces ammonium as an end product (Tiedje, 1988). These metabolic processes are carried out by methanogens, sulphate-reducing, iron-reducing, and nitrate-reducing bacteria respectively (Magonigal *et al.*, 2004).

#### 2.4.2 Facultatively Anaerobic and Anaerobic Groundwater Biota

Enteric bacteria are facultatively anaerobic and their presence in groundwater systems normally indicates the presence of faecal contamination, either from humans or animals (Keswick *et al.*, 1982). However some members of the Enterobacteriaceae are indigenous to soils such as *Aerobacter aerogenes*. *E.coli* is an enteric bacterium that can be distinguished from an indigenous *A. aerogenes* by fermentation products. *E.coli* is a mixed-acid fermenter whereas *A. aerogenes* is an example of a butylene glycol fermenter.

Methanogens are a dominant group within Archaea that utilise hydrogen and carbon in the form of carbon dioxide or acetate which serves as the electron acceptor with the production of methane. Methanogens are morphologically indistinguishable from bacteria but must be cultured under strict anaerobic conditions because of their sensitivity to oxygen (Woese, 1981). Methanogens proliferate in very anoxic conditions (Leenheer, *et al.*, 1976; Godsy, 1980) and therefore extensively colonise subsurface environments with characteristically low redox conditions. A study by Ludvigsen *et al.*, 1999 investigated the effects on microbial biomass and/or microbial community composition from a shallow landfill-leachate polluted aquifer. Samples were analysed for total numbers of bacteria by use of the acridine orange direct count method (AODC). Numbers of dominant, specific groups of bacteria were measured by the most probable number method (MPN). Viable biomass estimates were obtained from measures of ATP and ester-linked phospholipid fatty acid (PLFA) concentrations. Methanogens were found to be restricted to the most polluted and reduced part of the aquifer. These results led to the conclusion that the landfill leachate induced an increase



in methanogen cell numbers by altering the subsurface aquifer so that it was conducive to methanogenic growth.

Methanogens have also been isolated from deep hydrothermal systems using hydrogen as the primary energy source in an environment with negligible carbon concentrations. This study demonstrated that hydrogen-based methanogenic communities can occur in the Earth's subsurface. It was suggested that this provided an analogue for possible subsurface microbial ecosystems on other planets (Chapelle *et al.*, 2002).

Sulphate-Reducing Bacteria have traditionally been classified as anaerobic bacteria as they are ubiquitous in anoxic environments and are generally associated with more reduced conditions (Ludvigsen *et al.*, 1998). Other studies have demonstrated that sulphate reducing bacteria can tolerate low concentrations of oxygen (Jones *et al.*, 1989) whereas recent evidence now suggests that some sulphate reducing bacteria can mineralise carbon at significant rates under fully oxic conditions (Jonkers, et al., 2005). Sulphate-reducing bacteria are widely distributed in groundwater systems (Dockins *et al.*, 1980; Chapelle *et al.*, 1987). Some sulphate reducing gram positive rods demonstrate resilience to cellular rupture thus hindering DNA extractions which can be problematic for environmental studies (Robertson *et al.*, 2002). Sulphate reducing bacteria typically utilise hydrogen or simple organic compounds such as acetate, formate and lactate as energy sources. These electron donors are coupled with sulphate reduction by using sulphate as a terminal electron acceptor (Megonigal *et al.*, 2004). Sulphate reducers can display metabolic diversity as a sulphate reducing bacterium has been isolated from heavy metal contaminated sediments. This sulphate-reducing bacterium was able to grow with metals such as ferric III as a sole electron acceptor (Tebo and Ya Obraztsova, 1998). Their ability to adapt to extreme physical and chemical conditions enables them to play an important role in global geochemical cycles.

Fe (III)-Reducing Bacteria are capable of respiratory growth on organic compounds coupled to the reduction of Fe (III) oxyhydroxides and produce Ferrous II (Megonigal *et al.*, 2004). Fe (III) oxyhydroxides are present in a broad range of hydrologic systems. It is a principal cause of high concentrations of dissolved iron in groundwater. Microbial ferric III reduction is considered to play an important role in a variety of processes of environmental concern such as degradation of aromatic compounds

(Lovely, 1997). Iron reducing bacteria are receiving a great deal of attention because of their ability to precipitate soluble metals and thus their potential for bioremediation of metal polluted sites (Lovely et al, 1993). Geochemically, Fe (III) reduction is one of the most important microbially mediated redox processes that occur in groundwater systems as these microbes and associated enzymatic reactions are required for its transformation (Lovely *et al.*, 1991). Some species are also capable of reducing Mn (IV) and U(VI) in addition to Fe (III) (Lovely *et al.*, 1993). The largest known group of ferric iron reducers is the *Geobacteraceae* family which appear to be widely distributed in groundwater systems (Lovely, 1997; Lovely *et al.*, 1990).

*Gallionella sp* is a chemolithotrophic bacterium that is an obligate anaerobe that obtains energy from oxidising dissolved ferrous iron to ferric oxyhydroxides. Ferrous iron is largely absent from aerobic water therefore *Gallionella sp.* generally lives where aerobic and anaerobic waters mix such as bores which penetrate anaerobic aquifers. The growth of *Gallionella sp.* in bores is a significant problem because the ferric oxyhydroxides that it produces will eventually clog the well (Hanert, 2006).

Nitrate-Reducing Bacteria include a wide variety of microbes that are able to couple oxidation of organic substrates with the reduction of nitrate in order to obtain energy for growth (Megonigal *et al.*, 2004). Many bacteria only reduce nitrate as far as nitrite, while others can reduce nitrate to ammonia (Tiedje, 1988). Denitrification (nitrate to nitrogen or nitrous oxide) and nitrate ammonification (nitrate to ammonium) are competitive bacterial processes (Tiedje *et al.*, 1982 and Gilbert *et al.*, 1997). Nitrate reductase is inhibited by the presence of molecular oxygen and thus these bacteria are restricted to anaerobic environments (Megonigal *et al.*, 2004). Nitrate reduction is rarely an important respirative process in pristine groundwater systems which characteristically contain low concentrations of nitrate (Robertson, 1979). Anthropogenic pollution such as septic effluent can therefore provide advantageous conditions for the proliferation of nitrate-reducing bacteria in groundwater (Smith and Duff, 1988; Bulger *et al.*, 1989).

Fermentative bacteria are able to extensively proliferate in the subsurface and examples include *Bacillus* and *Clostridia* (Chapelle *et al.*, 1996). Fermentative bacteria are able to directly utilise complex organic matter, carbohydrates and lignins present in anoxic subsurface environments to supply energy for growth (Megonigal *et al.*, 2004). They

are therefore essential to ecosystems which contain refractory material as they are able to initiate the biotransformation of complex organic matter. Fermentation contrasts with respiration in that fermentative bacteria cannot completely oxidise organic compounds. Fermentative bacteria are only able to partially oxidise carbon to carbon dioxide with the production of reduced compounds such as hydrogen, formate and acetate (by utilising acetogenic or hydrogen-producing metabolic pathways). Electrons are retained within these organic carbon compounds therefore the remaining energy can be used as substrate for sulphate-reducing, iron-reducing, nitrate-reducing bacteria and methanogenic respiration (Migonigal *et al.*, 2004).

#### 2.4.3 Aerobic respiration

Aerobic respiration uses oxygen as an electron acceptor. Oxygen reduction is the most energetically favourable mechanism by which microbes oxidise organic carbon material. Therefore aerobic respirers produce greater levels of ATP whilst using the least amount of energy (Madigan and Martinko, 2005).

#### 2.4.4 Aerobic Groundwater Biota

Protozoa are abundant in aerobic groundwater (Sinclair and Ghiorse, 1989; Sinclair *et al.*, 1990). The grazing of bacteria by Protozoa and its effect on aquatic populations have been reported (Hahn and Hofle, 2001; Kinner *et al.*, 1990). Moulds and other fungi are also common in groundwater near the land surface, where oxygen concentrations are greater than deeper groundwater they may be anaerobic (Sinclair and Ghiorse, 1989). Groundwater fauna, or stygofauna, are animals that reside in groundwater. Stygofauna predominantly consists of many kinds of crustaceans but also consists of worms, snails, insects and other invertebrate groups. Additionally in Australia two species of blind fish have been detailed. Most species are unique to groundwater and these communities typically are species that have evolved independently within aquifers and thus have important consequences for evolutionary studies. As a consequence of geographical isolation stygofauna may be susceptible to variation in environmental parameters. It has been suggested that groundwater fauna contribute substantially to the biodiversity of Australia and that they may be functionally important in aquifers (Boulton, 2000; Hancock, 2002). Most eukaryotes are restricted to environments which receive oxygen and are therefore not ubiquitous in anaerobic groundwater systems.

Aerobic bacteria are widely distributed in soils and shallow aerobic groundwater systems. Common genera include *Pseudomonas*, *Alcaligenes*, *Gallionella*, *Acinetobacter* and *Flavobacterium* (Chapelle *et al.*, 1988; Stetzenbach *et al.*, 1986). In particular *Pseudomonas* appears to be particularly common in groundwater systems (Chapelle *et al.*, 1988; Balkwill and Ghiorse, 1985; Balkwill and Boon, 1997). A lithotrophic metabolic process which utilises oxygen in some groundwater systems includes nitrification of ammonia to nitrate which is performed by microbial species such as *Nitrosomonas* and *Nitrobacter* (Bothe *et al.*, 2000; Herbert, 1999).

Microbes contrast with higher level groundwater organisms such as stygofauna because the microbes described are often ubiquitous in other ecosystems other than groundwater. Despite the ubiquitous nature of many microbes molecular ecology techniques have isolated unique microbial species from aquatic ecosystems (Souza *et al.*, 2006).

#### 2.4.5 Viruses in groundwater

Viruses are particularly associated with wastewater and their survival in groundwater systems has been well documented (Toze, 1997). Bacteriophages have also been demonstrated to exert a species specific control of bacteria in natural waters (Bratbak *et al.*, 1994) and may therefore have a significant affect on groundwater microbial populations. Groundwater viral studies have predominantly focused on enteric viruses associated with wastewater which cause a threat to humans (Berg, 1983; Abbazadegan *et al.*, 1993). Viruses are prevalent in many aquatic systems (Weinbauer, and Rassoulzadegan, 2004) and therefore it is anticipated that they also colonise the subsurface other than from anthropogenic pollution such as septic tanks.

## 2.5 Microbial Ecology

The study of microbial ecology is important because microbes have major roles in determining global warming, water quality and biogeochemical cycling. Microbial ecology studies the processes which influence competitiveness interactions, diversity and function of microbes in their natural and managed habitats.

### 2.5.1 Influence of Free-living and Attached Biofilm Niches on Microbial Population Dynamics

Microbes can be found in groundwater either suspended in the water phase (free-living) or more commonly attached to the solid phase of the porous medium. Bacteria that attach to surfaces produce extracellular materials called polysaccharides. The term biofilm describes the coating of a surface and is made up primarily of polysaccharides, bacteria, organic matter and other nutrients (Costerton, *et al.*, 1987). Adsorption of nutrients to biofilms occurs, thus creating a more advantageous niche within oligotrophic aquatic environments. Biofilms are highly diverse and in aqueous environments such as groundwater, bacteria will adhere to any solid surface such as clays and sand particles, living plants or animals or organic remains (Marshall, 1988). Therefore polysaccharides produced by bacteria enable them to colonise fine to coarse-grained sand and particulate organic matter (POM) which provides access to concentrated nutrients. Aquifer sediments are considered to be more representative of aquifer conditions (Phelps and Fredrickson, 2002), thus implicating the potential importance of attached microbes in groundwater systems. A study by Crump *et al.*, (1999) demonstrated that particle attached bacteria from an aquatic environment grew more rapidly and formed a unique population compared with free-living aquatic bacteria.

### 2.5.2 Effect of Competition, Antagonism, Predation and Synergistic Relationships on Microbial Population Dynamics

Heterogenous microbial populations are often adapted to different niches which are determined by microbial genotype. Infinite versatility is biologically unrealistic as an endless amount of energy would be required in order to be successfully adapted to very contrasting niches. Thus microbes are generally adapted to survive and reproduce in a selection of niches, with each niche having upper and lower tolerance thresholds. Examples include adaptation to certain temperature ranges such as mesophiles and osmotic tolerance within a defined range (Bradley and Chapelle, 1996; Wunsch, 1988; Troussellier *et al.*, 2002). These physiological adaptations are required in order to successfully survive within a selection of defined niches which require energy (ATP) expenditure in order to compete. Despite the ability of microbes to survive and reproduce within a range of conditions with upper and lower thresholds it has been

shown that microbial success is often limited by microbial competition (Veldkamp *et al.*, 1984).

Microbes that possess the physiological characteristics suited to the conditions of a given environment have a competitive advantage. These microbes have the ability to thrive and dominate an advantageous environment, competitively excluding other microbial members (Chapelle and Lovely, 1995). Competitive relationships between different terminal electron-accepting microbes, each of which attempts to use the same carbon sources are very common in subsurface environments (Lovely and Klug, 1983; Lovely and Klug, 1986; Raskin *et al.*, 1996). Sinclair and Ghiorse, (1989) and Chapelle and Lovely, (1990) showed that sand contained higher numbers of microbes than clay and that the diversity of bacteria correlated with sediment type. Sandy sediments displayed the greatest diversity. In addition the constituent nature of organic matter within groundwater directly affects microbial respiration as microbes preferentially exploit more readily bioavailable carbon first (easily assimilable) before utilising refractory carbon compounds (Jorgenson 1990). Different microbial populations are thus better suited to utilising these different forms of bioavailable carbon. The differential affinity for hydrogen uptake by anaerobic respiratory bacteria results in competition for a single resource (Lovely and Goodwin, 1988). These studies thus indicate the ecological importance of the available resource types present within an environment as it affects microbial abundance and diversity.

Microbes actively compete for available resources whether they inhabit nutrient-rich or nutrient-deprived environments. Microbes which are not adapted to the prevailing environmental conditions may become dormant or die. Subsurface microbial populations are typically subjected to low carbon concentrations compared with surface aquatic systems which are predominantly refractory in nature in addition to low nitrogen concentrations (White *et al.*, 1983). It has been suggested that uncontaminated oligotrophic subsurface environments contain bacteria with slow reproduction rates or microbes that are in a state of dormancy due to low nutrient concentrations and other adverse conditions (review Lappin-Scott and Costerton, 1990). In contrast studies by Ghiorse and Balkwill, (1983) and Harvey and George, (1987) demonstrated actively dividing cells *in situ*. Further support for microbial activity Chapelle *et al.*, (1988) demonstrated that subsurface carbon dioxide production occurred via microbial metabolism and not from abiotic processes. A study by Ghiorse and Balkwill, (1983)

indicated that many subsurface bacteria contained poly-beta-hydroxybutyrate (PHB), a substance used by bacteria as an energy storage compound. These results suggested that some microbial populations in groundwater are adapted for starvation stress.

Synergistic relationships occur within the subsurface between fermentative and respiring microbes (Meronigal *et al.*, 2004). The co-existence of these two metabolic pathways results in the complete oxidation of organic carbon. Respirative bacteria are dependent on associated fermentative bacteria to partially metabolise complex organic matter. Electrons are retained in the organic carbon compounds for subsequent bacterial respiration. The fermentative bacteria are reliant on the respirative bacteria to remove fermentation products from the environment thus aiding their metabolism (Chapelle, 2001). Antagonism in contrast is a form of competition whereby microbes produce an inhibitory substance such as antibiotics, organic acids and hydrogen sulphide. The production of these substances can inhibit specific microbial growth through competitive exclusion thus reducing diversity. Antibiotic resilience in groundwater microbes have been identified in subsurface environments (Fredrickson *et al.*, 1988) thus indicating the potential for antagonism within the subsurface.

Predation may also occur where one organism attacks and ingests the other such as bacterial grazing via protozoans and viral bacterial lysis. Predation plays an important role in the 'microbial loop' of oligotrophic aquatic environments as the microbial loop allows for further recycling of nutrients (Weinbauer and Rassoulzadegan, 2004; Wilhelm and Suttle, 1999). The subsurface is a unique environment as energy moves primarily through the microbial food chain due to the absence of energy associated with direct sunlight. Thus an increase in the complexity and variability of nutrient recycling within groundwater may play a potentially important role within the subsurface.

The affects of microbial interactions can thus have an affect upon microbial community structure within an ecosystem. Significant overlap between niches, with strong competition can result in a single organism being dominant within that ecosystem thus reducing diversity (Beeman & Suflita 1987). Conversely, weak competition can result in high diversity with or without an overlap of niches as microbial populations are not being competitively excluded or attacked by chemicals or direct lysis. The groundwater environment contains many different kinds of microbial populations that can interact

through competition, antagonism and predation. These interactions may have positive or negative effects.

## 2.6 Studying Population Dynamics Using Molecular Microbial Ecology

It is now recognised that only a small fraction of all bacteria have been isolated and characterised (Wayne *et al.*, 1987; Ward *et al.*, 1990 and Ward *et al.*, 1992). It is generally accepted that cultivation methods recover less than 1% of the total microbes present in environmental samples (Amann *et al.*, 1995; Ward *et al.*, 1990). However, molecular studies suffer from the drawback that physiology can only rarely be inferred from the DNA sequence alone. In addition molecular techniques that use traditional polymerase chain reaction (PCR) are unable to discriminate between living and dead microbes. In contrast molecular techniques such real-time PCR can provide both qualitative and quantitative information and with the use of mRNA allows 'living cells' to be identified and quantified. Cultivation and molecular methods may complement each other for the investigation of microbial structure and function within an ecosystem.

Ribosomal RNAs (rRNAs) and the genes encoding those molecules (rDNA) are commonly used in molecular biodiversity studies. The RNA sequences contain variable and highly conserved regions and thus organisms can be distinguished on all phylogenetic levels. There are many molecular techniques available to study the diversity of environmental samples such as DNA-DNA hybridizations, restriction length fragment polymorphisms (RFLPs), microsatellites, nucleotide sequencing, single nucleotide polymorphism (SNP), random amplification of polymorphic DNA (RAPD) and denaturing gradient gel electrophoresis (DGGE) (Anderson and Anderson, 1998). Genetic fingerprinting techniques provide a pattern or profile of the genetic diversity in a microbial community (Muyzer and Ramsing, 1996).

DGGE is a method that separates DNA fragments according to their mobilities under increasingly denaturing conditions (Muyzer *et al.*, 1993). The DNA fragments are obtained from universal primers targeted at conserved regions of the RNA in order to obtain the maximum diversity possible within a given sample. Electrophoresis through a denaturing gradient separates the DNA fragments. Separation occurs as DNA migration is halted through denaturation. Differential separation occurs because of the



different melting behaviour inherent to fragments that contain dissimilar DNA sequences. As a result, a band pattern is obtained, which reflects the complexity of the microbial community structure within a given sample based on the total genomic DNA retrieved from that sample. The sensitivity of DGGE analysis can be refined with the targeting of precise taxonomic groups using specific PCR primers or by hybridization of blotted DGGE gels with group-specific oligonucleotide probes (Heuer *et al.*, 1999). By excising individual DGGE bands from the gel and reamplifying the DNA, it is possible to get sequence information of single community members (Muyzer *et al.*, 1993; Muyzer and Smalla, 1998).

The DGGE/DNA profiles can be analysed by computer-assisted pattern analysis of molecular fingerprints and database construction (Rademaker *et al.*, 1999). Multivariate statistical analysis of the binary matrix data generated from the DNA profiles can then be used to assess the microbial community structure but also in relation to environmental parameters (Fromin *et al.*, 2002). Therefore fluctuations in microbial populations due to environmental perturbations can be rapidly assessed. DGGE is relatively rapid to perform, and many samples can be run simultaneously. The method is therefore particularly useful when examining time series and population dynamics. DGGE represents a powerful tool for monitoring microbial communities.

Subsurface environments typically contain low microbial numbers (Chapelle *et al.*, 1987). Thus DNA isolated from oligotrophic environments may contain low DNA yields due to insufficient microbial numbers. Additionally microbes vary in tolerance to cellular rupture and thus DNA extractions are always a trade-off between obtaining the maximum amount of DNA possible from cells without degradation through extraction processes (Roose-Amsaleg *et al.*, 2001). It is also sometimes difficult to obtain DNA of sufficient purity for reliable molecular analyses. PCR inhibitors which include substances such as salts, pigments, exopolysaccharides, humic acids and other unknown substances are ubiquitous in environmental samples (Roose-Amsaleg *et al.*, 2001). There are also some disadvantages specifically associated with DGGE. Direct sequencing of manually excised bands does not always allow reliable phylogenetic analyses due to the short sequence length (200-500bp). Furthermore, co-migration of several different 16S rDNA sequences, which have the same melting behaviour and therefore the same position in the gel, leads to overlapping DGGE bands which cannot be sequenced directly (Rolleke *et al.*, 1996). Similar band migration of different

microbial species also reduces the observed total biodiversity of the sample. It is also very important to standardise gels which can be problematic if microbial populations need to be compared over a series of gels for statistical analyses (Fromin *et al.*, 2002).

## 2.7 Wastewater treatment methods

The constituents of sewage typically contains: semi-liquid wastes from households such as washing and laundry water, faeces, urine, liquid and industrial waste. Compared with surface and groundwater, sewage chemically contains high concentrations of organic carbon, nitrogen and phosphorous. Sewage also contains microbes, heavy metals, trace organics and suspended solids.

Conventional wastewater treatment processes involve pre-treatment by screening, primary sedimentation, secondary treatment by activated sludge or trickling filters and tertiary treatment processes such as sand filtration, land application, ozonation, chlorination, reverse osmosis and ultrafiltration (Shishida *et al.*, 2000; Jolis *et al.*, 1996; Feachem, 1983). The quality of sewage effluent is primarily determined by the level of treatment (i.e. primary, secondary or tertiary). Contaminants in wastewaters are removed by physical, chemical and biological means. The term used to describe wastewater which is diverted into recycling schemes is known as reclaimed water.

## 2.8 Wastewater reuse

Most large-scale wastewater re-use schemes are in arid/dry climatic locations where alternative sources of water are limited (Thomas *et al.*, 1997). Currently, interest in wastewater reuse is increasing in many parts of the world in response to growing pressures on high quality natural water resources. Current uses of freshwater supplies by both developing and developed countries have been deemed unsustainable with worldwide water usage reported to be growing at more than three times the world's population increase (UN Commission on Sustainable Development 1997). It has been predicted that water will become one of the scarcest resources in the 21st century. Wastewater reuse provides a means of reducing the uses of natural freshwater reserves as well as providing an alternative to disposal into sensitive river and marine ecosystems. Wastewater reuse for drinking purposes has now become a reality in South East Queensland, Australia which hopes to return treated wastewater into the drinking

supply by 2008 (ABC News online, 2007). Sustained drought over ten years is to blame and it is being speculated that all major cities within Australia will eventually follow suit to meet freshwater demands. Wastewater recycling schemes include technologies such as Managed Aquifer Recharge (MAR), Reverse Osmosis (RO), Microfiltration and Advanced Oxidation. These technologies can be used in unison or combined to improve the quality of wastewater.

### *2.8.1 Reverse Osmosis*

Reverse osmosis is the process of forcing a solvent from a region of high solute concentration through a membrane to a region of low solute concentration by applying a pressure in excess of the osmotic pressure. The membrane is semipermeable and therefore water flows through whereas solutes are trapped by the membrane. This process is best known for its use in desalination by removing the sodium and chloride from seawater to obtain freshwater. Reverse osmosis has also been introduced to purify treated effluent as the method effectively improves water quality by removing wastewater solutes. High quality water can be obtained which has subsequently been supplied to industries that benefit from receiving water that is of above drinking water standards. Problems associated with reverse osmosis are the subsequent corrosive properties of the water after reverse osmosis as removal of ions effectively destroys its neutral pH properties.

### *2.8.2 Managed Aquifer Recharge (MAR)*

The term Managed Aquifer Recharge (MAR) describes the intentional recharge and treatment of water in aquifers. MAR was previously called artificial recharge (Pavelic and Dillon, 1997) and has also been called enhanced recharge, water banking and sustainable underground storage (Dillon, 2005). Most commonly, MAR is a means of enhancing groundwater supplies for subsequent extraction in times of peak demand. Storing water underground offers several advantages when compared with the more traditional use of surface water reservoirs such as significantly reduced evaporation and improvements in water quality (Tuinhof and Heederik, 2002). Further benefits of MAR include reducing native groundwater salinity, precluding seawater intrusion, controlling floods, purifying surface waters, alleviating land subsistence and storage and treatment of reclaimed water (Pavelic and Dillon 1997). Recovered water is particularly beneficial for the irrigation of green open spaces such as parks, ovals golf courses and

agricultural land which generally use large quantities of water. The type of water to be used for aquifer recharge and the level of pre-treatment required varies with its intended end use which can range from virtual potable standards (Pyne, 1995) to poor quality reclaimed water and stormwater. Water type and quality of pre-treatment required for MAR also largely depends on the hydrology and geology of the aquifer and the technique of MAR being used.

There are many MAR techniques which can be used to inject or infiltrate water into an aquifer and then retrieve it (Dillon, 2005):

- Aquifer storage and recovery (ASR) - injection of water into a well for storage and recovery from the same well.
- Aquifer storage transfer and recovery (ASTR) – injection of water into a well for storage and recovery from a different well, generally to provide additional water treatment.
- Bank filtration – extraction of groundwater from a well or caisson near or under a river or lake to induce infiltration from the surface water body thereby improving and making more consistent the quality of water recovered.
- Dune filtration – infiltration of water from ponds constructed in dunes and extraction from wells or ponds at lower elevation for water quality improvement and to balance supply and demand.
- Infiltration ponds – ponds constructed usually off-stream where surface water is diverted and allowed to infiltrate (generally through an unsaturated zone) to the underlying unconfined aquifer
- Percolation tanks – a term used in India to describe harvesting of water in storages built in ephemeral waddies where water is detained and infiltrates through the base to enhance storage in unconfined aquifers and is extracted down-valley for water supply or irrigation.
- Rainwater harvesting – roof runoff is diverted into a well or a caisson filled with sand or gravel and allowed to percolate to the water table where it is collected by pumping from a well.
- Soil aquifer treatment (SAT) – treated sewage effluent, known as reclaimed water, is intermittently infiltrated through infiltration ponds to facilitate nutrient and pathogen removal in passage through the unsaturated zone for recovery by wells after residence in the aquifer.

### 2.8.3 MAR of Secondary Treated Effluent using Infiltration Galleries, Perth, Western Australia

A three-year pilot research project was commissioned in July 2006 to trial the infiltration of reclaimed water to a shallow superficial aquifer in Perth using secondary treated effluent (Bekele *et al.*, 2006). The infiltration galleries are connected by subsurface pipes to the source of reclaimed water at Subiaco Waste Water Treatment Plant. The subsurface galleries are covered engineered structures that facilitate the infiltration of water into the soil. The reclaimed water percolates from the infiltration gallery through six to eight meters of sand of the Spearwood Dune System to Tamala limestone which is a lime-sand eolinite (Playford *et al.*, 1976). It is thought that MAR via infiltration to the subsurface can use wastewater of a poorer quality as during percolation the water infiltrates the soils biological, chemical and physical processes (Dillon, 2005). This ‘filtering’ process continues whilst the water infiltrates, resides and laterally moves through the aquifer to recovery wells. During migration to the recovery well the water quality improves from many mechanisms including volatilization, precipitation of metals, sorption within the soil matrix, biological breakdown of organics, nitrification and denitrification (Martin and Dillon, 2005). Pathogen attenuation also significantly improves during the infiltration process (Asano and Levine, 1996). MAR using infiltration galleries extracts recovered water at distance and depth from the infiltration gallery allowing sufficient residence time within the aquifer for water quality improvements. To date both the geochemical and microbial changes within the aquifer have not been characterised.

### 2.8.4 Aquifer Storage and Recovery (ASR) of Tertiary Treated Effluent, Adelaide South Australia.

A Bolivar Aquifer Storage and Recovery (ASR) research project (1997 to 2005) fully investigated water quality improvements using tertiary treated effluent which was introduced into a confined aquifer (Martin and Dillon, 2005). The sandy-limestone aquifer used for storage was the lower of two confined aquifers (T2) and is brackish and anoxic in nature. ASR was achieved by injection of tertiary treated effluent over time. Cessation of injected wastewater occurred during a storage phase. During the storage phase, extensive biogeochemical transformation of organic and inorganic chemicals associated with wastewater significantly improves the quality of extracted

water. Geochemical studies demonstrated that after a storage period the quality of injected waters had returned to ambient groundwater conditions (Martin and Dillon, 2005). Both geochemical and microbial interactions were implicated in contributing to significant improvements in the quality of reclaimed water (Greskowiak *et al.*, 2005). In addition pathogen decay studies demonstrated that microbial pathogens associated with wastewater significantly declined within the environment (Toze and Hanna, 2004). ASR is a proven water management technique being practised in Australia (Martin and Dillon 2001), Europe (O'Shea and Sage 1999), Israel (Harpaz 1971), Netherlands (Peters 1989) and USA (Pyne 1995). To date the microbial population dynamics associated with the described geochemical changes during ASR have not been described.

## 2.9 Groundwater Biogeochemistry of a Contaminated Aquifer.

Numerous geochemically important processes in subsurface environments are carried out exclusively by enzymatically controlled microbial processes. In particular ferric iron reduction can only occur via ferric iron-reducing bacteria that possess the enzyme iron reductase in natural groundwater systems (Lovely *et al.*, 1991). Consequently it is clear that microbes are able to change the chemical nature of groundwater.

Groundwater chemistry data suggests that an aquifer is segregated into discrete redox zones dominated by different physiologic microbial processes (Stumm and Morgan, 1981; Lovely, 1991; Lovely and Goodwin, 1988; Chapelle, 2001). The redox zones can be described as a succession of predominant terminal electron accepting processes (TEAPs) (McGuire *et al.*, 2000). Much of the work undertaken on groundwater geochemistry (e.g., Bennett *et al.*, 2000; Chapelle *et al.*, 1995; Chapelle *et al.*, 1996; McGuire *et al.*, 2000; Vroblesky and Chapelle, 1994; Vengosh and Keren, 1996; Kass *et al.*, 2005; McGuire *et al.*, 2005) and microbial (e.g., Doijka *et al.*, Madsen, 2000; Bekins *et al.*, 2001; Pickup *et al.*, 2001; Roling *et al.*, 2001; Davis *et al.*, 2002; Kinner *et al.*, 2002) analyses have focused on contaminated aquifers. Only two of these studies have combined molecular techniques to describe microbial community structure with multivariate statistics to investigate microbial and chemical characteristics (Fahy *et al.*, 2005; Haack *et al.*, 2004). The groundwater of these various studies had been polluted with various substances such as crude oil, landfill leachate, benzene, petroleum, aromatic hydrogens, waste fuel, chlorinated solvents and organically rich waste.

Subsurface pollution is described as a migrating plume within groundwater which is essentially a chemical gradient which is strongest at the point of source.

Davis, (1967) and Pedersen and Ekendahl, (1990) both recognised that groundwater geochemistry gave important information as to the *in situ* microbial processes. Later, Cozzarelli, *et al.*, (2000) highlighted the importance of using a combination of indicators for the verification of active biogeochemical transformation of TEAPs. These indicators are shown in Table 2.1

**Table 2.1 - Indicators of Active Terminal Electron-Accepting Processes**

TEAP	Electron acceptor (Conc. Decrease)	Reduced product (Conc. Increase)	Microbial activity present	Hydrogen conc. (mM)	Potential standard free energy
CO <sub>2</sub> reduction	CO <sub>2</sub>	CH <sub>4</sub>	Methane production	>4.0	34
Sulphate reduction	SO <sub>4</sub> <sup>2-</sup>	H <sub>2</sub> S	Sulphate reduction	1.0-4.0	38
Iron reduction	Solid-phase Fe(III)	Fe <sup>2+</sup>	Iron reduction	0.1-0.8	50
Nitrate reduction	NO <sub>3</sub> <sup>-</sup>	NH <sub>4</sub> <sup>+</sup>	Nitrate reduction	<0.10	224

Adapted from Lovely and Goodwin, (1988) and Cozzarelli, *et al.*, (2000)

Table 1 clearly shows that nitrate reduction is the most favourable energy reaction. Nitrate reduction is thus generally favoured in non-polluted aquifers that are oxygenated at the recharge site in groundwater which contains nitrate. Redox zonation in the subsurface has been shown to vary according to the most favourable energy reaction. Therefore an iron reducing zone follows the nitrate zone which progresses into a sulphate reducing zone terminating in a methanogenic zone (Lovely and Goodwin, 1988). Methanogenesis is not a thermodynamically favourable reaction therefore this reaction generally only occurs in environments that lack other inorganic electron acceptors such as sulphate, iron and nitrate in pristine groundwater. Subsurface environments which contain unlimited electron acceptors and donators display redox zonation that progressively move towards a more anoxic and reduced environment

further down the flow path from the recharge site culminating in a methanogenic zone (Lovely and Goodwin, 1988).

In contrast, the enhanced nutrient concentration caused by contamination creates a polluted plume. Pioneering investigations of groundwater suggested that subsurface microbes played an important role in the mobility and fate of many contaminants (McNabb and Dunlap, 1975; Freeze and Cheery, 1979; Matthess, 1982). Initially, it has been shown that the enhanced nutrient supply of a polluted aquifer results in oxygen depletion due to an increase in microbial numbers and respiration thus creating anoxic conditions. Wilson *et al.*, (1983) demonstrated that toluene was rapidly degraded, with about 90% of the toluene disappearing within a week. Microbial activity leads to increasingly reduced conditions within the polluted plume (Haack and Bekins, 1999). Thus the redox zones were reversed (Champ *et al.*, 1979) with methanogenesis occurring at the most concentrated part of plume (Ludvigsen *et al.*, 1999). Geochemical evaluation of the storage period at the ASR site in Adelaide observed methanogenic and sulphate-reducing zones at the injection and 4m well respectively (Vanderzalm *et al.*, 2006). Therefore the ASR site reacted similarly to that proposed for a polluted aquifer with a characteristic chemical plume. During the ASR injection phase oxygen was introduced into the aquifer thus methanogenic conditions were not attained. However a nitrate-reducing zone was detected in the vicinity of the injection well during the injection cycle consistent with successional redox zones based on standard free energy as indicated Table 1. The type, concentration and range of electron acceptors and donors created from the contaminants therefore have the potential to influence the receiving ecosystem as available resources generally determine the structure and function of microbial communities (Beaman and Suflita, 1987).

Groundwater flow results in solutes being transported along the aquifer flow path.

Consequently an increase in a metabolic product such as methane does not necessarily indicate the precise location of a methanogenic redox zone. Microbiologically driven TEAP processes occur at characteristic hydrogen concentrations as a result of differing efficiency for hydrogen uptake (Chapelle *et al.*, 1996). These concentrations are less prone to variations by groundwater flow as fermentative bacteria constantly produce hydrogen in all redox zones whilst respirative bacteria are continually utilising hydrogen. Thus hydrogen concentrations can help to verify the location of the redox zones (Chapelle, 2001). Exceptions can occur when electron donors are plentiful as



may arise from pollution. It has been shown under these circumstances a contaminated aquifer does not always segregate into characteristically distinct redox zones due to several microbial processes occurring in unison (Cozzarelli *et al.*, 2000). These included methanogenesis, sulphate reduction and ferric iron reduction. Consequently the hydrogen concentrations were inconclusive (Cozzarelli *et al.*, 2000). None-the-less the indicators shown in Table 1 can be used to support evidence for groundwater microbial activity that has directly affected groundwater chemistry (Chapelle *et al.*, 1996). The gathering of microbial and chemical data thus assists with locating groundwater redox zones.

Multivariate analysis of bacterial phospholipid fatty acid analyses (PLFA) profiles were used to distinguish between microbial communities present in near surface soils, vadose zone sediments, and deep subsurface communities (Rigelberg *et al.*, 1997). Results from Balkwill and Ghiorse, (1985) suggested that indigenous microbial populations from deeper subsurface samples were adapted to carbon sources characteristic to their environment. The study showed that the transition from surface waters to the unsaturated zone and subsequently to the saturated zone resulted in decreasing microbial numbers, activity and changes in the types of carbon available.

It has also been shown that metabolic activity decreases with depth (Chapelle *et al.*, 1988; Lovely, 1990) thus indicating that the ability to reproduce quickly is less important in oligotrophic environments due to adaptation to their environment. Numerous studies have indicated that subsurface environments contain low microbial diversity (Balkwill *et al.*, 1989; Hirsch and Rades-Rohkohl, 1983; Stetzenbach *et al.*, 1986; Pedersen and Ekendahl, 1990; Ekendahl, 1990). In addition Balkwill *et al.*, (1989) showed that soil bacteria were able to metabolise a wider range of substrates than subsurface bacteria. He suggested that the differences in metabolic flexibility reflected the more limited kinds of organic matter present in the subsurface than in the soil.

No comprehensive studies have been undertaken on MAR microbial population dynamics consequently there is no direct literature to review. Despite this, indirect comparisons can be made with environments undergoing similar stress or environmental conditions. Many studies have been carried out on the microbial population dynamics of soil where it has been shown that microbial community structures are very diverse and can vary considerably over time (Review: Torsvik *et al.*,

1998). It has been suggested that the dynamic variation of microbial populations in soil are a result of the constant flux of nutrients being added from fertilisation and removed by plant uptake (McCaig, Glover and Prosser, 2001). The introduction of treated wastewater into the subsurface via MAR is in some ways analogous to this concept. The introduction of treated wastewater into an aquifer creates a eutrophic plume (Russell and Dillon, 2005). It is proposed that MAR results in a cyclic flux of nutrient enrichment via continuous injection or infiltration of treated wastewater and removal through the microbial food web from microbes that have been shown to occur in groundwater systems.

The type of organic matter can vary along a chemical gradient particularly in relation to easily assimilable and refractory carbon. A study by Skjemstad *et al.*, (2002) for the ASR site in Adelaide, demonstrated that DOC decreased over distance to the 75m observation well. Not only was there a DOC gradient but the carbon analyses demonstrated that the assimilable organic carbon (AOC) was preferentially lost from the system. When full penetration of the injectant reached 4m there was a discrepancy in AOC between the two distances. The results suggested that the increased microbial activity associated with biofilm formation at the injectant well resulted in the rapid utilisation of preferential AOC via microbial metabolism. Full penetration of the injectant assessed by a chloride stable tracer to 75m (Vanderzlam *et al.*, 1996) determined that the groundwater at this distance predominantly contained refractory material. Additionally it was demonstrated that DOC increased during the storage phase. A study by McMahan, 1990 demonstrated that subsurface microbes are able to metabolise refractory carbon. This study also demonstrated that microbial processes changed the constituent make-up of carbon in the subsurface as they preferentially oxidised the more easily assimilable carbon. Bradley *et al.*, (1998) and Coates *et al.*, (2002) also demonstrated that bacteria were capable of utilising humic substances as electron acceptors and donors for anaerobic respiration. These results indicate microbial activity and nutrient recycling therefore it is possible that microbial loop cycling plays an important role in the biogeochemical processes during MAR. Allochthonous microbes are at a disadvantage as they are not 'adapted' to local conditions (Roszak and Colwell 1987).

The transformation of nutrients along a chemical gradient provides the opportunity for increased competition compared with a homogenous pristine aquifer. The addition of a

continuous and enhanced nutrient supply provided by MAR may result in metabolic stress for indigenous groundwater microbial species which are possibly adapted to a nutrient poor refractory environment. Therefore MAR may create an environment for substantial dynamic changes in microbial populations as has been shown to occur for nutrient enrichment and pollution studies (Schafer *et al.*, 2001; Torsvik *et al.*, 1998). In Addition studies have also shown that there is a decrease in microbial diversity within polluted environments (Larrick *et al.*, 1981; Wassel and Mills, 2005). Population changes may thus result from opportunistic species that are able to rapidly take advantage of the new environmental conditions thus decreasing species diversity. Therefore it is possible that microbial populations isolated from ambient groundwater may differ to microbial populations adapted or associated with wastewater.

In summary the subsurface is an environment rich in mineral oxidants. The microbial populations of groundwater are therefore reliant on the geology of their environment and thus groundwater microbial processes are shaped by geologic and hydrologic factors (Fredrickson *et. al.*, 1989). Conversely the microbial populations which cycle organic matter directly influence the carbon, oxygen, hydrogen, sulphur and iron cycles within an aquifer. Therefore groundwater microbial activity directly affects groundwater geochemistry due to changes in the constituents of water (Lovely and Goodwin, 1988). Thus the subsurface is a unique environment where organic and inorganic carbon, sulphur, nitrogen and iron species moves primarily through the microbial food chains in the form of energy storage and utilisation. Therefore understanding the nature of biogeochemical cycles is fundamental to understanding the distribution of chemical species in microbiologically active groundwater subjected to MAR.

## **2.10 Concluding remarks**

There are many potential advantages of MAR to enhance freshwater supplies and to store water at a time when freshwater predictions for the future are bleak. Despite these advantages it is fundamental that environmental and public health concerns are addressed with sound scientific research.

Environmental concerns relate to stygofauna where it has been suggested that the techniques used by MAR could pose a potential threat to these ancient species which

are sensitive to changes in environmental conditions (Hancock *et. al.*, 2005). Research is required to understand these threats but to also investigate the potential for MAR to assist groundwater dependent ecosystems which may be under threat from declining groundwater supplies. There is also concern that wastewater may be lost from the intended aquifer due to an increase in hydraulic head which has the potential to contaminate down-gradient groundwater if not correctly managed. Increased nutrient loads into an aquatic environment have been shown to affect biodiversity. It is therefore important that appropriate scientific studies be undertaken to demonstrate that recharged water/treated effluent is not lost into the aquifer but is fully recovered after recharge. Studying the impact of wastewater on groundwater microbial populations endeavours to evaluate the changes in biodiversity at the lowest trophic level. It is hoped that this work will path the way for elucidating chemical and microbial indicators that can be used as 'standards' to track plume migration and assess water quality changes pertinent to environmental impact assessment and regulations.

Public health issues relate to the pathogens associated with wastewater and their potential for survival within the subsurface and subsequent infection of humans through drinking and irrigation supplies. Groundwater micro-organisms are influential in removing pathogens from recharged water (Gordon and Toze, 2003). Characterisation of microbial communities in MAR schemes therefore hopes to assist in the improved prediction of the removal of pathogens from the recharged water in addition to contaminants such as nutrients and trace organics. Identification of indigenous groundwater microbial populations will help to assist with studies aimed at identifying the microbial populations responsible for enhanced removal of pathogens from wastewater.

The economic viability of MAR schemes also need to be addressed as MAR as a method to supplement freshwater supplies needs to be financially sustainable. Therefore technical issues need to be investigated such as the management of recharged water which has the potential to overwhelm the ability of the aquifer to process the waste. Changes in microbial biodiversity may increase or decrease the potential of the aquifer to process treated wastewater. Aquifer clogging of the extraction well can also be problematic due to a reduction in hydraulic conductivity thus reducing the efficiency of extracted water. Microbiologically, biofilms associated with enhanced microbial growth at the extraction well have been implicated. Thus identification of microbial

species at the extraction well may provide insight into the characterisation of microbial biofilms associated with clogging. Therefore these technical issues clearly demonstrate the need for MAR schemes to be appropriately managed and studied.

Studying the biogeochemical characteristics of MAR can be used to fully investigate water quality changes in conjunction with microbial and chemical processes which occur during MAR. Multivariate statistics considerably simplifies vast quantities of chemical and biological data so that the overall microbiological and chemical trends can be evaluated.

## 2.11 Hypotheses and Main Aims

Hypotheses – That:

- I. A spatial and temporal chemical plume develops from the infiltration gallery (Perth) and injection well (Adelaide).
- II. The nutrient plume from MAR changes groundwater bacterial diversity.
- III. Microbial and chemical changes correspond over distance and time thus demonstrating aquifer biogeochemical changes/trends in response to MAR.
- IV. Changes in groundwater nutrients from MAR will cause a shift in microbial community structure and species dominance.
- V. Groundwater microbial populations unaffected by plume migration are more similar in community structure than groundwater microbial populations subjected to MAR nutrients.
- VI. The development of microbial communities in response to MAR is different between attached compared with free-living bacterial populations.

Aims- To:

1. Determine the complexity of microbial populations via DGGE for (i) non-culture water samples and (ii) cultures targeted at biogeochemical cycles namely, Sulphate-reducing bacteria, nitrate-reducing bacteria and fermentative bacteria.
2. Analyse a range of chemical parameters via multivariate analysis to evaluate the migrating nutrient plume over time
3. Analyse individual chemical parameters to determine redox zonation
4. Determine if the microbial populations that were free-living and attached differed in their spatial and temporal patterns.
5. Relate changes in microbial diversity to changes in geochemical data over distance and time.

## SECTION 3: MATERIALS AND METHODS

### 3.1 Adelaide ASR aquifer and site details.

This trial ASR site at Bolivar targeted the lower of two main Tertiary limestone aquifers (T2). This aquifer was within the Port Willunga Formation which approximately extends from 100 to 160m below ground surface. The aquifer is overlain by a 7 m confining layer of Munno Para Clay. A pilot ASR scheme was undertaken at this site between 5<sup>th</sup> March 2002 and 8<sup>th</sup> October 2002 Table 3.1

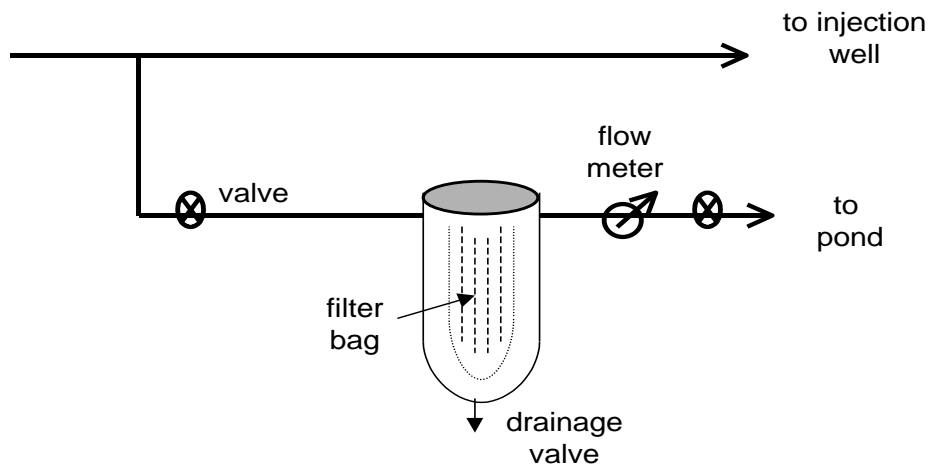
**Table 3.1** Operational details of the ASR cycle undertaken in the Bolivar ASR field trial.

Reclaimed water treatment	Activity	Period	Duration (days)	Net. Volume (ML)	Ave. flow rate (L s <sup>-1</sup> )
Activated sludge, lagoons, DAFF, chlorination	Injection	05 Mar 02 - 27 Jun 02	111	114.3	11.9
	Storage	28 Jun 02 – 24 Jul 02	27	n/a	n/a
	Recovery	25 Jun 02 – 08 Oct 02	70	90.9	15.0

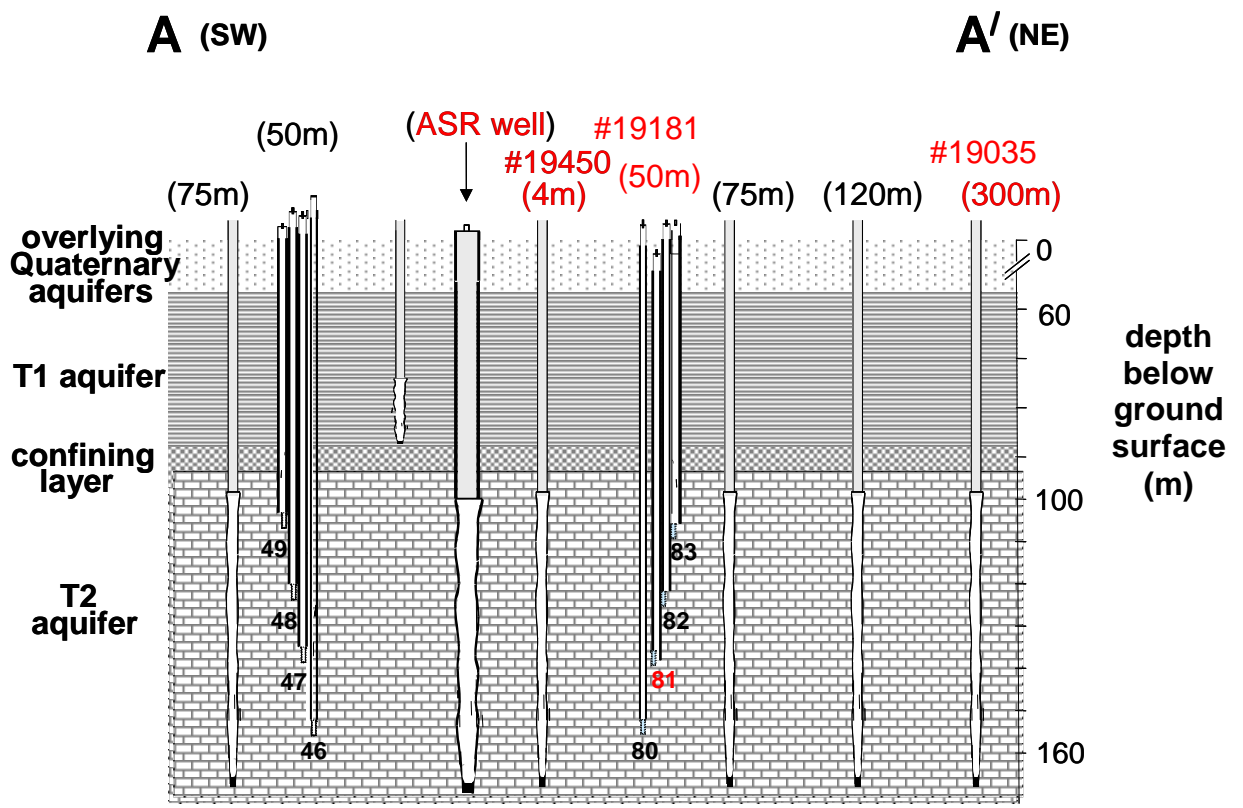
During the duration of this study a total volume of 114.3 ML of tertiary treated effluent was injected into the confined aquifer from 5<sup>th</sup> March to 27<sup>th</sup> June 2002. Cessation of injection occurred during a storage period from 28<sup>th</sup> June to 24<sup>th</sup> July 2002. A total volume of 90.9 ML of water was recovered from 25<sup>th</sup> June to 8<sup>th</sup> October 2002. Thus slightly less water was recovered than was put into the aquifer. Within the zone of treatment in the aquifer, three sampling stations were monitored for the microbial population studies. These include the on-line filter (surrogate for injection well) (Figure 3-1), 4m well (19450) and 50m well (19181) (Figure 3-2). All sampling stations fully penetrated the confined aquifer. The numerical values denoted by metres reflect the distances (m) of each sampling station from the injection well.



**Figure 3-1** On-Line Filter (Surrogate for Injection well sampling stations)

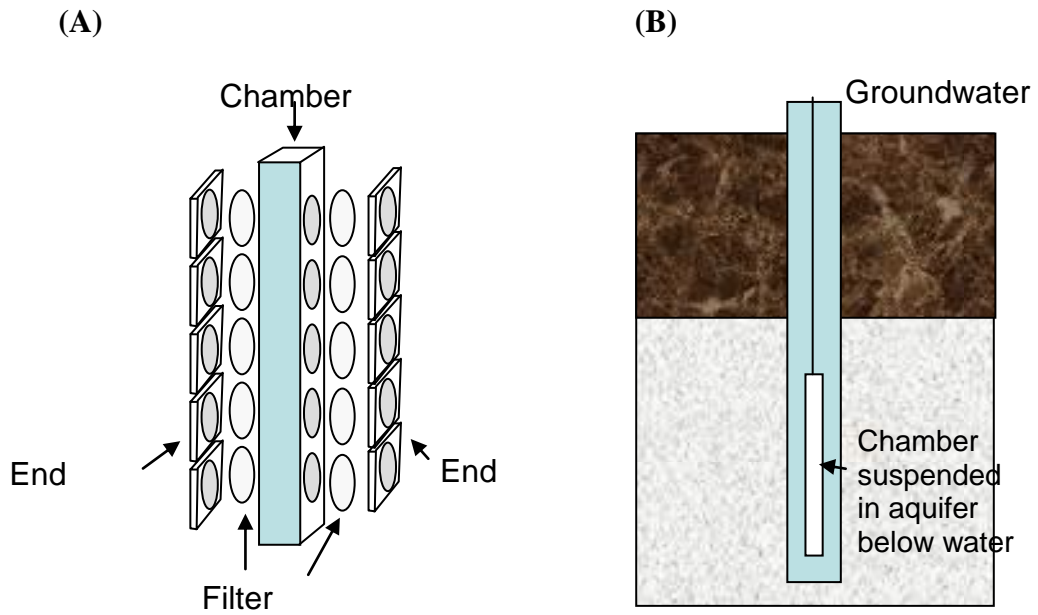


**Figure 3-2** Simplified vertical section along transect A-A' showing location of four sampling stations monitored (ASR well, 19450, 19181 and 19035)



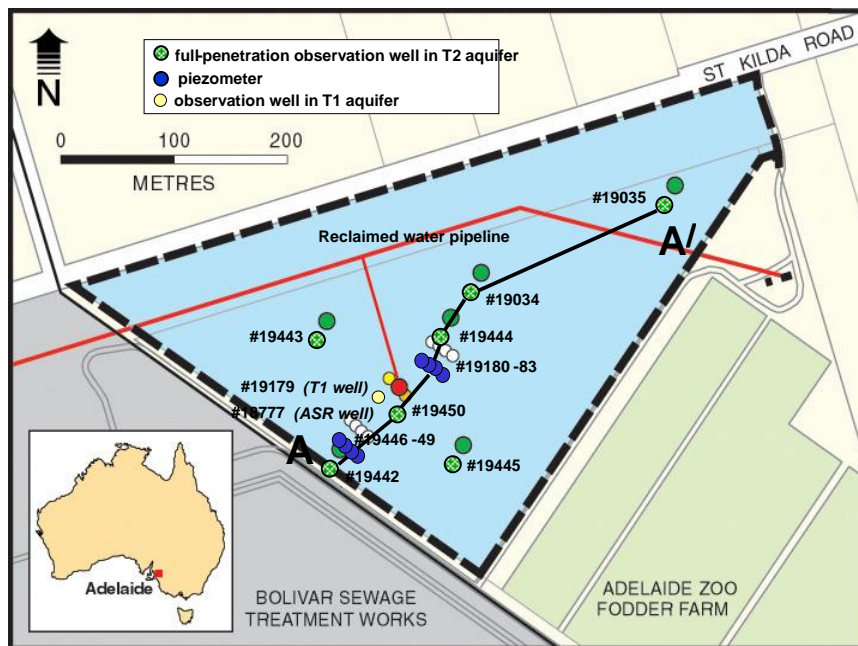
ASR	Well = Injection and Recovery Well
4m	Well = 4m from ASR Well
50m	Well = 50m from ASR Well
300m	Well = 300m from ASR (Background Well)

**Figure 3-3** (a) Diffusion Chamber design and (b) Chamber Assembly in Groundwater



(Pavelic et al., 1998)

**Figure 3-4** Northern Adelaide Plains horticultural region indicating location of the Bolivar ASR trial site.



Background water quality was monitored from a bore located 300m from the injection well (19035) which was previously shown to be unaffected by ASR activities (Martin and Dillon, 2006).

### 3.2 Chemical Analysis of Adelaide ASR Site

The chemical analytes measured in the ASR monitoring wells at the Adelaide ASR site were as follows:

**Table 3.2** Chemical analytes measured at the Adelaide ASR site

Chemical (Field) Measurements	Abbrev.	Units	Chemical Measurement (Chem. Centre)	Abrev.	Units
pH	pH	pH	Total Organic Carbon	TOC	mg/L
Electrical conductivity	EC	ms/m	Chloride	Cl	mg/L
Dissolved Oxygen	DO	mg/L	Dissolved Organic Carbon	DOC	mg/L
Temperature	Temp	°C	Sulphate	S04	mg/L
Redox	Eh	mV	Iron	Fe	mg/L
			Ammonia	N-NH3	mg/L
			Nitrate	N-N03	mg/L
			Total Nitrogen Kjeldahl	N-TK	mg/L
			Soluble Phosphate	P-SR	mg/L

Chemical measurements were undertaken by Vanderzalm, (2004). Chemical analysis was part of a separate research activity within a cohort of related research projects. This thesis analysed the chemical measurements differently to Vanderzalm, 2004 and used the results of chemical measurements specific to microbial ecology only. The methods for chemical analyses were undertaken using standard methods which are also fully described in Martin and Dillon, (2005) and Vanderzalm, (2006).

**Table 3.3** Calendar Date of ASR cycles and sampling with designated ‘Sampling Event number’ used for all graphical analyses

Sampling Event	Calendar Date	Chemical	Chamber Microbial	Time period between samples	Injection Cycle
01	22/01/02	X		Background	Background
	05/03/02				Start of Injection
29	20/03/02	X		01	
	25/03/01		*	05	
43	03/04/02	X	X	14	Injection
57	17/04/02	X	X	14	Injection
71	01/05/02	X	X	14	Injection
116	15/05/02	X	X	14	Injection
159	27/05/02	X	X	14	Injection
188	25/06/02	X	X	29	Injection
					Storage Phase
218	25/07/02	X	X	30	Start of Recovery
232	08/08/02	X		14	Recovery
282	27/08/02	X		19	Recovery
324	08/10/02	X		42	Recovery

Microbial and chemical samples were collected at the same time.

\* Chambers suspended into on-line filter (surrogate for injection well – Figure 3-1), 4m, 75m and 300m bores. (x) indicates sample types taken for each sampling event.

### 3.3 Chamber Design for Microbial Analysis at Adelaide ASR site

Chambers containing 300m background groundwater were suspended into four locations namely the on-line filter (surrogate for injection well), 4m, 50m and 300m bores (Figure 3-3). The chambers used for the study were modified McFeters chambers which were assembled as described by Pavelic et. al., (1998). Each chamber was enclosed within the wooden frame by two 0.45µm pore-size polycarbonate membrane filters (Millipore Type VS). The chambers contained catheter single lumen PVC tubing

(0.58 mm ID) for injecting background groundwater. Liquid flowing silicon glue provided watertight seals along all joints. Each chamber has a diameter of 25 mm, depth of 16 mm, volume of 7.9 ml. Aquifer material was added to some chamber assemblies which filled approximately half the chamber volume.

### **3.4 Groundwater Sampling of Adelaide ASR site**

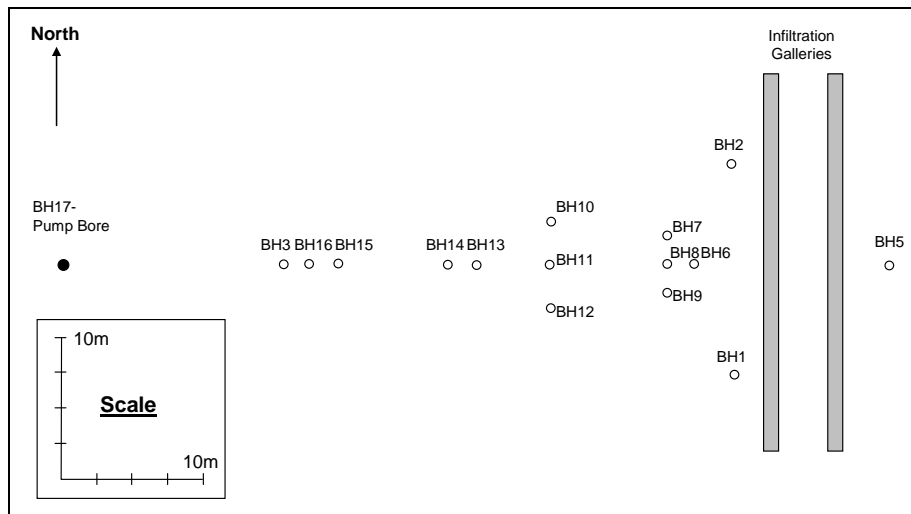
The chambers were filled with native background groundwater (approximately 7.9mL). This groundwater was collected from the 300m well. The background groundwater was collected into a nitrogen-flushed sterile 500mL glass bottle sealed with a silicone septum. This sample was then used to fill the chambers using a syringe flushed with the 300m collected groundwater. The 300m groundwater was transferred to the chambers immediately prior to use at each sampling station. The chambers were lowered and suspended into the on-line filter (surrogate for injection well), 4m, 50m and 300m bores. Each sampling station received one assembly containing nine chambers with aquifer matrix material included and one assembly containing nine chambers without aquifer matrix included. Chambers were sampled on numerous occasions as indicated in Table 3.3. The chambers were brought to the surface on these sampling events and the water removed from the chamber. A syringe flushed with a little of the sample was used to transfer chamber water into nitrogen-flushed sterile 100mL sterile glass bottle sealed with a silicone septum. The chambers containing the aquifer material were shaken to remove as many of the attached microbes as possible. A 24 gauge needle was used so that some of the aquifer material was also collected with the water sample. All samples were packed in a cooled biohazard transportation box and air transported overnight to the CSIRO microbiology laboratory in Perth, WA, where they processed immediately on receipt.

### **3.5 Perth Infiltration Galleries aquifer and site details.**

The MAR site in Perth is located in an unconfined aquifer within 6 to 8 meters of sand of the Spearwood Dune System overlying Tamala Limestone; a lime-sand eolianite (Playford *et al.*, 1976). The water table depth below the site varies depending on the season and location, but is generally between 9.7 and 11m below ground in the Tamala Limestone (Bekele *et al.*, 2006).

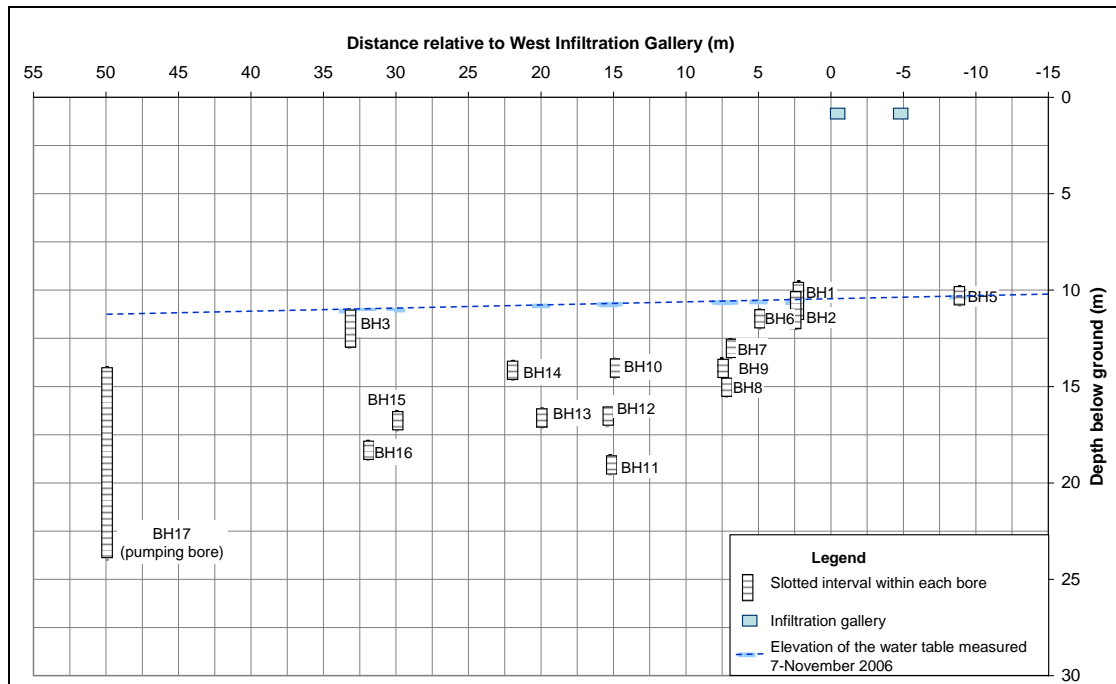
The managed aquifer recharge system consists of two infiltration galleries buried between 0.5 to 1m below ground with the base of the galleries 1m below ground. The infiltration galleries receive a total of 50 KL/day of secondary treated sewage effluent from the Subiaco Wastewater Treatment Plant. The treated effluent percolates through the unsaturated sand into the underlying Tamala Limestone. A forced hydraulic gradient in the Tamala Limestone is imposed by pumping continuously from a bore located a distance of 50m to the west (Bekele *et al.*, 2006; Figure 3-5). The pumping bore extracts 250 KL/day, which maintains a water table gradient of approximately -1.6% to the west (Bekele *et al.*, 2006; Figure 3-6). Thus a greater volume of water is extracted than is put into the aquifer. Within the zone of treatment in the aquifer, extending from the infiltration galleries to the recovery bore, there are 15 monitoring bores with slotted intervals at different depths below the water table. An off-site bore, located 177m northeast and up-gradient from the infiltration galleries, has been monitored for background water quality in the Tamala Limestone (Bekele *et al.*, 2006). The stratigraphic variability of the Perth MAR aquifer site is shown in Figure 3-7.

**Figure 3-5** Map-view of the Floreat MAR showing all sampling stations



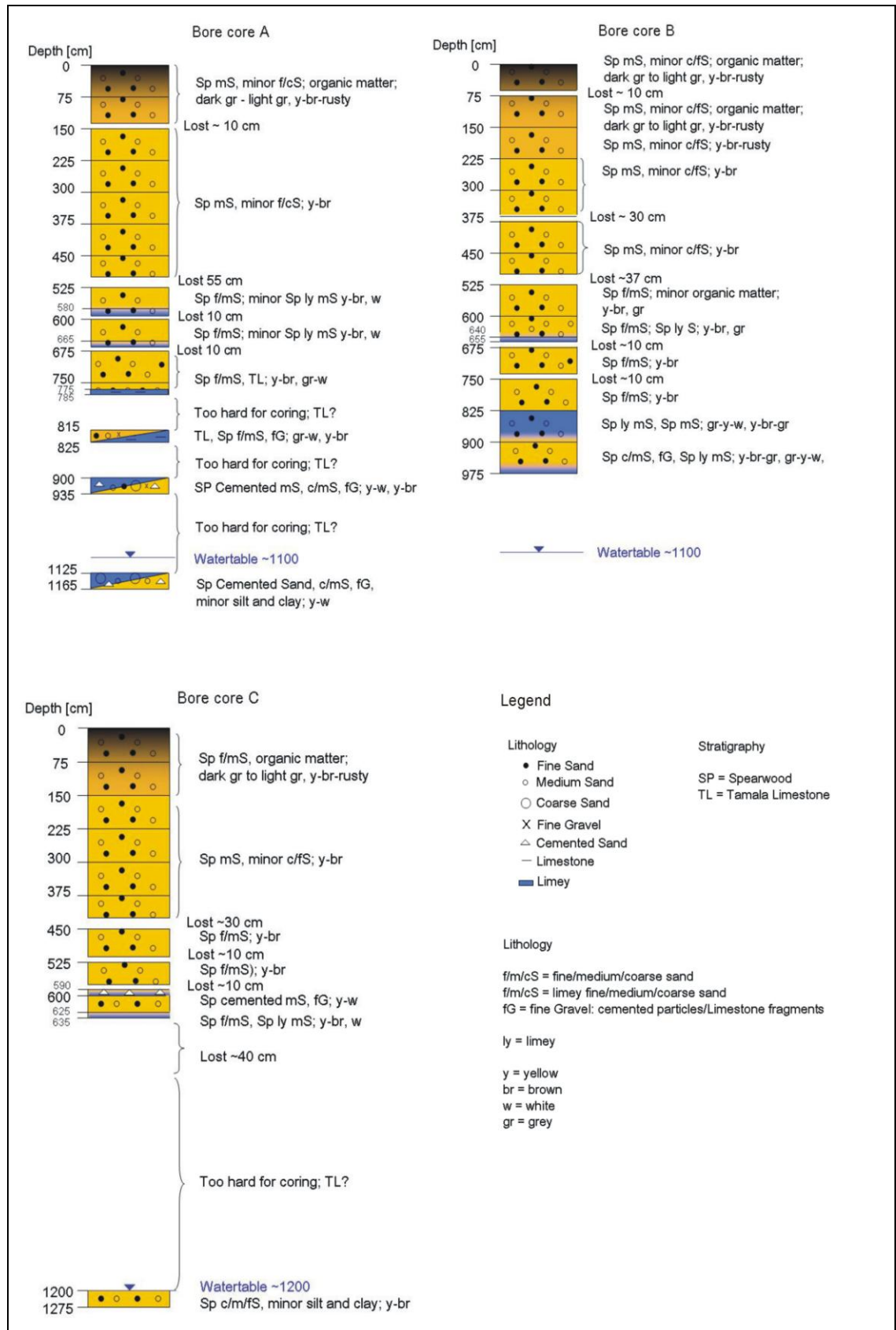
(Bekele *et al.*, 2006).

**Figure 3-6** East-west transect of the field site, showing the location and screened intervals of the monitoring bores and pumping bore



(Bekele *et al.*, 2006).

**Figure 3-7 Stratigraphic variability of Perth MAR aquifer site**





### 3.6 Groundwater Sampling Perth Infiltration Galleries

Water samples were taken using a LF QED pro micropurge pump. All water samples were taken after stabilization of pH, temperature, dissolved oxygen (DO), electrical conductivity (EC) and redox potential (Eh), in accordance with appropriate well purging (approximately three well-volumes) (ASTM International, 1999; ASTM International, 2001). Between sampling events the bladder pump was stripped and cleaned and the bladder replaced. Samples were collected starting from the least contaminated e.g. furthest away from the infiltration gallery progressing to the wells closest to the galleries. Thus sampling progressed towards the infiltration gallery in order to minimise cross contamination between samples. Transport blanks were also used to demonstrate proper QAQC had been undertaken and that contamination of samples did not occur during sampling.

Samples for microbial population studies were collected in 500mL acid-washed autoclaved schott bottles. Bottles were opened immediately before sample collection and were rinsed with the groundwater from the sampling station. Microbial samples for culture analysis were transferred from 500mL schott bottle samples whilst in the field using anaerobic 'field' techniques. A syringe flushed with groundwater from the relevant observation well was used to transfer 50 mL of groundwater sample to a 100mL nitrogen flushed sterile anoxic bottle sealed with a silicone septum. Chemical samples were collected in containers prepared by Chemistry Centre, Western Australia (CCWA). 1L polyethylene bottles were rinsed with the sample prior to collection. 250mL bottles were treated with a preservative. The bottles for water samples which were for chemical analysis that required filtering were not rinsed prior to water being added by passing through 0.45 µm filter. Clear glass vials for TOC/DOC were rinsed with sample before collection. Water samples for both microbial and chemical analysis were immediately placed on ice and in the dark after collection. All samples were maintained below 4°C and transported for analysis within 24 hours. Sampling, preservation and analysis methodology was based on the Standard Methods for the Examination of Water and Wastewater (American public Health Association (APHA) 1998). The methods were performed by the Chemistry Centre of Western Australia.

### 3.7 Chemical Analysis of Perth Infiltration Galleries MAR site

A number of chemical analytes were measured at the Perth MAR site from November 21<sup>st</sup> 2005 to 3<sup>rd</sup> April 2006. The chemical analytes used for the analyses are shown in Table 3.4.

**Table 3.4** Chemical analytes measured at the Perth Infiltration Galleries MAR site

Chemical Measurements	(Field)	Abbrev	Units	Chemical Measurement (Chemistry Centre)	Abrev.	Units
pH		pH		Alkalinity	Alk.	mg/L
Electrical conductivity		EC	ms/m	Chloride	Cl	mg/L
Dissolved Oxygen		DO	mg/L	Dissolved Organic Carbon	DOC	mg/L
Temperature		Temp	°C	Electrical Conductivity	Econd	ms/m
Oxidation_Reduction Potential		ORP	mV	Iron	Fe	mg/L
				Ammonia	N-NH3	mg/L
				Nitrate	N-N03	mg/L
				Total Nitrogen Kjeldahl	N-TK	mg/L
				Soluble Phosphate	P-SR	mg/L
				Sulfate	S04	mg/L
				Total Organic Carbon	TOC	mg/L

Chemical measurements were undertaken as detailed in Bekele, (2006). Chemical analysis was part of a separate research activity within a cohort of related research projects. This thesis analysed the chemical measurements differently to Bekele, 2006 and used the results of chemical measurements specific to microbial ecology only. The methods for chemical analyses were undertaken using standard methods.

**Table 3.5** Calendar Date of Perth Infiltration Gallery activity and sampling with designated ‘Sampling Event number’ used for all graphical analyses

Sampling Event	Calendar Date	Culture analysis	Non-culture analysis	Chemical analysis
	<b>*29/09/05</b>			
2	22/11/05		x	x
13	05/12/05		x	x
32	19/12/05		x	x
33	20/12/05		x	x
68	24/01/06		x	x
69	25/01/06	x	x	x
81	06/02/06	x	x	x
95	20/02/06	x	x	x
110	07/03/06	x	x	x
123	20/03/06	x	x	x
137	03/04/06	x	x	x

Microbial and chemical samples were collected at the same time.

**\* Indicates commencement date for infiltration of treated effluent**

(x) indicates sample types taken for each sampling event.

### 3.8 Microbial Media

#### 3.8.1 Basic Anaerobic Medium for Nitrate-Reducing and Fermentative Bacteria

Components illustrated in Table 3.6 were added to 1L of ddH<sub>2</sub>O and mixed until dissolved. Additionally 1.7 g/L of NaNO<sub>3</sub> (20mM) was added for nitrate-reducing medium. The medium was adjusted to pH 7.0 for both fermentative and nitrate-reducing medium.

Media was heated to near boiling point in a 2L flask and nitrogen gas was purged through the liquid until the media cooled. 10mL tubes were then purged with nitrogen and 10 mL of media subsequently transferred to the tubes using anaerobic techniques. A rubber septum and screw cap was then fixed to maintain anoxic conditions. The tubes were autoclaved at 121°C for 15 min.

**Table 3.6** Basal Anaerobic Medium

Chemical	g/L
NaHCO <sub>3</sub>	2.5
CaCl <sub>2</sub> .H <sub>2</sub> O	0.1
KCl	0.1
NH <sub>4</sub> Cl	1.5
NaH <sub>2</sub> PO <sub>4</sub> .H <sub>2</sub> O	0.6
Sodium Acetate	6.8

(Balch *et al.*, 1979)

### 3.8.2 Vitamin Solution

Reagents detailed in Table 3.7 were added to 1L of ddH<sub>2</sub>O and mixed until dissolved. The vitamin solution was filter-sterilized (0.2 µm) into a sterile 100 mL serum bottles. The solution was gently heated and then purged with nitrogen using a 0.2 µm filter and sterile needle, until anoxic conditions were accomplished. A sterile rubber septum was fitted to maintain anoxic conditions and crimp sealed. The solution was maintained in the dark at 4°C.

**Table 3.7** Vitamin Solution

Chemical	g/L
Biotin	2.0
Folic acid	2.0
Pyridoxine hydrochloride	10.0
Thiamine hydrochloride	5.0
Riboflavin	5.0
Nicotinic Acid	5.0
DL-Calcium Pantothenate	5.0
Vitamin B <sub>12</sub>	0.1
p-aminobenzoic acid	5.0
Lipoic acid	5.0

(Balch *et al.*, 1979)

### 3.8.3 Trace Element Solution

Nitrilotriacetic acid was dissolved with KOH to pH 6.5. Minerals detailed in Table 3.8 were added to a final volume of 1L with ddH<sub>2</sub>O. The solution was mixed thoroughly until dissolved. The trace element solution was filter-sterilized (0.2 µm) into a sterile 100 mL serum bottle. The solution was gently heated and then purged with sterile nitrogen using a 0.2 µm filter and sterile needle, until anoxic conditions were accomplished. A sterile rubber septum was fitted to maintain anoxic conditions and crimp sealed. The solution was maintained in the dark at 4°C

**Table 3.8** Trace Element Solution

Chemical	g/L
Nitrilotriacetic acid	1.5
MgSO <sub>4</sub> ·7H <sub>2</sub> O	3.0
MnSO <sub>4</sub> ·2H <sub>2</sub> O	0.5
NaCl	1.0
FeSO <sub>4</sub> ·7H <sub>2</sub> O	0.1
C <sub>0</sub> Cl <sub>2</sub> or C <sub>0</sub> S <sub>0</sub> <sub>4</sub>	0.1
CaCl <sub>2</sub> ·2H <sub>2</sub> O	0.1
ZnSO <sub>4</sub>	0.1
CuSO <sub>4</sub> ·5H <sub>2</sub> O	0.01
AlK(SO <sub>4</sub> ) <sub>2</sub>	0.01
H <sub>3</sub> BO <sub>3</sub>	0.01
Na <sub>2</sub> M <sub>0</sub> 04.2H <sub>2</sub> O	0.01

(Balch *et al.*, 1979)

### 3.8.4 Lactate/Acetate Solution

Components detailed in Table 3.9 were added to 1L of ddH<sub>2</sub>O and mixed until dissolved. The lactate/acetate solution was transferred to 100 mL serum bottles and gently heated and purged with nitrogen until anoxic conditions were accomplished. A

rubber septum was fitted to maintain anoxic conditions and crimp sealed. The lactate/acetate solution was autoclaved 121°C, 15 min and stored at 4°C.

**Table 3.9** Lactate/Acetate Solution

Chemical	g/L
(70%) Sodium/Lactate Solution	35.0
Sodium/Acetate	25.0

(Atlas, 1996)

### 3.8.5 Carbohydrate solution for Fermentative medium

Components detailed in Table 3.10 were added to 1L of ddH<sub>2</sub>O and mixed until dissolved. Once dissolved the carbohydrate solution was filter sterilised through a 0.2 µm filter into a sterilised 100 mL bottle. The solution was then heated and purged with nitrogen using a 0.2 µm filter using a sterilised needle. A sterilised rubber septum was fitted and crimp sealed to maintain anoxic conditions. The carbohydrate solution was stored at 4°C.

**Table 3.10** Carbohydrate Solution

Chemical	g/L
Glucose	60.0
Pancreatic digest of casein/tryptone	150.0
Bromthymol blue	0.1

(Atlas, 1996)

### 3.8.6 Addition of vitamin, trace elements, lactate/acetate

Vitamin, trace element, lactate/acetate and carbohydrate solutions were added after autoclaving of basal medium just prior to inoculation. Needles and syringes were purged with nitrogen using a 0.2 µm filter for solution transfer. A volume of solution (Table 3.11) was added to 10mL of medium prior to inoculation.

**Table 3.11** Volume of Vitamin, Trace element, Lactate/acetate and Carbohydrate solutions added to basal medium

Volumes (mL) added per 10 mL medium				
Medium	Vitamin solution	Trace element solution	Lactate/acetate solution	Carbohydrate Solution
Nitrate-reducing	0.1	0.1	0.35	n/a
Fermentative	0.1	0.1	n/a	1.0

### 3.8.7 Sulphate-Reducing Medium

Components illustrated in Table 3.12 were added to 1L of ddH<sub>2</sub>O and mixed until dissolved.

Media was heated to near boiling point in a 2L flask and nitrogen gas was purged through the liquid until the media cooled. 10mL tubes were then purged with nitrogen and 10 mL of media subsequently transferred to the tubes using anaerobic techniques. A rubber septum and screw cap was then fixed to maintain anoxic conditions. The tubes were autoclaved at 121°C for 15 min.

**Table 3.12** Sulphate-Reducing Medium

Chemical	g/L
60% Sodium Lactate	4.08
MgSO <sub>4</sub> .7H <sub>2</sub> O	2.0
NH <sub>4</sub> Cl	1.0
CaSO <sub>4</sub>	1.0
Yeast Extract	1.0
KH <sub>2</sub> PO <sub>4</sub>	0.5
FeSO <sub>4</sub> .7H <sub>2</sub> O	0.5
Ascorbic acid	0.1
Thioglycolic Acid	0.1
Resasarin	A few grains

(Atlas, 1996)

### **3.9 Isolation of Single Fermentative Colonies via anaerobic plates for DGGE single colony standards.**

500mL of basic anaerobic medium was prepared using 3% agar in a schott 1L bottle. The medium was heated to near boiling and cooled via nitrogen purging to obtain anoxic conditions and immediately autoclaved. Whilst still hot the medium was transferred to an anaerobic cabinet (COY – maintained under a 90% nitrogen/10% hydrogen atmosphere) with the lid kept loose. As the temperature decreased to approximately 50°C additional solutions were added. Vitamin and trace element solutions at a volume of 5 mL and an additional 50 mL of carbohydrate solution were added to the medium. The media was then thoroughly mixed. The medium was poured onto agar plates and left to solidify in the anaerobic chamber. The agar plates were stored in plastic bags within the anaerobic chamber. Fermenting broth cultures were used to ‘streak’ the agar plates in the anaerobic chamber using standard microbiological methods to obtain single colonies and incubated in the anaerobic chamber. Single colonies were then used to inoculate fermenting broth cultures and incubated at 28°C. The procedure was repeated until single colony broth cultures were obtained.

### **3.10 Sample Processing for both Adelaide ASR and Perth Infiltration Gallery sites for Microbial Population Culturing**

For all selective media 1 mL of collected water sample was inoculated into the various medium in an anaerobic chamber and incubated at 28°C. Cells were harvested when medium displayed substantial growth determined by a visually significant change in medium from a negative control. The cultures were transferred to 15mL sterile centrifuge tubes and spun at 4,500 rpm for 20 min and the supernatant removed leaving 2mL. The 2 mL was resuspended and transferred to a 2 mL tube and centrifuged at 13,000 rpm for 2 mins. The supernatant was removed and the concentrated cells stored at -80°C.

### **3.11 Groundwater concentration for Perth Infiltration Gallery site**

A maximum groundwater volume of 1.2L was filtered through a 0.2µm polycarbonate filter (Millipore). Smaller volumes were filtered if the filter became clogged. The filters were transferred onto autoclaved foil in a biohazard cabinet to reduce contamination



from airborne microbes. All equipment for handling filters were flame sterilised or autoclaved. Additionally the biohazard cabinet underwent ultra violet sterilization between samples to reduce cross contamination. The filters were chopped into tiny sections (approximately 5mm x 5mm) with sterile scalpels and placed into 2mL beadbeating tubes and stored at -80°C for further processing. A transport control was also incorporated into this process to ensure field samples were not contaminated from field techniques and that the procedure for microbial collection onto filters did not introduce contamination.

### **3.12 Molecular Analysis of Population Dynamics**

#### *3.12.1 DNA extraction from Groundwater Microorganisms in Filter Concentrates*

Zirconia beads were placed into a McCarthey bottle with foil overlaid and baked at 180°C for at least 48 hours in order to sterilise beads and degrade DNAase and RNAse enzymes. DNA was obtained using a QIAamp Stool Mini Kit (Qiagen) using the provided method with modifications as follows. A volume of 1.4 mL ASL buffer (QIAamp stool mini kit) and 0.5 µl of zirconia/silica beads (0.5 mm diameters; Biospec) was added to the tubes containing the polycarbonate filter used to concentrate microorganisms from groundwater samples. The filter and ASL buffer was incubated at 95°C for 5 min to lyse and/or partially weaken bacterial cell walls. The beadbeating tubes were immediately placed in ice for 1 min followed by beadbeating in a MINI-BEADBEATER™ (Biospec) set at homogenize rpm for 3 min and placed on ice for 1 min. The supernatant was then removed. An additional volume of 1.4 mL ASL buffer was added to the polycarbonate filter to maximize the recovery of cells from the filter and the process repeated. The second volume of supernatant was added to the first to produce a combined supernatant of approximately 2.5 mL. An inhibitex tablet (QIAamp stool mini kit) was then added and fully homogenised via vortexing. The inhibitex tablet was added to remove PCR inhibitors. The solution was then spun at 13,000 rpm for 6 min. All the supernatant was then used for subsequent steps which followed the manufacturer instructions. The volumes of reagents described in the manufacturer instructions were proportionately increased to coincide with the increased volume of sample which resulted from using the combined supernatant of 2.5mL. The concentration of DNA was then estimated in each sample using a NanoDrop ND-1000 Spectrophotometer (BIOLAB) and stored at -80°C until further processing.

### 3.12.2 DNA extraction of Concentrated Groundwater Mixed Cultures

A QIAamp Stool Mini Kit was also used and the protocol followed according to that described for the concentrates (3.12.1) except only one volume of 1.4 mL ASL buffer was used to resuspend concentrated cells from the cultures. The extra volume used for concentrates (3.12.1) was primarily used to maximize recovery of cells attached to the filter. In contrast with the manufacturers instruction all supernatant was always recovered and the volumes of reagents recommended were proportionately increased. The QIAamp Stool Mini Kit is optimised by the manufacturer for recovery of DNA from stools which are anticipated to contain higher levels of microbial numbers than expected for groundwater samples. Thus all supernatant was always recovered to maximise the amount of DNA recovered from groundwater samples.

### 3.12.3 PCR and DGGE profiling

Each reaction mixture contained approximately 50-100 ng of DNA, 0.4 µl of bacterial forward primer V3-F-GC (20µM) and 0.4µl of 907-R (20µM) reverse bacterial primer (Muyzer et al., 1993), 25µl of BioRad iQ<sup>TM</sup> supermix and autoclaved ddH<sub>2</sub>O to a total volume of 50µl. Cycling was provided by a PTC-200 (MJ Research) thermal cycler. A PCR cycle with the following cycle program was used: An initial melting cycle at 94°C for 5 min was used to activate DNA polymerase, 63°C annealing for 1 min and 72°C elongation for 2 min followed by 18 cycles of 94°C for 1 min, 63°C for 1 min and 72°C for 2min. The annealing temperature was decreased 1°C every other cycle. Nine cycles were then performed using an extension temperature of 53°C; and 10 min at 72°C for final elongation.

DGGE was performed using the Bio-Rad D-Code<sup>TM</sup> System as described previously (Muyzer *et al.*, 1993). To separate bacterial PCR fragments, 30-70% linear DNA-denaturing gradients were used for Perth infiltration gallery samples and 40-60% linear DNA-denaturing gradients were used for Adelaide ASR samples. The percentage of DNA-denaturing gradient used was optimised for each of the two sites in order to maximise DNA band diversity over a range of samples. This optimisation occurred in conjunction with an objective to recover clear DNA band separation with minimisation of DNA smearing across the DGGE gel. Working denaturing solutions were created

using ddH<sub>2</sub>O where 100% denaturant is equivalent to 7mol/L urea and 40% deionized formamide. An overlay of 0% denaturing solution was used for good structural well formation. The DNA-denaturing gradients were formed in 8% polyacrylamide gels for Perth SAT samples and 6% polyacrylamide gels for Adelaide ASR samples using a Bio-Rad Gradient delivery system (model 475). Gels were polymerized using 12.8µl of Tetramethylethylenediamine (Sigma-Aldrich) and 128µl of 1% Ammonium persulfate (Sigma-Aldrich) in 15mL volume denaturing solution.

TO run a DGGE gel the full 50µl of PCR product for each sample was mixed with 8µl of 6x gel loading buffer (Amresco) and loaded into individual lanes. In addition each DGGE gel contained two 50µl PCR bacterial reference ladders from 'in-house' groundwater sample strains which were obtained from isolating single fermentative bacteria. A further non-PCR reference ladder was used namely a 100bp ladder (K180-amresco). This ladder was diluted 1:2 with 6x gel loading buffer (amersco). A volume of 25 µl was added to three lanes of each DGGE gel. DGGE was performed at 200 V for 4 hrs at 60°C in 1x TAE buffer. Gels were stained for at least 1 hour in 250mL of 1X TAE buffer with ethidium bromide (0.5mg/L) and visualized by UV illumination (254 nm), and the gel images were acquired by using the transilluminator MultiImage™ Light cabinet (Alpha Innotech Corporation).

### 3.13 DGGE Gel analysis

DGGE Tiff files were processed by GelCompar II, version 3.5 (Applied Maths). An overall standard was created from the 'in house' culture standards and the 100bp ladder standards from a representative DGGE gel. All gels were subsequently normalised in accordance with this generated overall standard as stringent standards are required for statistical analyses of DGGE gels (Fromin, et al., 2002). Optimisation of 4% was used for automatic band assignment, a value which visually appeared to detect most bands. Manual editing was undertaken on bands to remove incorrectly assigned bands and editing was also used to assign any bands missed by the program. Band matching between samples was also set at 4%. Optimisation of band matching was determined by comparison of the DGGE standards over a range of gels so that gel to gel comparisons could be reliably evaluated as shown in the results. A binary matrix table was generated for presence and absence of bands (band class type) belonging to defined band groups at 5% tolerance level and exported into an Excel spreadsheet.

### 3.14 Statistical Analyses for Microbial and Chemical Data

A statistical methodology flow chart is shown in Figure 3-8. This chart demonstrates the tactical flow of methods used to analyse data. Section 3.14 is an overview of the multivariate statistical methods used. A full description of each statistical method is detailed in Section 3.15.

#### 3.14.1 *Microbial Analyses*

The microbial DGGE binary matrix table was imported into PRIMER6 and PERMANOVA+ statistical package and a Bray Curtis similarity matrix created. The similarity matrix was used for multidimensional scaling (MDS), permanova and principal coordinate analyses (PCO). The permanova experimental design included distance and sampling events as independent fixed factors and an interaction of distance and sampling event as a variable factor. A permanova overall significance table was generated based on this experimental design. Additionally pair-wise comparisons used to generate the overall significance table were produced by the PRIMER6 and PERMANOVA+ program. These tests indicated the significance of these pair-wise comparisons tests which contributed to the overall significance table. Pair-wise tests were generated from each of the experimental designs used to generate the overall significance table, namely distance and sampling events as independent fixed factors and an interaction of distance and sampling event as a variable factor.

A further reduced Bray Curtis similarity matrix was also created from the original Bray Curtis similarity matrix using PCO centroid analysis based on distance.

An ANOSYM was also used to substantiate results from the permanova for testing significance differences in microbial community structures between observation wells at different distances from the infiltration gallery and over time.

Bubble plots of individual band class types (Perth MAR) were also examined individually by overlaying these variables for matched sampling events onto the microbial MDS to visually evaluate microbial band class types which contributed to the MDS spatial arrangements. .

### 3.14.2 Chemical Analyses

Chemical data was also transferred into PRIMER6 and PERMANOVA+ from Excel where data was normalised and a principal component analysis (PCA) undertaken. Excel line graphs were also undertaken on individual chemical parameters. Correlation analysis between chemical parameters was undertaken by PRIMER6 and PERMANOVA+ program.

### 3.14.3 Combined Microbial and Chemical Analyses

Bubble plots of individual normalised chemical parameters (Adelaide ASR) and non-normalised concentrations (Perth MAR) were also examined individually by overlaying these variables for matched sampling events onto the microbial MDS to visually evaluate microbial and chemical interactions.

## **3.15 Detailed Summary of Statistical Tests used, Explained in Relation to a Microbial Population Study.**

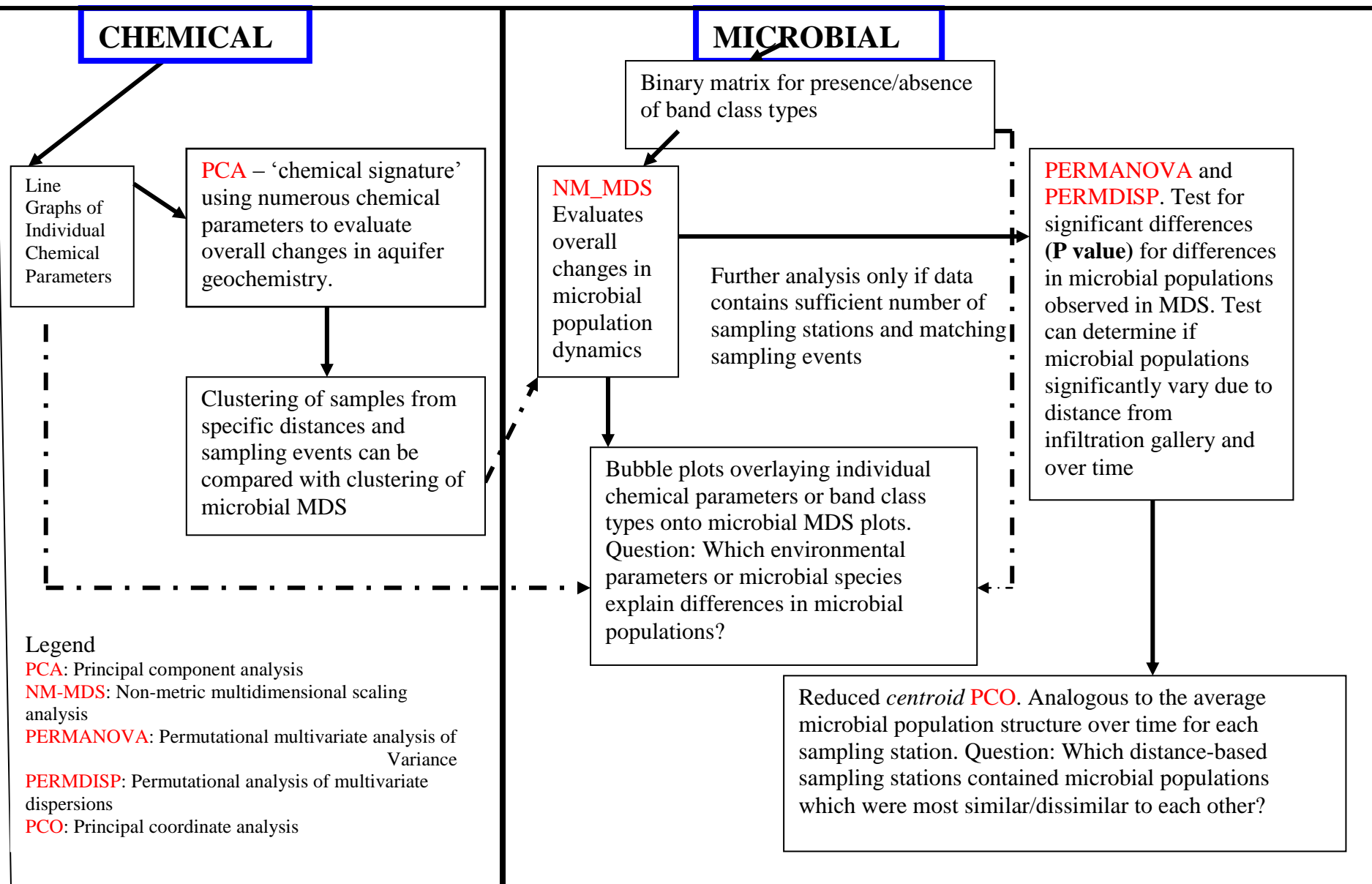
The summaries provided below are detailed to provide in depth details on multivariate statistical methods. These methods are explained in relation to this study and can be used throughout the following results and discussion. Section 3.15 is predominately aimed at providing assistance for interpretation of results that can be used as a reference during the results and discussion sections. Please also refer to: Ramette, 2007 for a detailed description for the use and interpretation of multivariate analyses in microbial ecology.

### 3.15.1 Principal Component Analysis (PCA) versus Multidimensional Scaling (MDS)

It is statistically more correct to use a principal component analysis (PCA) for analysing environmental parameters whereas it is statistically more correct to use an MDS for analysing biological parameters. A PCA uses Euclidean distance to measure the variability in environmental parameters for each distance over time. In contrast a MDS uses Bray Curtis to determine a similarity matrix which measures the variability between biological components e.g. presence/absence of band class types between different distances and over time within the groundwater.



**Figure 3-8 Statistical Methodology Flow Chart for Microbial and Chemical Analyses for Biogeochemical Evaluation of Groundwater**



### 3.15.2 Interpretation of Principal Component Analysis (PCA)

Many measured individual chemical parameters are used for the PCA which measures changes in the overall chemical signature at each distance over time. Unlike the MDS plot PCA separates sample variation along an axis which explains a proportion of the total variation in a negative and positive direction. The individual chemical parameters used to determine the PCA can be overlaid onto the PCA graph as variable vector lines. A strong positive association to a chemical parameter from a cluster of samples results in a long line pointing towards the cluster. Thus a negative correlation results in a long line pointing away from the cluster of samples. Short lines do not have strong positive or negative associations e.g. the evidence for all samples having low or high concentrations of a particular chemical variable is not as strong as shown for long lines. Thus shorter lines tend to indicate variable results. In summary samples are placed according to their overall chemical signature based on all chemical variables analysed over time for each distance. The chemical vector lines relate only to individual chemical parameters which are associated to clusters of samples based on their overall chemical signature.

### 3.15.3 Interpretation of Multidimensional Scaling (MDS)

A MDS plot, which can also be called non-metric multidimensional scaling (NM-MDS) is a distance based interpretation of similarity between samples. The MDS separates the samples via distance similarity e.g. samples placed in close proximity are more similar to each other than samples placed further away. The stress of the MDS plot is important: approximately 0.2 equates to high stress and thus interpretation of data has to be cautious e.g. positioning of samples on the MDS plot may have been random, thus occurring by chance which do not reflect reliable grouping of samples. But using a substantial amount of data can cause high stress values. By undertaking the MDS several times and obtaining similar grouping patterns indicates that the grouping structure is fairly consistent. Further analyses by PERMANOVA and PCO can also help to determine if the interpretation of the MDS is correct.



#### 3.15.4 Permutational Multivariate Analysis of Variance (PERMANOVA)

PERMANOVA (Anderson, 2001) can be used to substantiate interpretations of the MDS plots. It is able to provide evidence that clustering of samples on the MDS plot represents statistically different populations between different sampling events or distances. The PERMANOVA test provides a pseudo F statistic with a significance value. There are different types of PERMANOVA. Type 3 PERMANOVA is more robust than Type 1 in that it has to adhere to greater rigidity with regards to similar sampling events for each sampling station over time. Type 3 is normally a prerequisite for journal publications. In some way PERMANOVA is analogous to an ANOVA however, PERMANOVA allows for more sophistication in experiment design and hypothesis testing with fewer statistical assumptions around the primary data.

#### 3.15.5 Manipulation of Research Data

##### **(a) Perth Infiltration Gallery Data**

During the course of the total sampling period (sampling events 2-137) various sampling stations at various distances and depths were sampled over time. The sampling strategy hoped to track and intersect the migrating plume within the subsurface. Additionally it was hoped that sampling stations would intersect any possible fissures and preferential flow paths between the infiltration gallery and extraction bore.

From hydraulic modelling (Bekele, 2007) the relative migration of groundwater was calculated and therefore a sampling strategy was based on this modelled groundwater migration. As the plume migrated samples were taken at greater distance from the infiltration site in order to capture plume migration over time. Therefore samples were taken in close proximity to the infiltration gallery at the beginning of the sampling regime. As time progressed with expected migration of the plume more samples at greater distance were sampled over time. Therefore each sampling station was not consistently sampled at each sampling event as this would have created unrealistic workloads for efficient processing of samples with available resources. Samples were strategically taken based on the outcomes of hydraulic modelling.

DNA was not recovered from all non-culture samples collected. Due to the nature of environmental science it was not always possible to obtain DNA from every sample. It is virtually impossible to optimise conditions for every sample when environmental conditions can vary greatly over distance and time in their chemical and microbial make-up. This is particularly an issue when nutrient rich treated effluent infiltrates groundwater which is nutrient poor. The quality of groundwater changed in response to the migrating plume. Therefore conditions were constantly changing at different sampling stations. Although there were varying conditions from nutrient rich (infiltration gallery) to nutrient poor (background) it was important to use the same DNA extraction method for all samples. Consistency in methodology is important so that variations in microbial and chemical populations could be contributed to microbial and chemical differences rather than experimental variation.

As a consequence of the distance-based sampling strategy over time and random missing data it was not possible to obtain an identical number of sampling events for each distance/sampling station over time. The Type 1 PERMANOVA is able to manage missing information but the results should be viewed with caution. Due to its flexibility the test is not considered particularly robust and is used primarily as an initial insight into data behaviour. In an attempt to obtain a Type 3 PERMANOVA which is considered more robust some sampling stations were removed and sampling events were amalgamated so that there could be greater matching of sampling events for each sampling station over time. The chemical data PCA analysis shown in graphs 3 (a&b) 4 (a&b) were used to determine how to amalgamate sampling events. Initial chemical changes were shown to occur sometime between sampling event 33-68. Microbial sampling periods were thus split into three time periods consisting of:

Time period 01, (22<sup>nd</sup> November to 19<sup>th</sup> December - day 2 – 33)

Time period 02, (24<sup>th</sup> January to 20<sup>th</sup> February - day 68 – 95)

Time period 03, (7<sup>th</sup> March to 3<sup>rd</sup> April -day 100 – 137)

The PERMANOVA test provides greater detail than the basic overall differences in microbial populations for distance and time. The PERMANOVA is also able to undertake pairwise tests on the experimental design variables on which the PERMANOVA was based which included time and distance as independent fixed factors and an interaction of time and distance as a variable factor. These pairwise tests

provide insight into the possible individual variables which cause the significant differences in the PERMANOVA test. The pairwise test provides a pseudo F statistic with a significance value.

#### **(b) Perth Infiltration Gallery Data**

Only four sampling stations were analysed and not all cultures grew for each sampling event. As a result there was insufficient data for comparative statistical tests via PERMANOVA. Thus MDS plots were primarily used to interpret differences between microbial populations. PERMANOVA could not be undertaken and therefore a P value could not be obtained to test the significance of any visual differences in MDS microbial populations.

#### *3.15.6 Permutational Analysis of Multivariate Dispersions (PERMDISP)*

PERMDISP is a distance-based test for homogeneity of multivariate dispersions. This test was used on the microbial data for individual time points (sampling event) and distance (distance from the infiltration galleries) based on the Jaccard similarity matrix to understand any potential dispersions affects in the data. PERMDISP was undertaken as it is understood that the PERMANOVA analysis is sensitive to differences in dispersion among groups. Therefore when significant values are obtained for PERMANOVA tests the null hypothesis of no differences in microbial populations due to distance and time can only be rejected on the basis of (i) location (ii) relative dispersion or (iii) both. To put this into context with this project: if the PERMANOVA test is positive this indicates that the different distances and sampling events of the experiment caused (i) a shift in microbial assemblage or (ii) the assemblages became more or less variable or (iii) the significant PERMANOVA result was due to shifting microbial populations but in addition the assemblages also become more or less variable during the experiment.

Importantly, if the overall PERMDISP and PERMANOVA are significant and the significant pairwise comparisons between the PERMANOVA and PERMIDSP do not match then the null hypothesis can be rejected in that the microbial populations are not similar over distance and time as a consequence of location (PERMANOVA) and dispersion (PERMDISP). This information therefore suggests that the different

distances and sampling days resulted in shifting microbial populations which were also heterogeneously dispersed.

Similar to the PERMANOVA, the Adelaide ASR site provided insufficient data for PERMDISP analyses to be undertaken.

### 3.15.7 Analysis of Similarity (ANOSIM)

ANOSIM is an analysis of similarities which was undertaken on all samples over distance and time. This test was undertaken as a precautionary step to substantiate results obtained from the PERMANOVA. The PERMANOVA is more flexible and has greater complexity for experimental design than ANOSIM and thus not so robust in its statistical assumptions. The experimental design of the ANOSIM used sampling day as Factor 1 and Distance as Factor 2 as it is unable to test for interactions. PERMANOVA was used as it can also test for an interaction of distance and time having an affect on microbial populations. It is also good practice to compare similar methods which project similar outcomes especially as in this instance the outcomes are mathematically determined in different ways.

### 3.15.8 Principal Coordinate Analysis (PCO) using centroid output on Microbial Data

Unlike the MDS plot PCO separates sample variation along an axis which explains a proportion of the total variation in a negative and positive direction. The centroid is analogous to the mean/median but refers to the centre of points in three dimensional space. This method was used as the MDS plots for microbial data showed high stress  $>0.2$  and samples from 2.5m to 32m were not clearly distinct forming one cluster in the middle of the MDS plot. Despite not forming distinct groups in the MDS, the PERMANOVA indicated that microbial populations were different between different distances and varied over time. Thus a PCO was used to reduce the overall variation at each distance by averaging the variation at each sampling station over time. Distance, NOT time was used as the variable for averaging microbial diversity as distance was determined as the main driving force for microbial variation as indicated by PERMANOVA where distance had the greatest 'F' value. The condensed information of centroid PCO provides clearer insight into the relationship between microbial communities between the different sampling stations e.g. different distances. The PCO plot is thus created from this centroid information giving a single position for a

particular distance along the PCO axes. In this way it could be assessed whether the microbial populations affected by plume migration were more similar to each other than microbial populations unaffected by plume migration. Consequently the relationship between samples based on distance becomes clearer and therefore provides an insight into which distance-based samples were most similar. This approach could therefore potentially determine differences between samples that the MDS had difficulty in determining due to the variation for each sampling station over time.

### **3.16 Methodology for Presentation of Results and Discussion for Adelaide ASR and Perth MAR geochemical and microbial results**

The results and discussion for the Adelaide ASR and Perth infiltration sites are separated into two sections due to the differences between the two MAR schemes. Examples of contrasts between the Adelaide verses the Perth MAR sites include: tertiary verses secondary treated effluent, confined verses unconfined aquifer, deep verses shallow aquifer, brackish verses fresh native groundwater, chamber verses non-chamber samples, sediment verses non-sediment samples. The MAR schemes also markedly differed with Adelaide using injection and storage phases with recovery from the same well verses infiltration and extraction from a different well in Perth. The Perth ASR also extracted five times the volume of water compared with the volume of secondary treated effluent which penetrated the aquifer. In contrast there was very little difference between the volumes of tertiary treated effluent which was injected into the aquifer compared with the volume of groundwater extracted. Additional contrasts include the number of sampling stations and frequency of sampling events that differed between the two sites which resulted in dissimilar multivariate statistical analyses used. Thus due to the differences in complexity between the results and discussion between the two MAR sites they are detailed in two separate sections. A summary of the differences between the Adelaide and Perth sites are shown in Tables 3-13, 3-14 and 3-15

Each section for Adelaide ASR and Perth infiltration sites are then further divided into a chemical and a microbial section with the results and discussion for each detailed separately. The geochemical results and discussion are first detailed to understand the impact of the migrating plume over distance and time. The microbial population structure results over distance and time are then presented. The change in microbial

populations over distance and time are then discussed with reference to the established spatial and temporal changes in aquifer geochemistry. Thus the sequential flow of results and discussion from aquifer geochemistry to microbial populations fully evaluates potential interactions between aquifer geochemistry and microbial population dynamics for each site. As the information of aquifer biogeochemistry from MAR develops through the analyses at each site notable comparisons are detailed between the sites.

**Table 3.13** Differences in Aquifer properties between the two sites studied

<b>Perth</b>	<b>Adelaide</b>
Shallow	Deep
Sand overlaying limestone	Limestone
Aerobic	Anaerobic
Unconfined	Confined

**Table 3.14** Differences in the two MAR techniques adopted between the two sites studied

<b>Perth</b>	<b>Adelaide</b>
Infiltration to groundwater using infiltration galleries	Injection into confined aquifer
Extraction well at distance from infiltration site	Extraction occurs from injection well
Extract 5x infiltration rate	Injection and extraction volumes are similar
Many observation bores at varying distances and depths to capture plum	Fewer observation bores were analysed which were similar in depth
Continuous infiltration	Injection phase, storage phase and recovery phase

**Table 3.15** Average geochemical parameters of wastewater, recovered water and background groundwater for the two MAR sites

MAR Site	Water Type	Eh mV	EC mS/m	DO mg/L	Cl mg/L	DOC mg/L	Fe mg/L	N_N0 3 mg/L	S04 mg/ L	TDS mg/ L
Floreath	Wastewater <sup>1</sup>	437	144	3	279	11	0	2.5	64	757
	Rec Water <sup>2</sup>	170	116	4	188	5	3	0.7	60	681
	Groundwater <sup>3</sup>	348	102	2	161	3	0	0.05	73	650
Bolivar	Wastewater <sup>1</sup>	853	3592	6.0	415	20	0.1	2.5	181	2006
	Rec Water <sup>2</sup>	73	2470	0.02	594	11	0.7	0.01	216	1267
	Groundwater <sup>3</sup>	86	2265	0.3	1024	1	1	0.05	301	1470

<sup>1</sup> Waster water used for recharge: tertiary treated for ASR and secondary treated for infiltration galleries; <sup>2</sup> Recovered Water; <sup>3</sup> ambient groundwater collected from background bores outside the influence of the MAR schemes.

## **SECTION 4 – Adelaide ASR RESULTS and DISCUSSION**

The Adelaide ASR site based in South Australia consisted of a deep confined brackish carbonate aquifer that was injected with 114.3 ML of tertiary treated effluent during the 5<sup>th</sup> March 2002 to 27<sup>th</sup> June 2002. A one month storage period then occurred between 28<sup>th</sup> June 2002 to 24<sup>th</sup> July 2002. Recovery of 90.9 ML of water took place during 25<sup>th</sup> June 2002 to 8<sup>th</sup> October 2002. Chambers were inoculated with background groundwater to determine changes in microbial populations in response to MAR. Four observation wells were monitored over distance and time, namely the injection well at 0m, the 4m well (19450), the 50m well (19181) and the background well at 300m distance from the injection well.

Statistics used to evaluate aquifer biogeochemistry include: principal component analysis (PCA) to determine overall changes in aquifer geochemistry. Multidimensional scaling (MDS) and principal coordinate analysis (PCO) are used to determine the overall changes in microbial population structures which consisted of culture analysis only. Bubble plots of individual chemical parameters are overlaid onto the microbial culture MDS plots in order to evaluate biogeochemical interactions. PERMANOVA and PERMDISP could not be used due to insufficient observation wells monitored and matching sampling events between observation wells.

The format of the Adelaide section consists of the: aquifer geochemical results followed by a discussion. The microbial results are then analysed which are subsequently discussed in conjunction with the outcomes of aquifer geochemistry. Table 4.1 details the observation well specifics which can be used as a reference for geochemical and microbial Figures. The exact dates of the sampling events detailed in all geochemical and microbial Figures are detailed in Table 3.3.

**Table 4.1** Sample information to be used for all MICROBIAL and GEOCHEMICAL graphical legends. Distance refers to distance from the injection well.

Distance from Infiltration Galleries (m)		Sample ID		Depth below ground (m)
300	=	19035	Background Bore	170
0	=	Injection	Injection Well	170
4	=	19450		170
50	=	19181		140

**Sampling events which are used for graphical legends refers to days from beginning of sampling regime detailed in Table 3.3**

## 4.1 Adelaide ASR Geochemical RESULTS and DISCUSSION

### 4.1.1 Adelaide ASR Geochemical Results

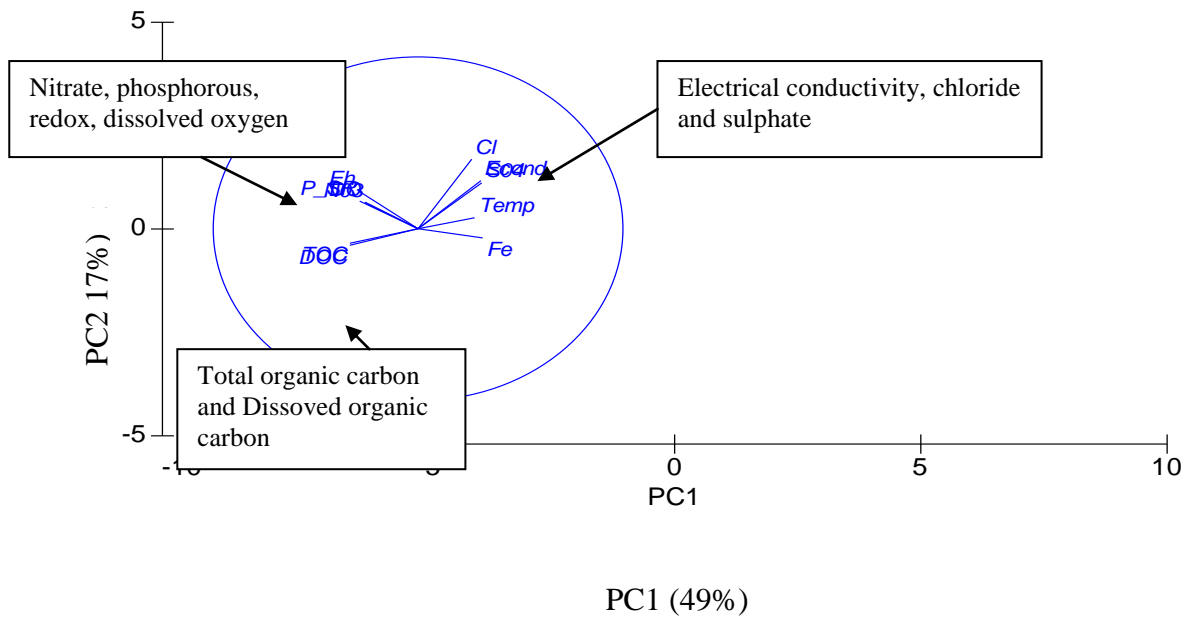
In order to assess the large amount of chemical data over time for all samples (Appendix 20) a Principal Component Analysis (PCA) was undertaken. The data from all chemical analytes for all samples from 22<sup>nd</sup> January 2002 to 8<sup>th</sup> October 2002 were normalised so that data could be compared statistically on a 'like-with-like' basis and the PCA calculate using Euclidean distance.

Figure 4-1 shows the chemicals (variable vectors) for PCA plot Figure 4-3. The variable vectors are shown separately here to reduce PCO plot congestion. Figure 4-2 highlights the interpretation of PCA plot Figure 4-3 in the form of a chemical gradient. The suggested nutrient gradient is shown here to again reduce PCO plot congestion.

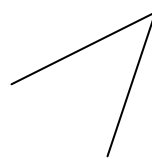


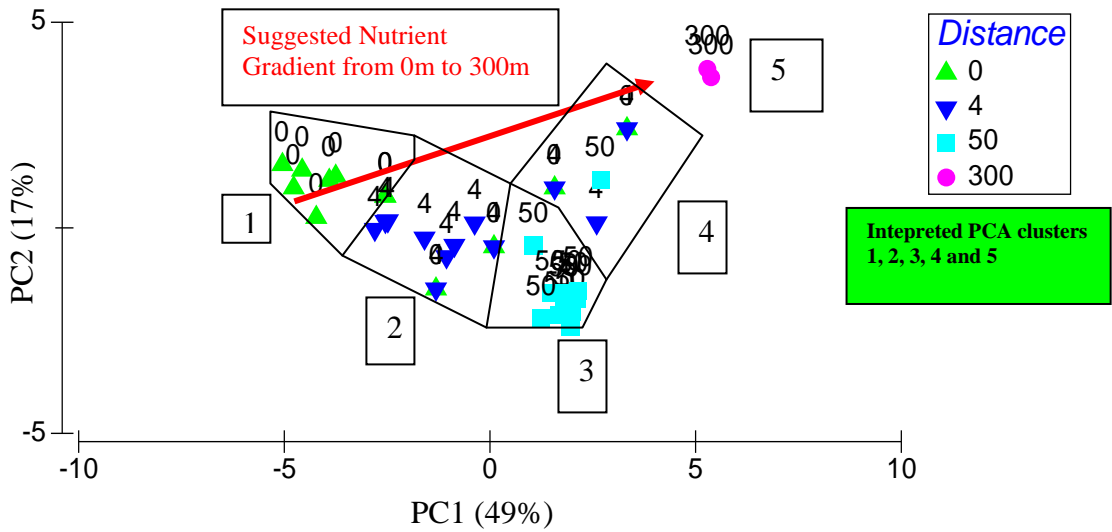
The suggested nutrient gradient formed five distinct clusters from the point of injection at 0m to the background samples at 300m. As can be clearly shown from Figure 4-2 the samples initially cluster at 0m (cluster 1) followed by 4m (cluster 2) and 50m (cluster 3). Between 50m and 300m there are a group of samples (cluster 4) that are from various sampling stations namely 0, 4 and 50m. Finally the suggested nutrient gradient terminates at the background sample which formed cluster 5.

**Figure 4-1** Chemicals (variable vectors) for PCA Plot of all Chemical Data (22nd January to 8th October 2002) from Water Samples

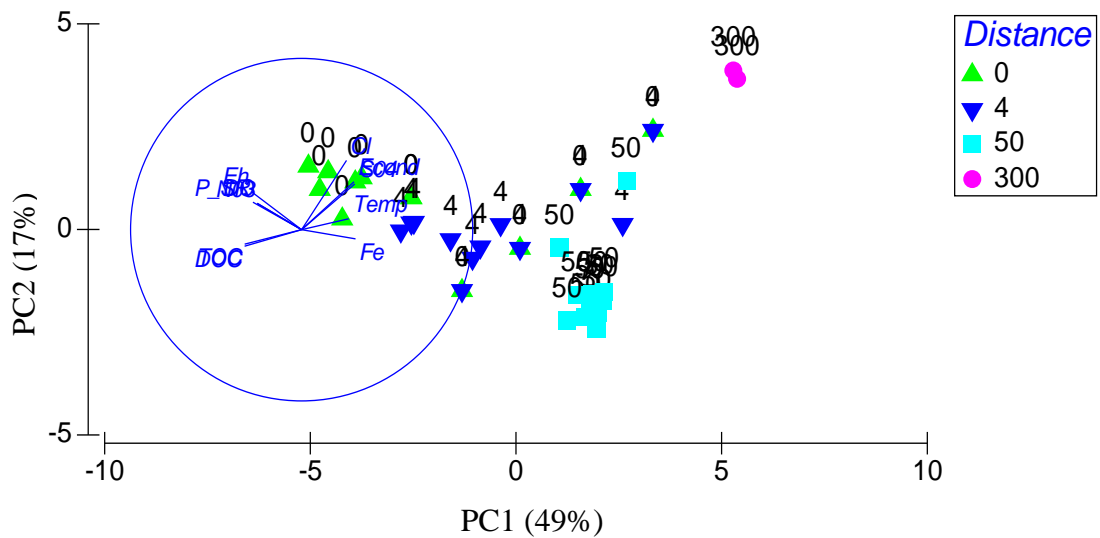


**Figure 4-2** PCA Plot of all Chemical Data (22nd January to 8th October 2002) from Injection, 4m, 50m and 300m Water Samples





**Figure 4-3** PCA Plot of all Chemical Data (22nd January to 8th October 2002) from the Injection, 4m, 50m and 300m Water Samples as shown in Figure 4.1 with additional overlaid chemical variables



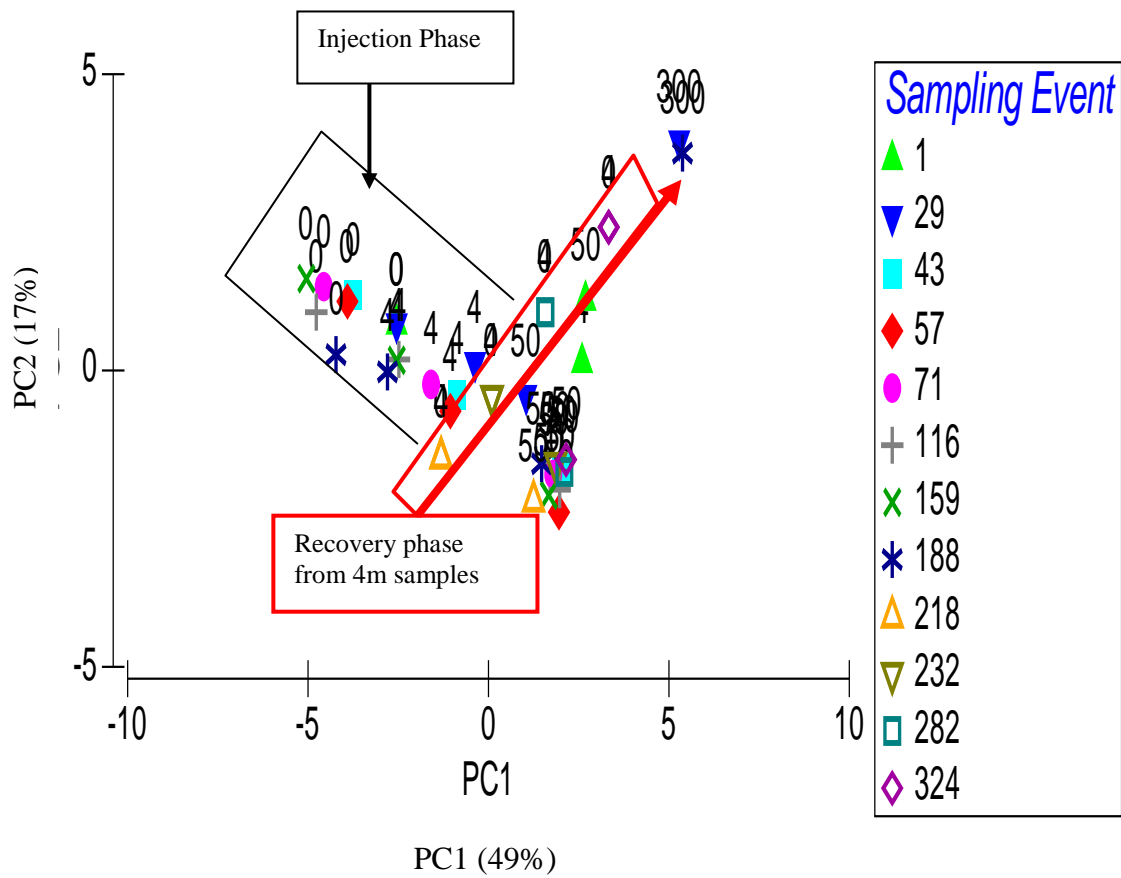
The interpretation of PCA plot Figure 4-3 should be used in conjunction with

Figure 4-1 and Figure 4-2 which are identical except each of the Figures emphasise different but related points. The chemical vectors for DOC and TOC shown in Figure 4-3 are pointing in the opposite direction to the direction of samples. However the order of samples placed closest to this variable vector are the injection well, followed by the 4m, 50m and finally 300m wells. These results therefore indicate that the injection well contains the highest concentrations of DOC and TOC whereas the background well at 300m contains the lowest concentrations of DOC and TOC. PCA clustering based on distance thus illustrates the potential nutrient gradient shown in Figure 4-2. This nutrient gradient is also time dependent as illustrated in Figure 4-4 which clearly separates the recovery phase injection and 4m well recovery samples from day 218.

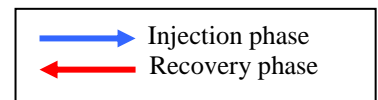
Electrical conductivity, chloride and sulphate were all highly correlated and very positively associated with injection samples. The analysis suggests that although concentrations of these chemicals were less for all other samples the concentrations did increase over time at 4m distance. The increase in these chemicals at 4m can clearly be shown in Figure 4-4 where 4m samples at day 116, 159 & 188 migrated towards the injection well samples. Nitrate, phosphorous, redox and dissolved oxygen were also all highly correlated. The analysis suggests that the injection well samples contained higher concentrations of these chemical analytes as they were positioned closest to these chemical vectors. In particular the 50m samples are placed in the most opposite direction of these chemical vectors suggesting that these samples were not greatly impacted by these chemical analytes over the course of the sampling period. In contrast the variation for 4m well samples as already discussed in conjunction with TOC and DOC are mostly affected at day 116, 159 and 188 from these chemical vectors. The sampling events are shown in Figure 4-4.

Figure 4-4 clearly shows a notable change for the recovery phase from 0 and 4m samples from sampling event 218 to 324. The plot demonstrates that these samples begin to more closely resemble the background samples over time when compared with 4m samples during the injection phase.

**Figure 4-4** PCA Plot of all Chemical Data (22nd January to 8th October 2002) from Injection, 4m, 50m and 300m Water Samples detailing the Injection and Recovery Phases



**Figure 4-5** PCA Plot of all Chemical Data (22nd January to 8th October 2002) from 4m Water Samples



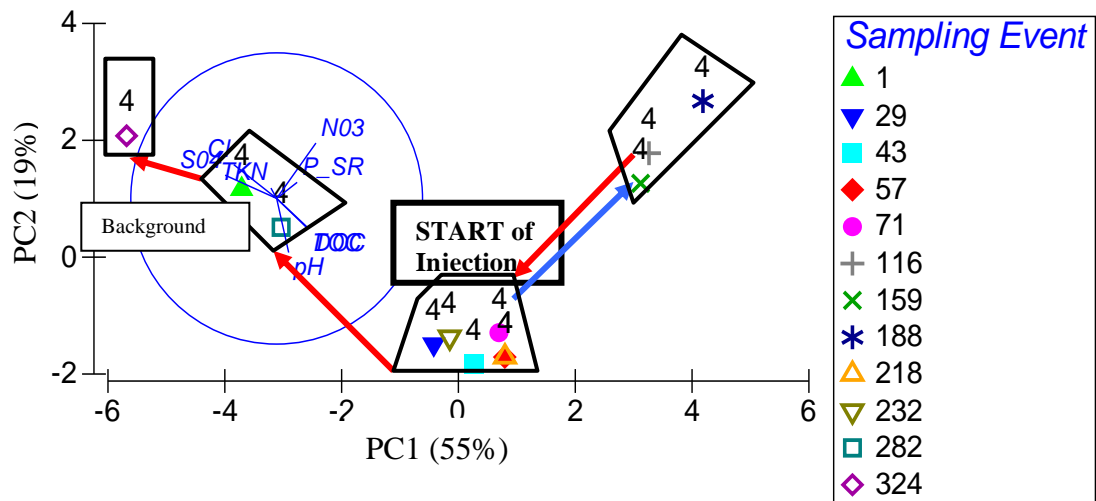


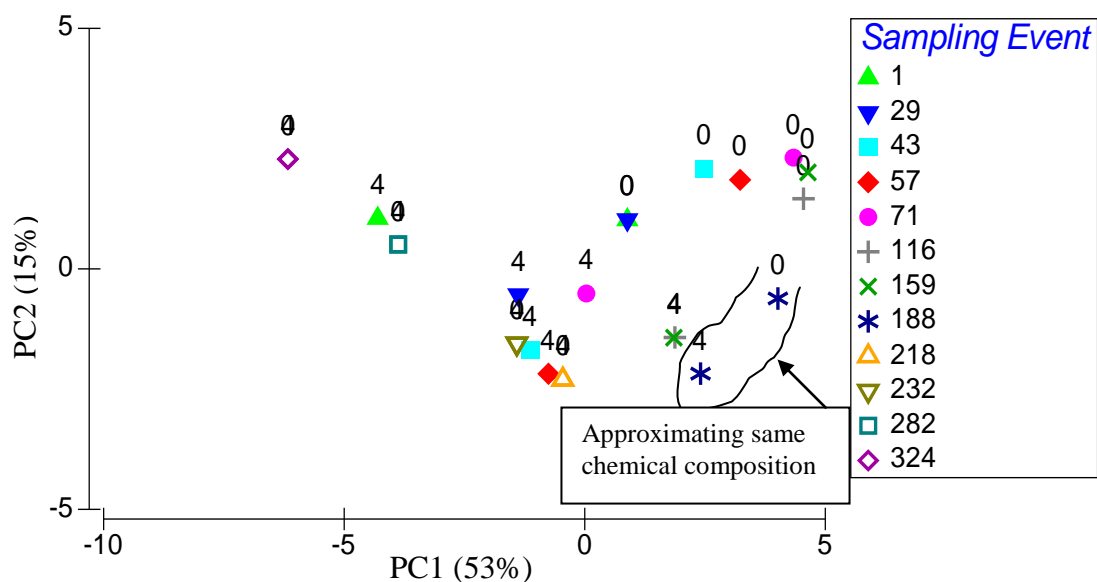
Figure 4-5 demonstrates the chemical change over time at the 4m well. The background reading is at sampling event 1. Injection begins and the chemical population rapidly changes from cluster 1 and remains stable from day 29 to day 71 forming cluster 2. After sampling event 71 the chemical population again changes and subsequently remains stable from sampling events 116 to 188. After the storage phase the chemical population returns to the cluster 2 position from sampling events 218 to 232 forming cluster 4. After sampling event 232 the chemical population returns to cluster 1 as at sampling event 282 forming cluster 5. Thus at this stage of recovery the chemical population has returned to its original starting position. The chemical population then changes again forming a final cluster which is very negatively associated with TOC and DOC but very positively associated with chloride, TKN and sulphate. The scale of temporal variation is quite large and ranges from 0 to +6 for PC1 during the injection cycle.

Figure 4-6 demonstrates that at day 188 (the starting phase for storage) the 4m and injection well approximated the equivalent chemical composition. Figure 4-7 shows the injection well data only. From day 188 it was impossible to obtain data from this injection source as recovery of recycled wastewater was obtained from the injection well. But as demonstrated in graph 50, the injection well and 4m well approximated the same composition from day 188 therefore from day 188, 4m well data was used. Figure 4-7 shows that there was chemical variation over time and that groundwater chemistry eventually returned to its original background status during the course of recovery. Interestingly pH appears to be associated with samples from day 218 to 282. The contrast in variation was quite large ranging from -2 to +4 for PC1.

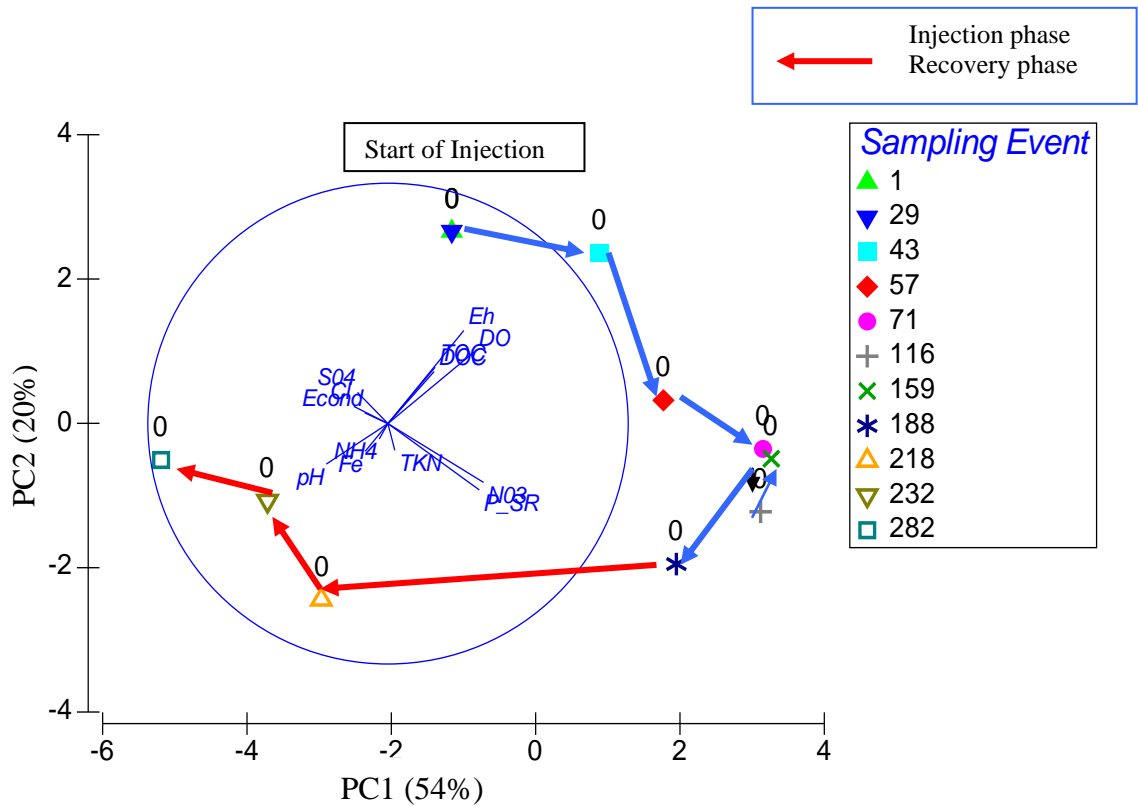
Figure 4-8 shows that the chemical population at 50m is very dynamic over time. The chemical variation over time during injection forms many clusters during the injection and recovery cycle. Despite the dynamic temporal variation the overall variation is quite small in that from the beginning of injection the variation for PC1 ranges from -1 to +2. In addition the groundwater chemistry did not return to its original starting position. In addition ammonia is positively linked to samples at sampling events: 71, 116 and 159 which appears to define this cluster.

Figure 4-9 (a-k) clearly show the temporal fluctuations in dissolved organic carbon, sulphate, nitrate, dissolved oxygen, redox, ammonia, total iron, phosphorous, temperature, chloride and pH for the injection, 4m, 50m and background bore at 300m over time. Time begins with the background reading for each distance at sampling event 1. Injection begins at sampling event 29 and the start of storage is at sampling event 188 and finally the start of recovery is at day 218.

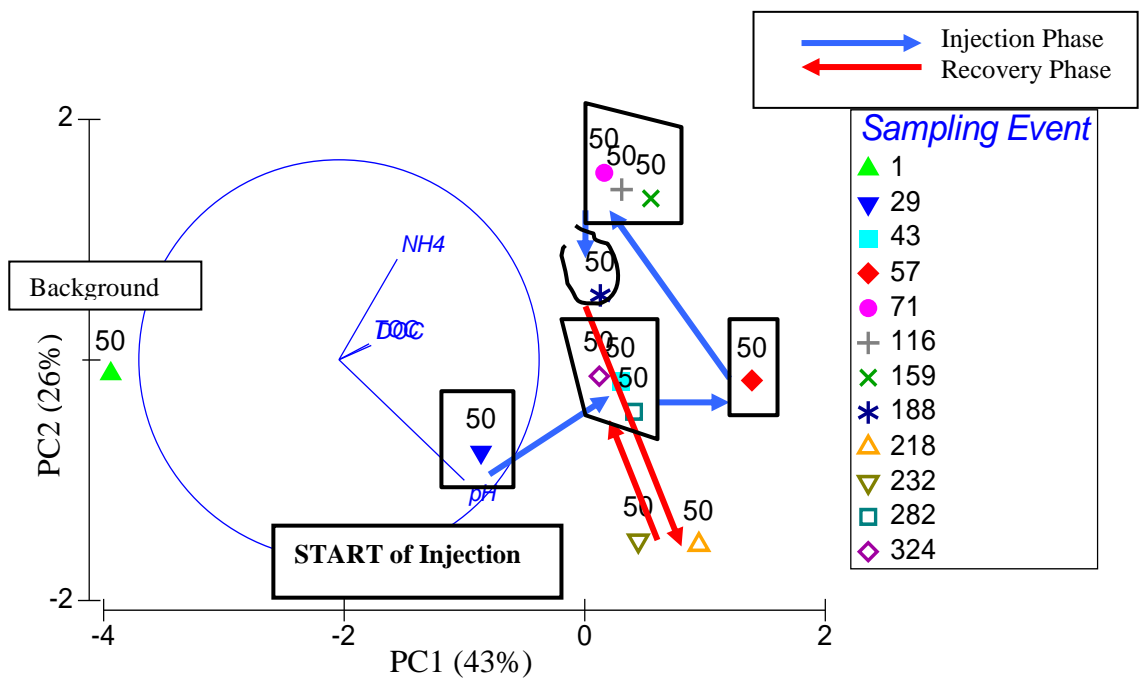
**Figure 4-6** PCA Plot of all Chemical Data (22nd January to 8th October 2002) from Injection and 4m Water Samples



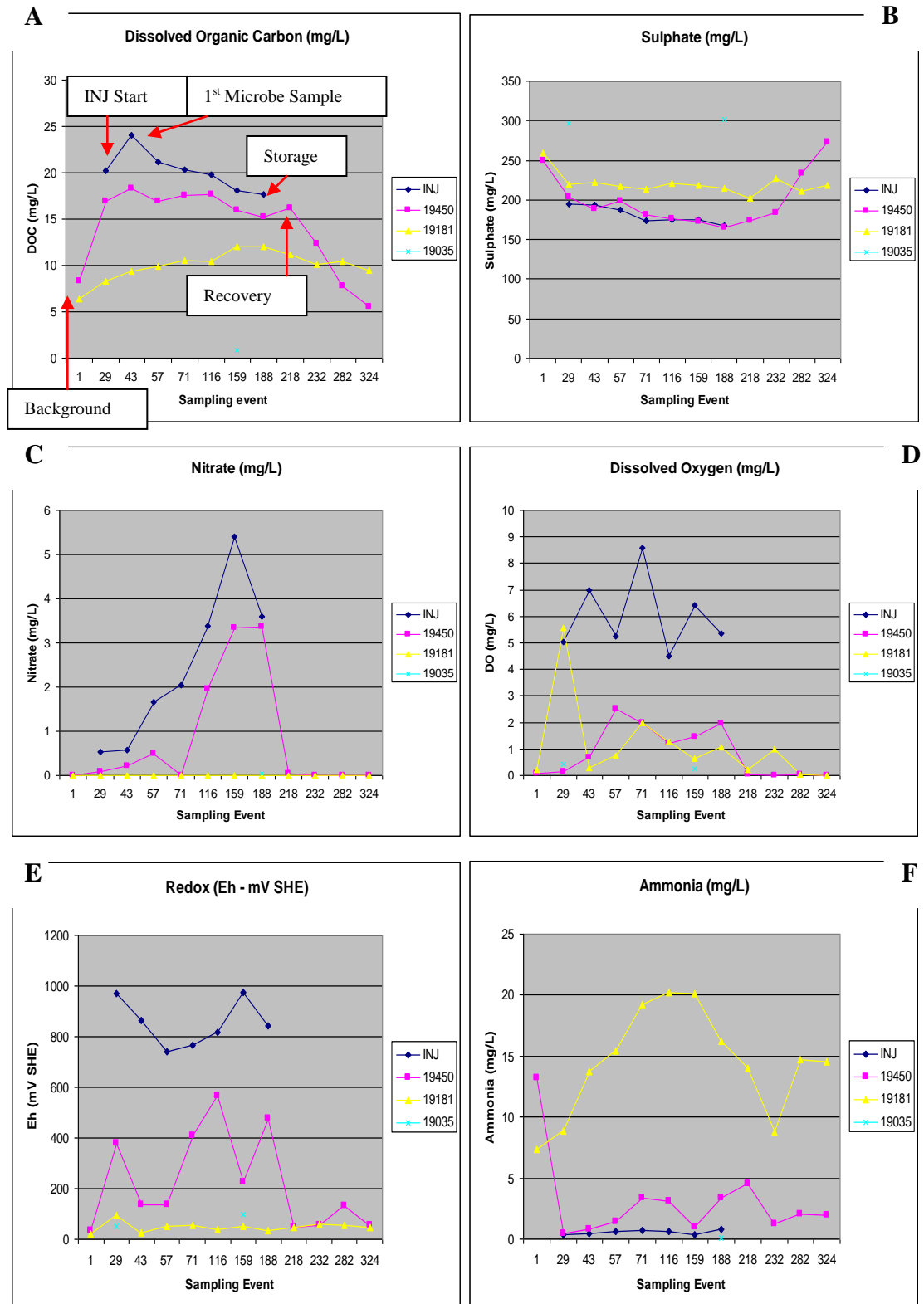
**Figure 4-7** PCA Plot of all Chemical Data (22nd January to 8th October 2002) from Injection Water Samples



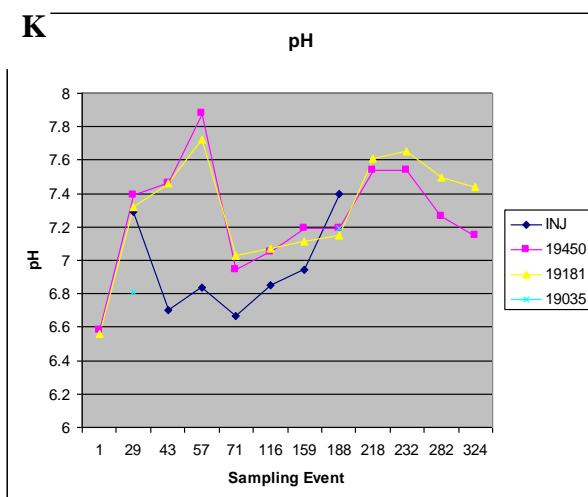
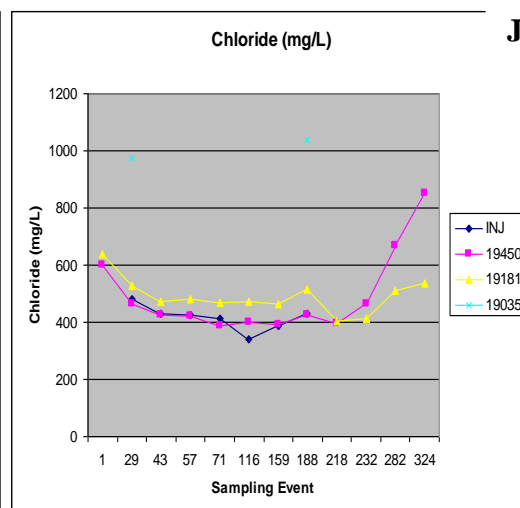
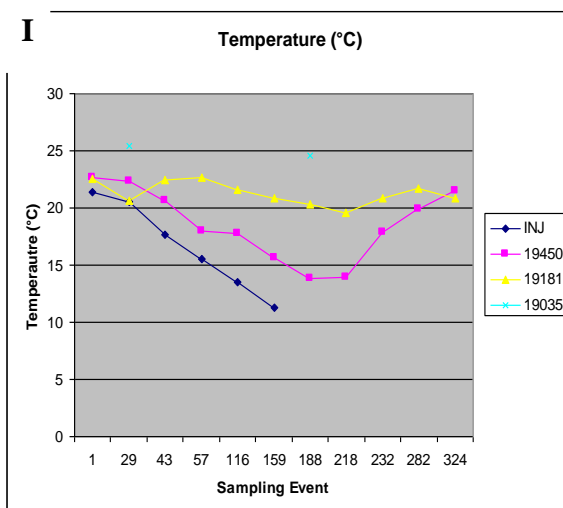
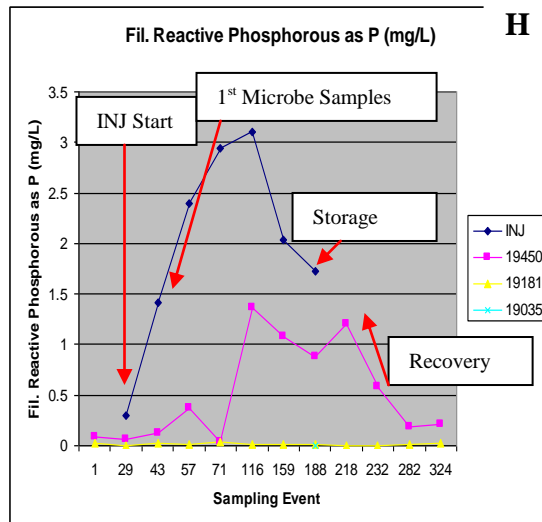
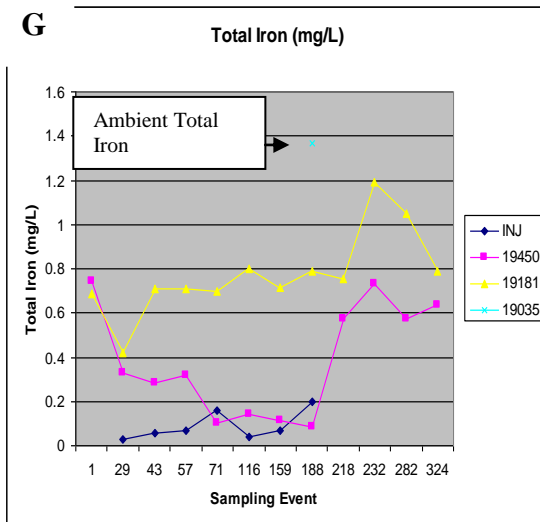
**Figure 4-8** PCA Plot of all Chemical Data (22nd January to 8th October 2002) from from 50m Water Samples



**Figure 4-9** Time course line graphs for chemicals used to calculate PCA plots for (a) dissolved organic carbon, (b) sulphate, (c) nitrate, (d) dissolved oxygen, (e) redox, (f) ammonia, (g) total iron, (h) phosphorous, (i) temperature, (j) chloride and (k) pH.







INJ = Injection well  
 19450 = 4m  
 19181 = 50m  
 19035 = 300m

#### 4.1.2 Adelaide ASR Geochemical Discussion

The results show that a chemical gradient was created by injection of treated effluent from the injection well (Figure 4-2) which was distance based. Figure 4-3 showed that the greater the distance from the injection well the more negative the association to total and dissolved organic carbon. Although there was a chemical gradient, the 4m well was similar to aquifer geochemistry at the injection well, although the two distances were distinct (Figure 4-2). The chemical similarity between the injection and 4m well suggests that the travel time for migration of injectant to 4m distance was relatively short. By sampling event 188 (the last day of injection) there was complete breakthrough of injectant at 4m as the injection and 4m well chemistry replicated each other (Figure 4-6). Additionally recovered water from the injection and 4m well returned to ambient groundwater conditions (Figure 4-4) during the recovery cycle. Therefore there was a significant change in groundwater chemistry between the injection and storage phase at the injection and 4m well.

The 50m well overall variation in chemical signature was less than that described for the injection and 4m well as determined by the tight cluster shown in Figure 4-4. The difference between the extent of overall change in chemical signature can be clearly seen when PCA plots for individual distances for the 50m well (Figure 4-8), injection well (Figure 4-7) and 4m well (Figure 4-5) are compared. The 4m well samples ranged from -4 to +6 for PC1 and -2 to +4 for the injection well whereas the 50m well PC1 only ranged from -1 to +2 from start of injection. Although the overall chemical variation was less at 50m distance the groundwater chemistry was more dynamic over time as determined by the number of clusters that developed from start of injection (Figure 4-8). The greater variation may be attributed to the greater lag phase for plume migration to this distance. Thus the 50m well was less impacted by the injectant over time. The overall difference in chemical signature variation was therefore less. In contrast there were greater instances of dynamic and slight changes in groundwater chemistry at 50m distance compared with the chemistry at the injection and 4m well.

**The smaller overall chemical signature variation at 50m may be attributed to declining concentrations of nitrate and phosphorous during plume migration (**

Figure 4-9 a-k). The 50m well was also different to the injection and 4m well in that the chemical population did not return to its original background chemical composition (Figure 4-4). In addition ammonia was very positively associated to a cluster of three

samples towards the end of injection namely sampling events 71, 116 and 159 (Figure 4-8). The positive association with ammonia for these dates suggests that an increase in ammonia is associated with plume migration.

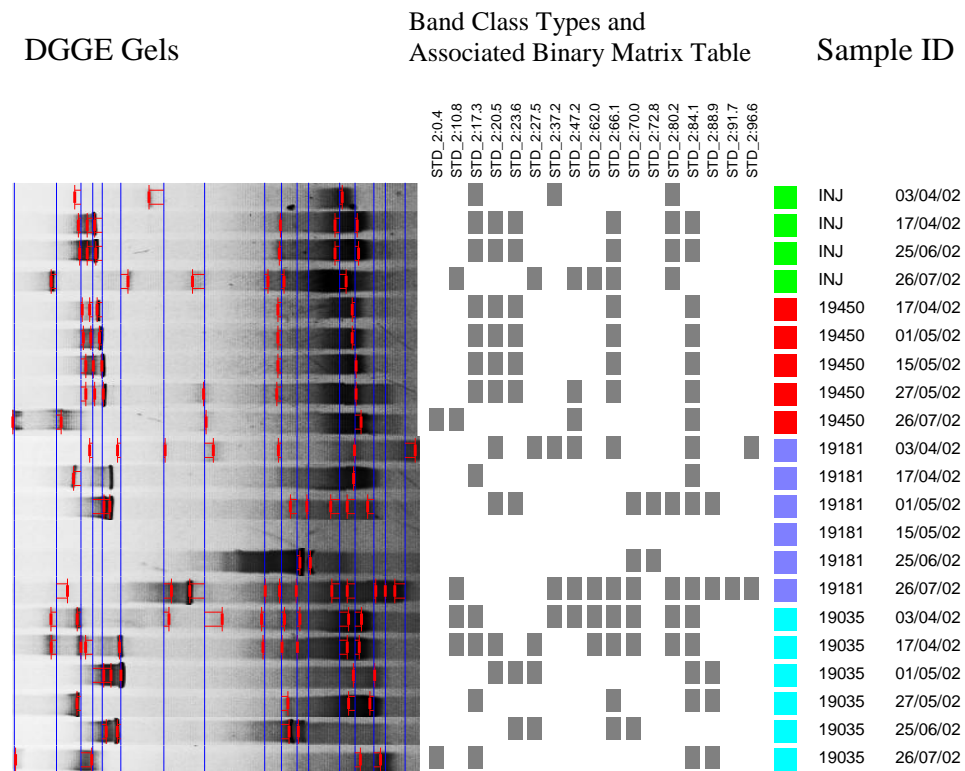
## 4.2 Adelaide ASR Fermentative Culture RESULTS for Water-only and Water-Sediment Samples

### 4.2.1 Adelaide ASR Microbial Results for Fermentative Cultures 'Water-Only' Samples

Figure 4-10 shows the DGGE DNA/PCR banding patterns for all fermentative cultures from Adelaide 'water-only' samples over time. This Figure illustrates that there were seventeen band class types ranging from (STD\_2):0.4 to (STD\_2):96.6. The majority of the samples containing these band class types did not vary greatly in band intensity between samples. A MDS was undertaken on the binary matrix data to ascertain similarities and differences in fermentative bacterial populations for 'water-only' samples. Figure 4-11 (a) indicates that there is some separation of microbial community structure into two distinct clusters illustrated as population groups i and ii. Although the MDS plot is separated into two clusters the analysis is unable to determine distinct fermentative bacterial populations between distances. Thus there does not appear to be distinct fermentative bacterial populations for 'water-only' samples between the injection, 4m, 50m and 300m wells. Although the fermentative community structures for 'water-only' samples are not distinct between distances the fermentative bacteria at the injection and 4m well are clearly different between the injection and recovery phase. Individual chemical parameters the concentrations of which were 'normalised' were overlayed onto the MDS plot (3.14.2). Chemical parameters between the injection and recovery phase at the injection and 4m well markedly change as shown in Figure 4-11 (b,c,d,e,f,g,h,i) where arrows indicate whether chemical concentrations decreased or increased between the injection and 4m well.

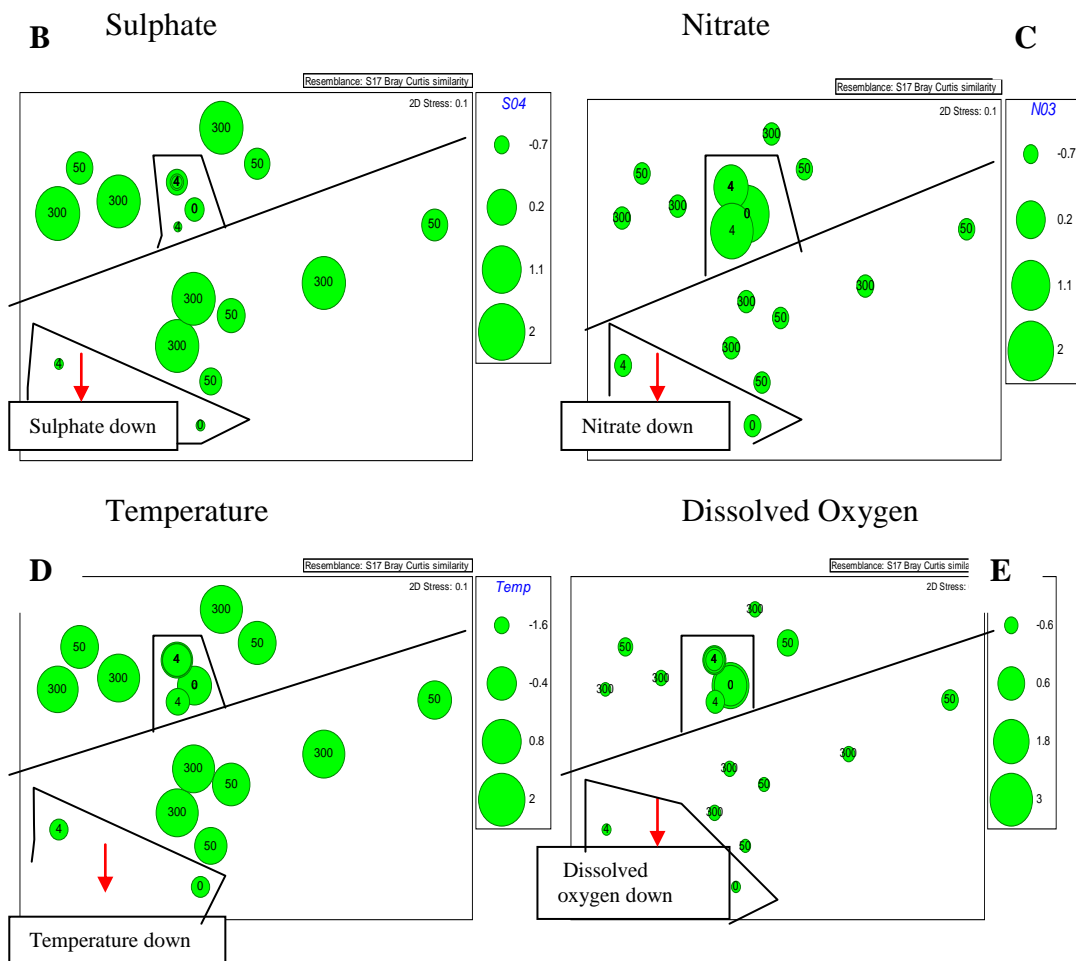
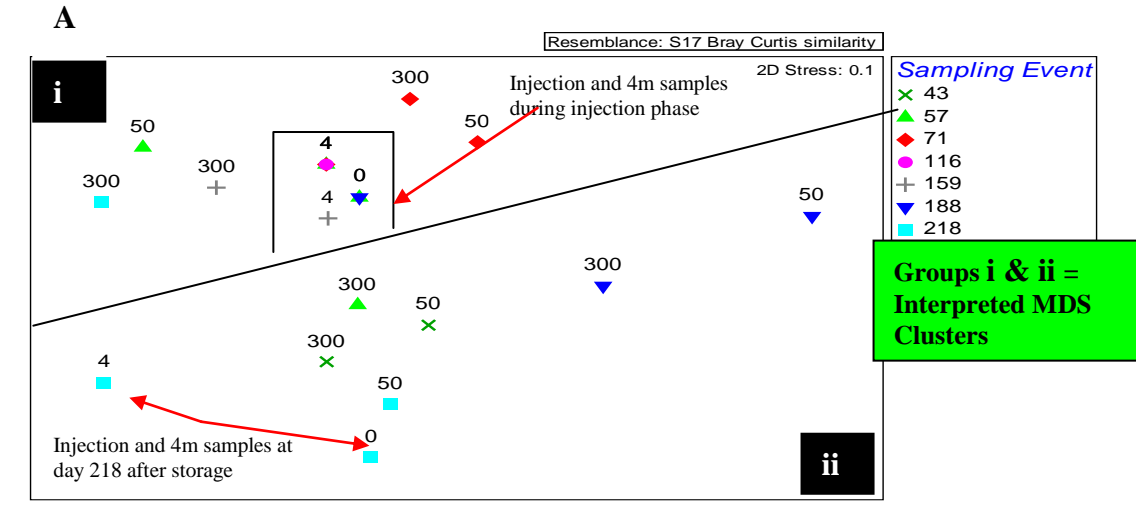
Figure 4-12 (a) clearly shows the stability in fermentative bacterial population structure at the injection and 4m well during the injection cycle. A notable shift in fermentative population structure occurs between the injection and recovery phase. The results from Figure 4-12 (b) in contrast show how diverse fermentative bacterial populations from the 50m and background well are over time.

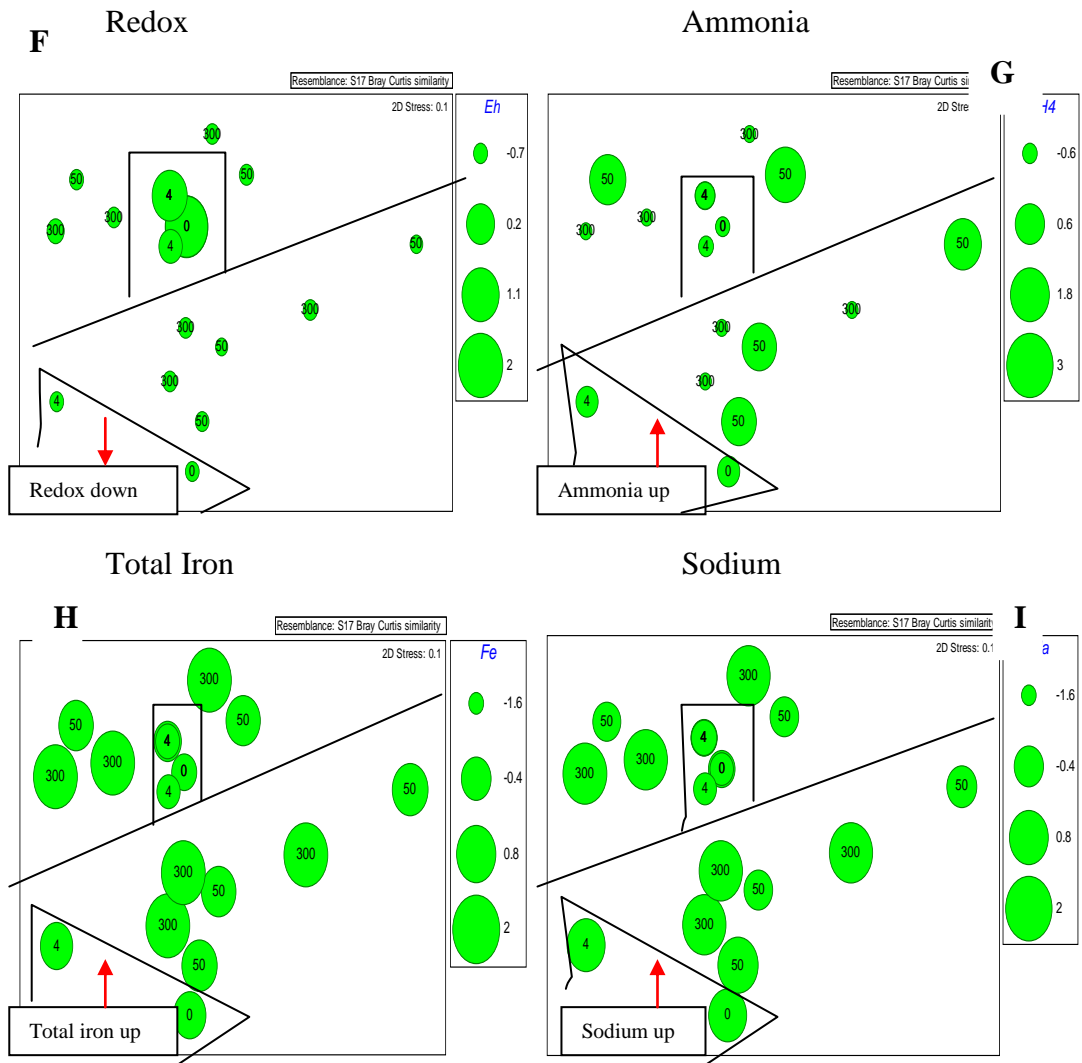
**Figure 4-10** DGGE of bacterial rDNA/PCR banding patterns and associated binary matrix table for band matching data for all fermentative cultures from Adelaide ASR ‘water-only’ samples over time





**Figure 4-11** 2D MDS Plot for amplified Microbial rDNA/DGGE banding patterns for (a) all Fermentative Cultures (water-only) over time with overlay of bubble plot for (b) sulphate, (c) nitrate, (d) temperature, (e) redox, (f) ammonia, (g) total iron and (h) sodium

Injection, 4m, 50m and 300m fermentative 'water-only' bacterial populations

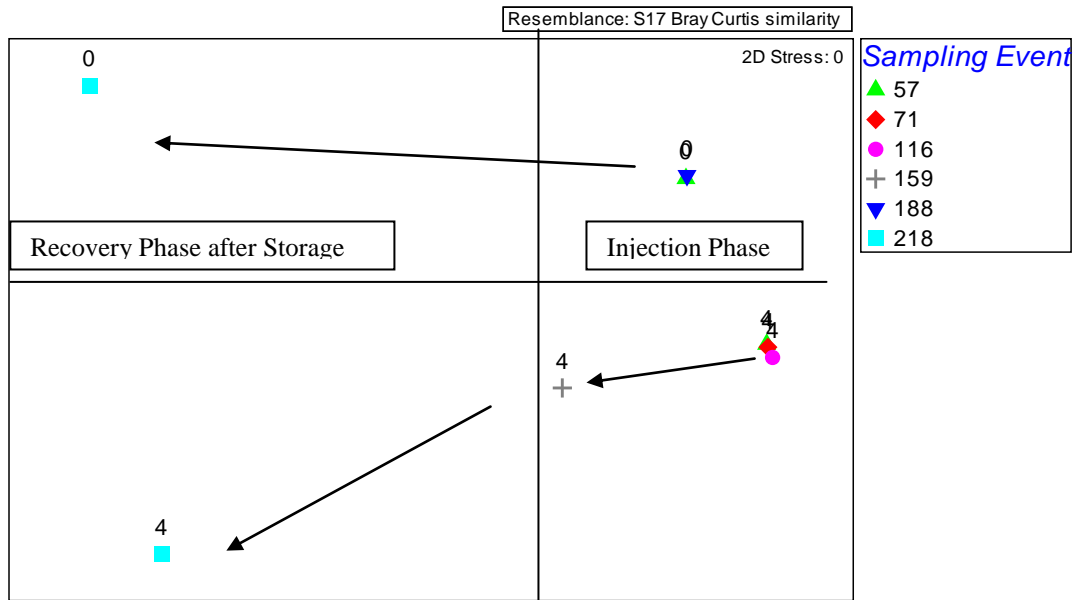




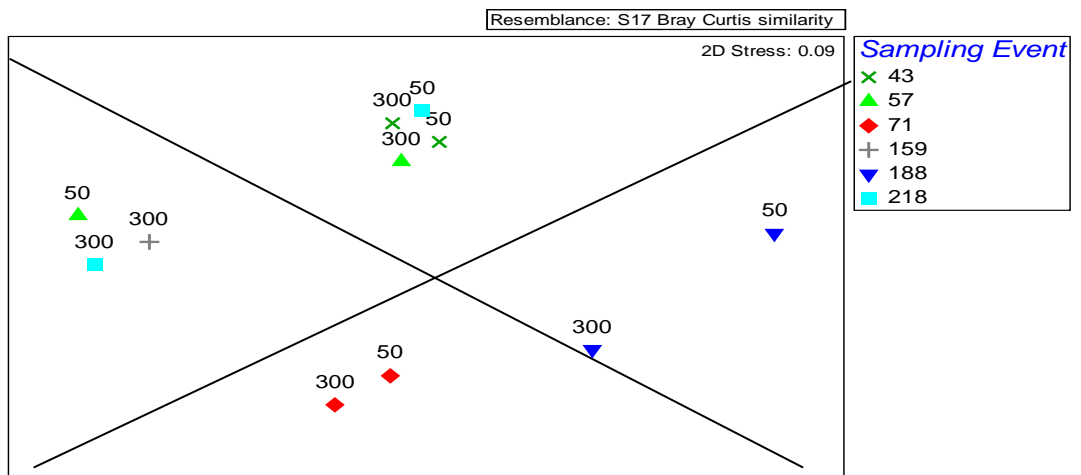


**Arrow = increase/decrease of individual chemical concentrations for distance-based sampling stations and ASR cycles indicated**

**Figure 4-12** 2D MDS Plot for amplified Microbial rDNA/DGGE banding patterns for all (A) Injection well, 4m and (B) 50m and 300m Fermentative Cultures from ‘Water-Only’ Samples

**A** Injection and 4m well fermentative ‘water-only’ bacterial populations

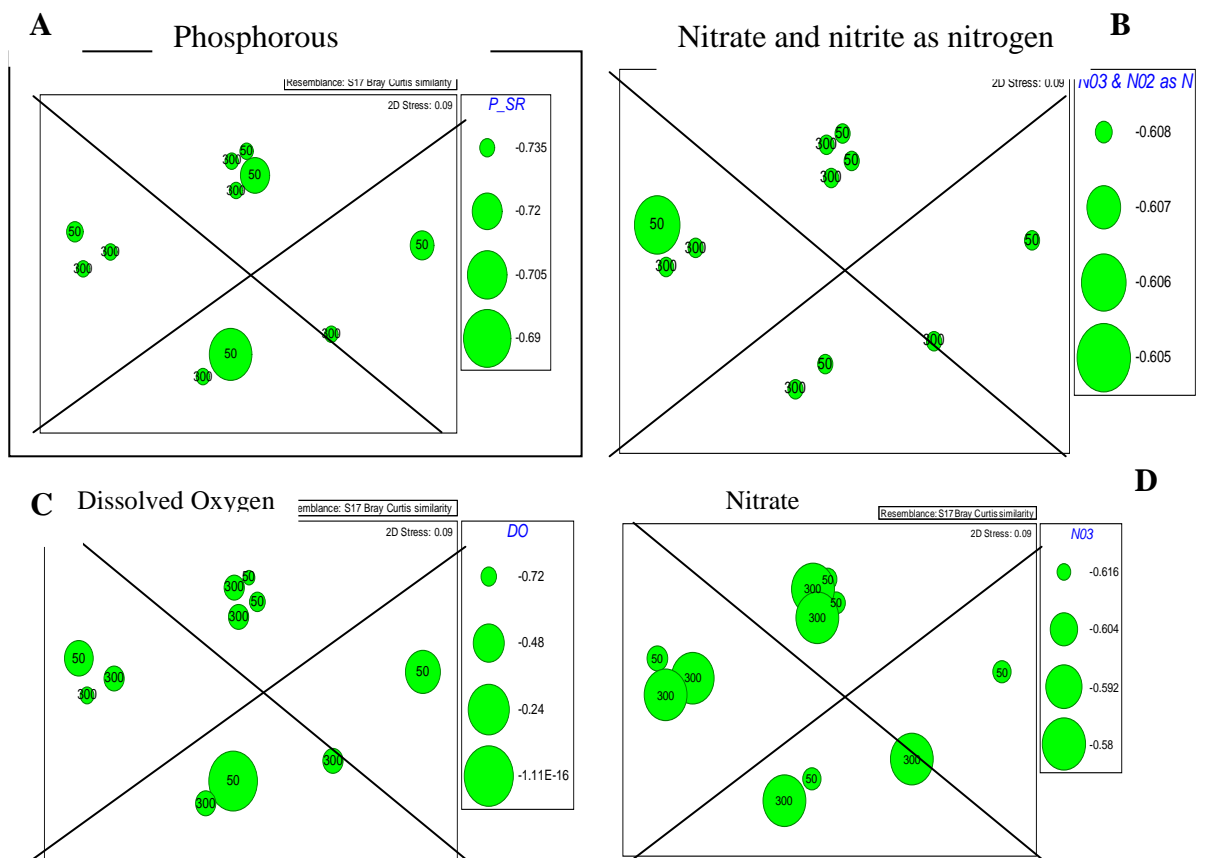


**B** 50m and 300m well fermentative ‘water-only’ bacterial populations





**Figure 4-13** 2D MDS Plot for amplified Microbial rDNA/DGGE banding patterns for all 50m well and 300m Fermentative Cultures (water-only) over time with overlay of bubble plot of (a) phosphorous, (b) nitrate and nitrite as nitrogen, (c) DO and (d) nitrate chemical



**Figure 4-13** (a.b.c.d) illustrates that phosphorous, dissolved oxygen, nitrate in addition to nitrate and nitrite as nitrogen concentrations vary over time at the 50m well. In contrast there is little variation in chemical concentrations at the 300m well which displays stable chemical concentrations over time. Despite these variations in concentrations of chemical parameters, it is unclear whether these differences separate the fermentative community structures over time between the 50m and 300m wells. It should be noted that despite the variation in chemical concentrations over time at 50m distance, the actual difference between the normalised concentrations is relatively small e.g. phosphorous variability is -0.69 to -0.735.

#### 4.2.2 Adelaide ASR Microbial Results for Fermentative Cultures 'Water-Sediment' Samples

### 4.3

Figure 4-14 shows the DGGE DNA/PCR banding patterns for all fermentative cultures from Adelaide 'water-sediment' samples over time. This Figure illustrates that there were twenty one band class types ranging from (STD\_2):9.0 to (STD\_2):95.0. The majority of the samples containing these band class types do not vary greatly in band intensity between samples. A MDS was undertaken on the binary matrix data to ascertain similarities and differences in the structure of fermentative bacterial populations from 'water-sediment' samples.

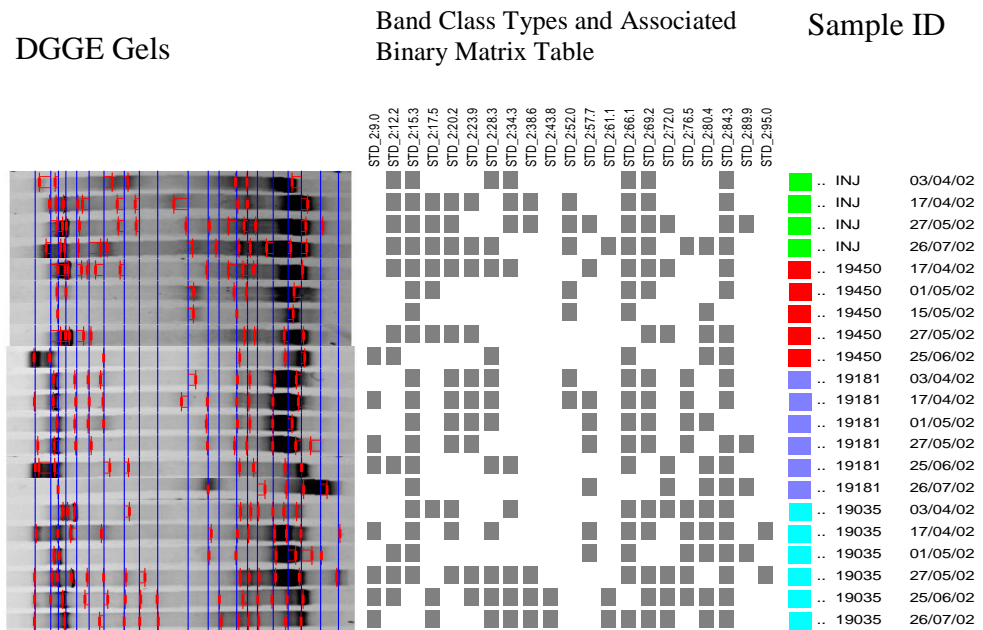
The results from Figure 4-15 show that all fermentative bacterial populations from 50m and 300m 'water-sediment' samples are contained within a cluster defined as population group i. Predominately all fermentative 'water-sediment' bacterial populations from the injection well and 4m well are clustered on the periphery of group i, defined as population group ii. Exceptions for the injection and 4m well include the initial injection phase (sampling event 43 and 57) and the recovery phase fermentative bacteria populations. These samples at sampling event day 218 clustered in group i with the 50m and 300m well samples. The injection and 4m well fermentative 'water-sediment' bacterial populations from group ii were not tightly clustered as described for 'water-only' fermentative communities shown in Figure 4-11.

Chemical parameters which may have separated the injection and 4m well 'water-sediment' fermentative bacterial populations during initial injection and from the recovery phase were investigated. The results from Figure 4-16 (b&i) show that nitrate and phosphorous concentrations were much lower during the recovery phase at the injection and 4m well. The results also show that redox Figure 4-16 (f) increased over time for the injection and 4m well but deeply declined during recovery. It was also shown that temperature decreased over time for the injection and 4m well. Other chemical parameters do not appear to have consistent trends over time for the injection and 4m wells.

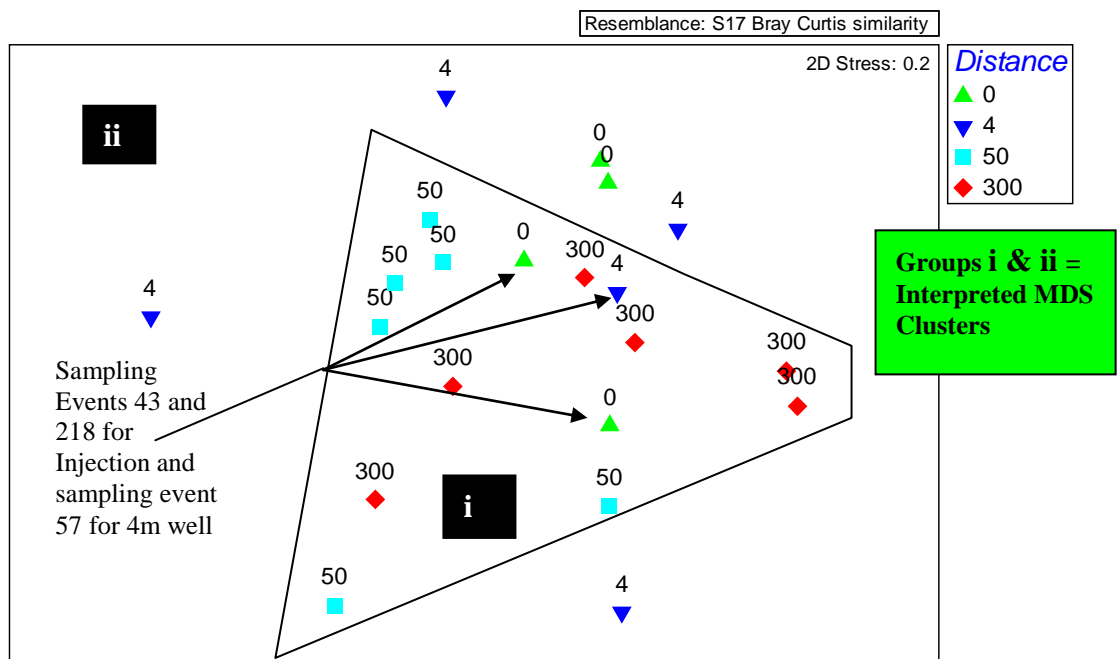
The results from Figure 4-17 (a) show that the fermentative 'water-sediment' bacterial community structures at the 300m well formed a distinct cluster which was different to

the microbial population structures described for the 50m well. Figure 4-17 (b,c,d,e and f) describe the variation in chemical concentrations for dissolved organic carbon, sulphate, ammonia, nitrate and total iron. Although there is great contrast between the chemical concentrations between the 50m and 300m wells there are no temporal variations apparent at each distance using the scales indicated. Because the 50m well were widely dispersed in Figure 4-17 (a) an MDS of these samples only was undertaken. Bubble plots were overlaid using individual chemical variables to determine which environmental parameter(s) were associated with these dynamic fermentative populations at 50m (Figure 4-18). Figure 4-18(a) indicates that the 50m well fermentative cultures from 'water-sediment' samples were predominantly clustered together. After this initial stability the fermentative bacterial population notably changed at sampling event 188. The fermentative bacterial population again changes during the recovery cycle. Thus the fermentative bacterial populations changed on the last day of the injection cycle and again during the recovery phase. The results therefore suggest there was temporal stability at the 50m well during most of the injection cycle. Changes in the fermentative population structure did not occur until a later time periods which is in contrast to the injection and 4m well population dynamics (Figure 4-16). Figure 4-18 (b,c,d,e,f,g) indicates the chemical parameter concentrations associated with the observation wells that the fermentative cultures were isolated from. These results indicate that there are differences in the chemical concentrations over time at 50m which may have separated the fermentative community structures.

**Figure 4-14** DGGE of bacteria rDNA/PCR banding patterns and associated binary matrix table for band matching data for all fermentative cultures from Adelaide ASR ‘water-sediment’ samples over time



**Figure 4-15** 2D MDS Plot for amplified Microbial rDNA/DGGE banding patterns for all Injection well, 4m, 50m and 300m Fermentative Cultures from ‘water-sediment’ samples

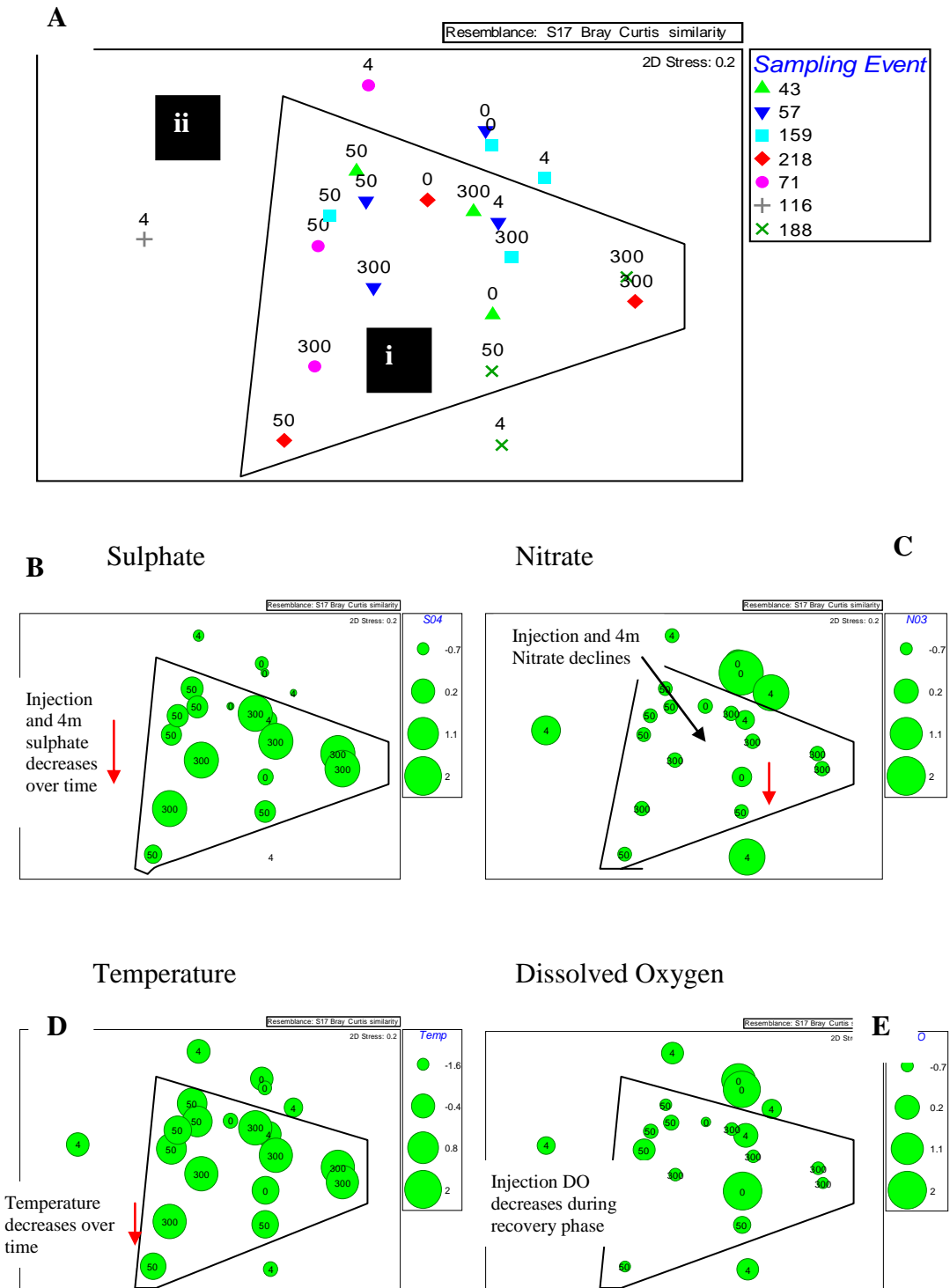


**i** = Cluster 1, contains the injection and 4m samples during INITIAL injection and recovery phase only, plus all 50m and 300m well samples.

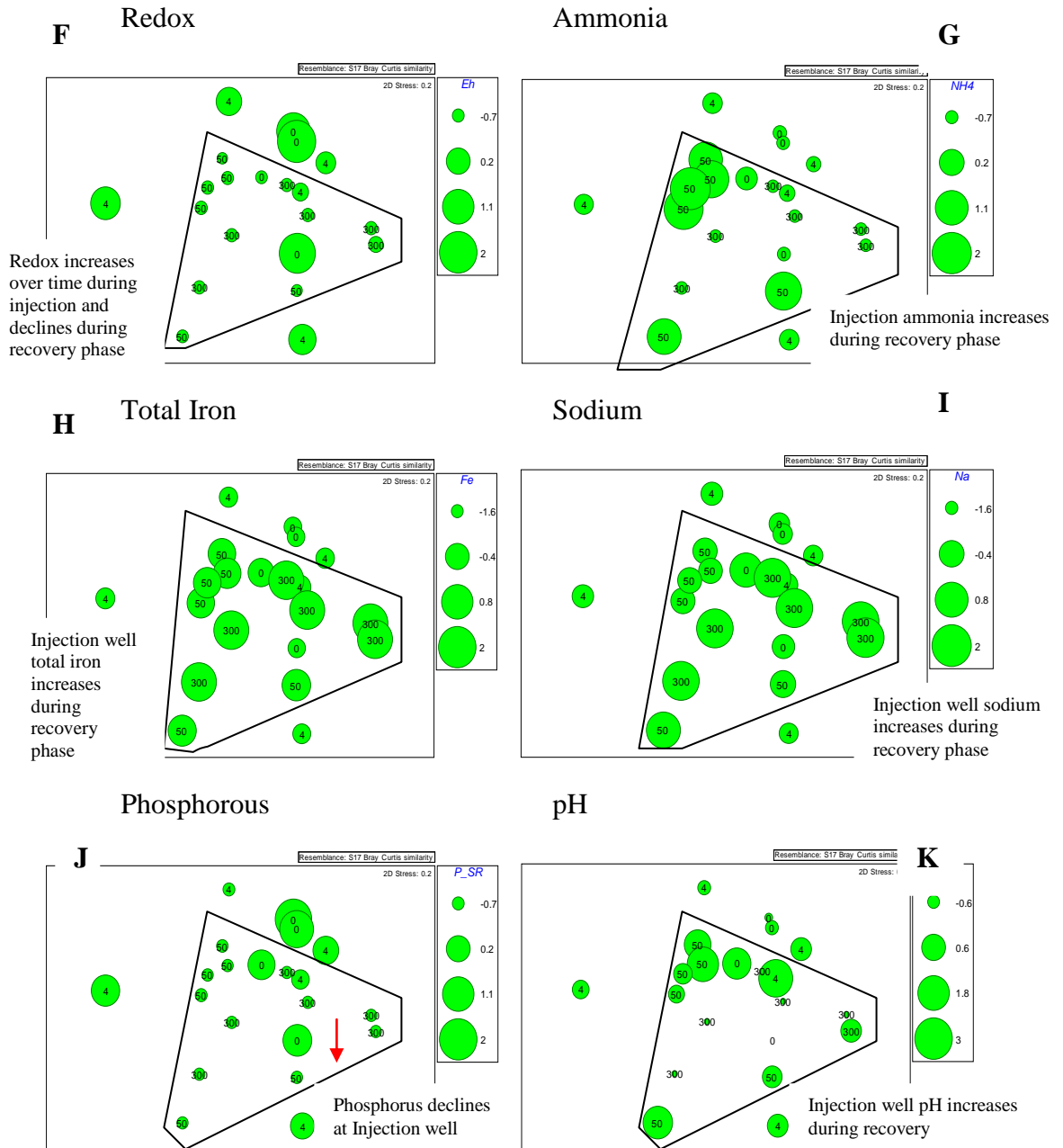
**ii** = Cluster 2, contains the remaining injection and 4m samples during the injection cycle.

**Figure 4-16** 2D MDS Plot for amplified Microbial rDNA/DGGE banding patterns for all Injection and 4m well Fermentative Cultures with overlaid chemical data for (a) sampling event (b)  $SO_4$ , (c)  $NO_3$ , (d) Temp, (e) DO, (f) Eh, (g)  $NH_3$  (h) total Fe (i)  $Na^+$  (j) P-SR and (k) pH

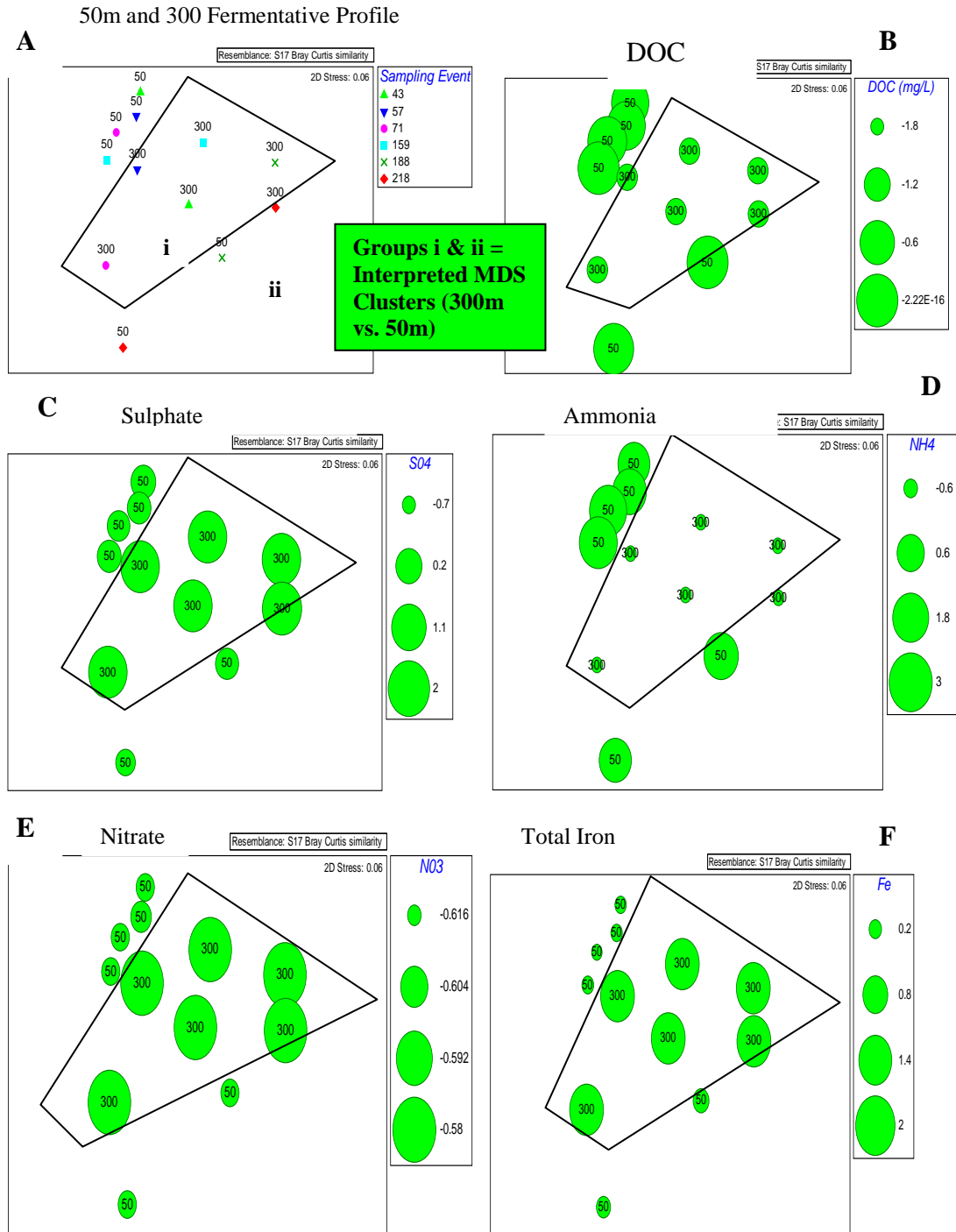
Injection, 4m, 50m and 300m fermentative 'water-sediment' bacterial populations



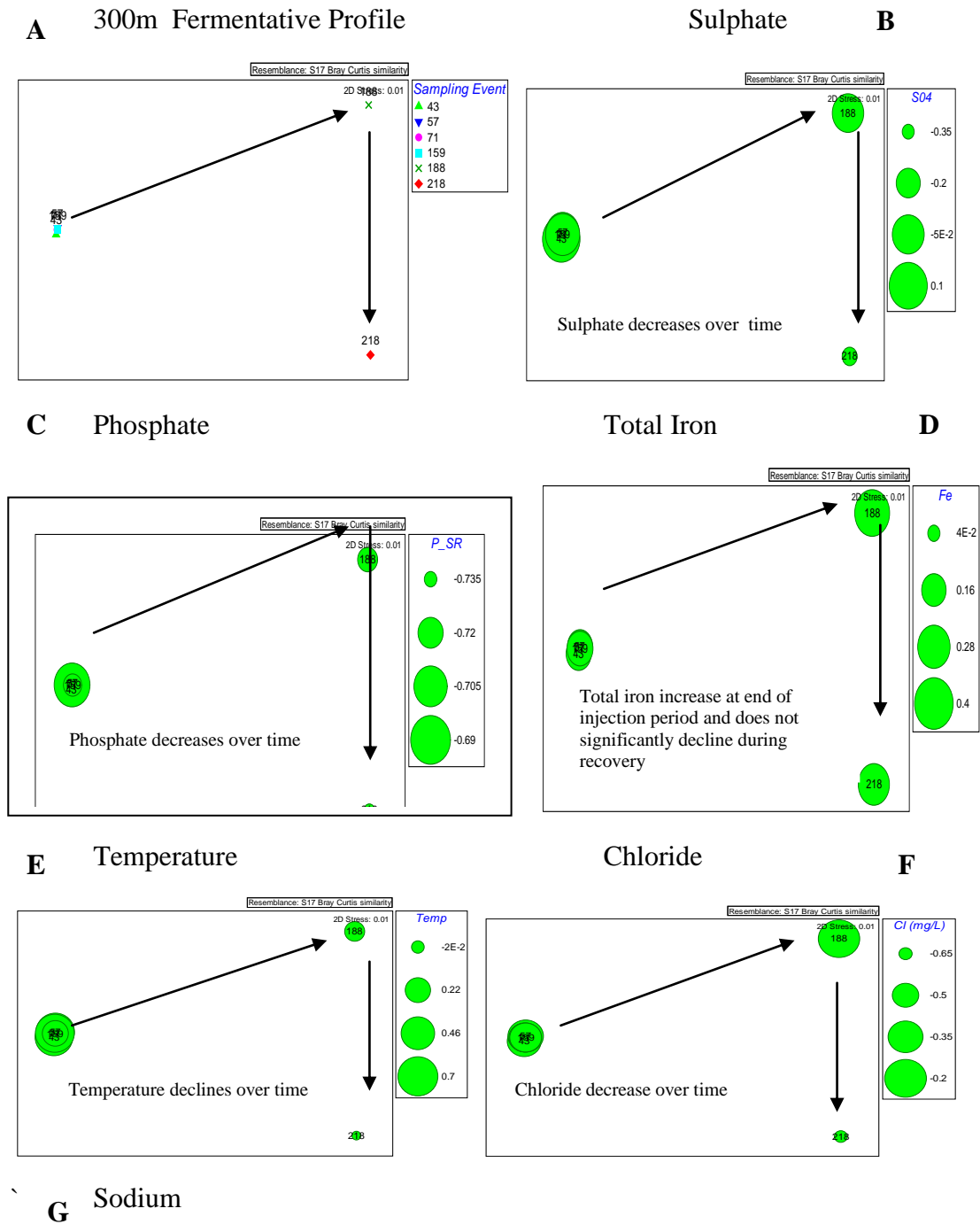
**Arrow = increase/decrease of individual chemical concentrations for distance-based sampling stations and ASR cycles indicated.**



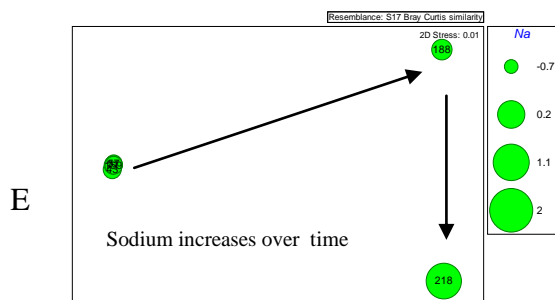
**Figure 4-17** 2D MDS Plot for amplified Microbial rDNA/DGGE banding patterns for (a) all 50m and 300m well Fermentative Cultures with overlaid chemical data for (b)DOC, (c)  $SO_4$ , (d)  $NH_3$ , (e)  $NO_3$ , and (f) total Fe.



**Figure 4-18** 2D MDS Plot for amplified Microbial rDNA/DGGE banding patterns for (a) all 50m well Fermentative Cultures from ‘water-sediment’ samples with overlaid chemical data for (b)  $SO_4$ , (c) P-SR, (d) total Fe, (e) Temp, (f) Cl<sup>-</sup> and (g) Na<sup>+</sup>





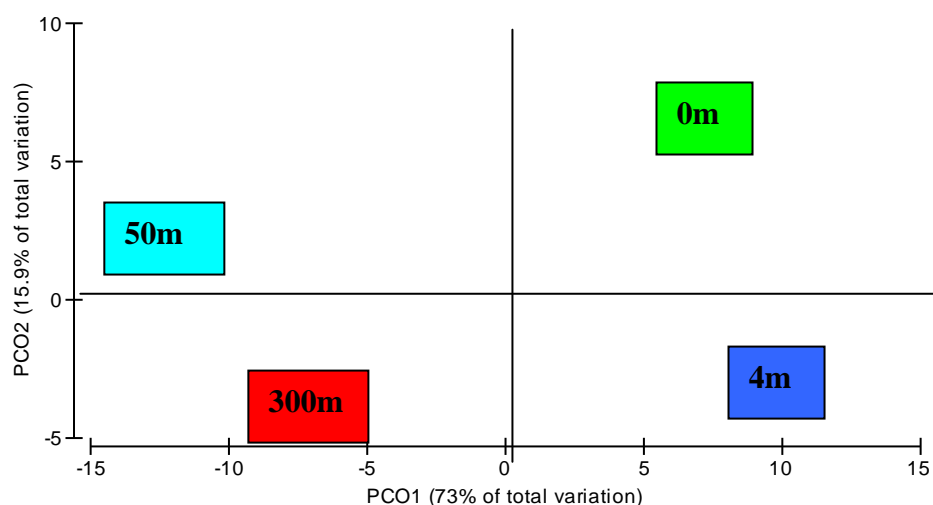


#### 4.3.1 Adelaide ASR Combined Microbial Results for Fermentative Cultures ‘Water-Only’ AND ‘Water-Sediment Samples

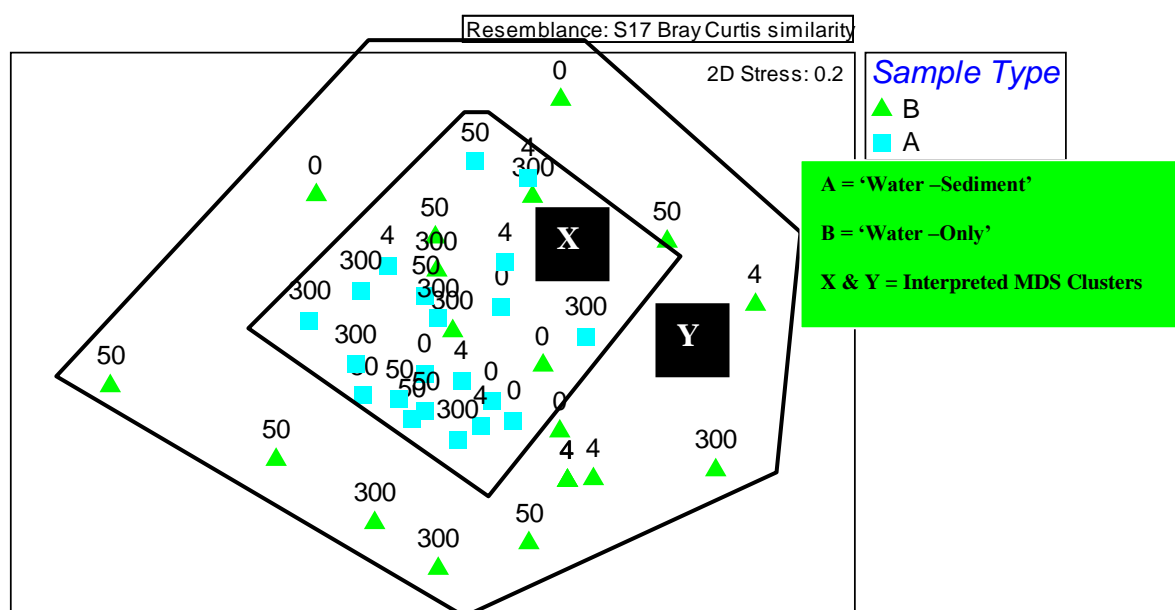
Figure 4-19 represents 88.9% of the total variation which separates the differences between the fermentative microbial community structures over time. This centroid PCO plot shows that the injection and 4m wells are more similar in microbial population structure than compared with the 50m and 300m well samples which more closely resemble each other.

The results from Figure 4-20, describes the differences in fermentative bacterial populations between samples A (water-sediment) and B (water-only) over time. The MDS plot clearly shows that the fermentative bacterial populations from the water-only samples (B) are more dynamic over time defined as population group Y. These results are indicating that ‘water-sediment’ samples are therefore more stable in microbial population structure over time defined as population group X.

**Figure 4-19** 2D PCO using the centroid reduced similarity matrix based on distance for the Injection, 4m, 50m and 300m well Fermentative culture samples



**Figure 4-20** 2D MDS Plot for amplified Microbial rDNA/DGGE banding patterns for all Injection, 4m, 50m and 300m Fermentative Cultures from (A) 'water-sediment' and (B) 'water-only'



## 4.4 Adelaide ASR Sulphate-Reducing Culture RESULTS for Water-only and Water-Sediment Samples

### 4.4.1 Adelaide ASR Microbial Results for Sulphate-Reducing Cultures 'Water-only' Samples

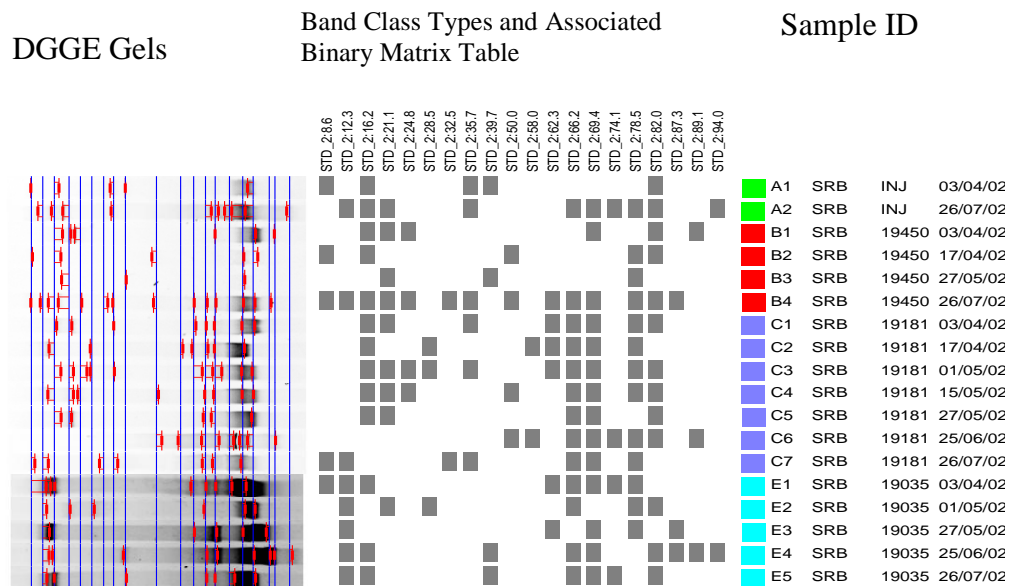
Figure 4-21 shows the DGGE DNA/PCR banding patterns for all sulphate-reducing cultures from Adelaide 'water-only' samples over time. This Figure illustrates that there were twenty band class types ranging from (STD\_2):8.6 to (STD\_2):94.0. The majority of the samples containing these band class types demonstrated faint band intensity except for background (300m) samples which showed much greater band intensity. A MDS was undertaken on the binary matrix data to ascertain similarities and differences in sulphate reducing 'water-only' population structures between samples.

Figure 4-22 (a) distinguishes the background (300m) and 50m sulphate reducing 'water-only' populations defined as group i from the majority of injection and 4m well communities (group ii). Exceptions include the injection and 4m well sulphate reducing bacterial populations during the recovery phase at sampling event 218. Figure 4-22 (b,c,d,e) visually describe the flux in chemical concentration at the injection and 4m well during the injection cycle. Sulphate-reducing bacteria from the 4m well could only be cultured during the injection cycle to the maximum sampling event day 159. The results from Figure 4-22 (b,c,d,e) show that as the injection cycle progresses to sampling event 159 sulphate concentration declines. This coincides with an increased concentration of nitrate as well as a decrease in temperature and oxygen. Sulphate-reducing bacteria could only be cultured from the injection well for the first sampling event and during the recovery phase. Figure 4-22 (f,g,h,i) visually highlights the difference between the injection and recovery cycles with regards to the chemical concentrations for the injection and 4m well samples. These Figures also highlights the different concentration of various chemicals between the injection and 4m wells verses

the 50m and 300m well samples. These results show how differences in chemical concentrations may have separated the sulphate reducing bacterial populations over distance and time.

Figure 4-23 shows that band class type 66.2 (Figure 4-21) is associated with the 50m and 300m sulphate reducing community structures. It also appears that this band class types also contributed to the separation between the injection and recovery phase sulphate-reducing community structure at the injection and 4m well.

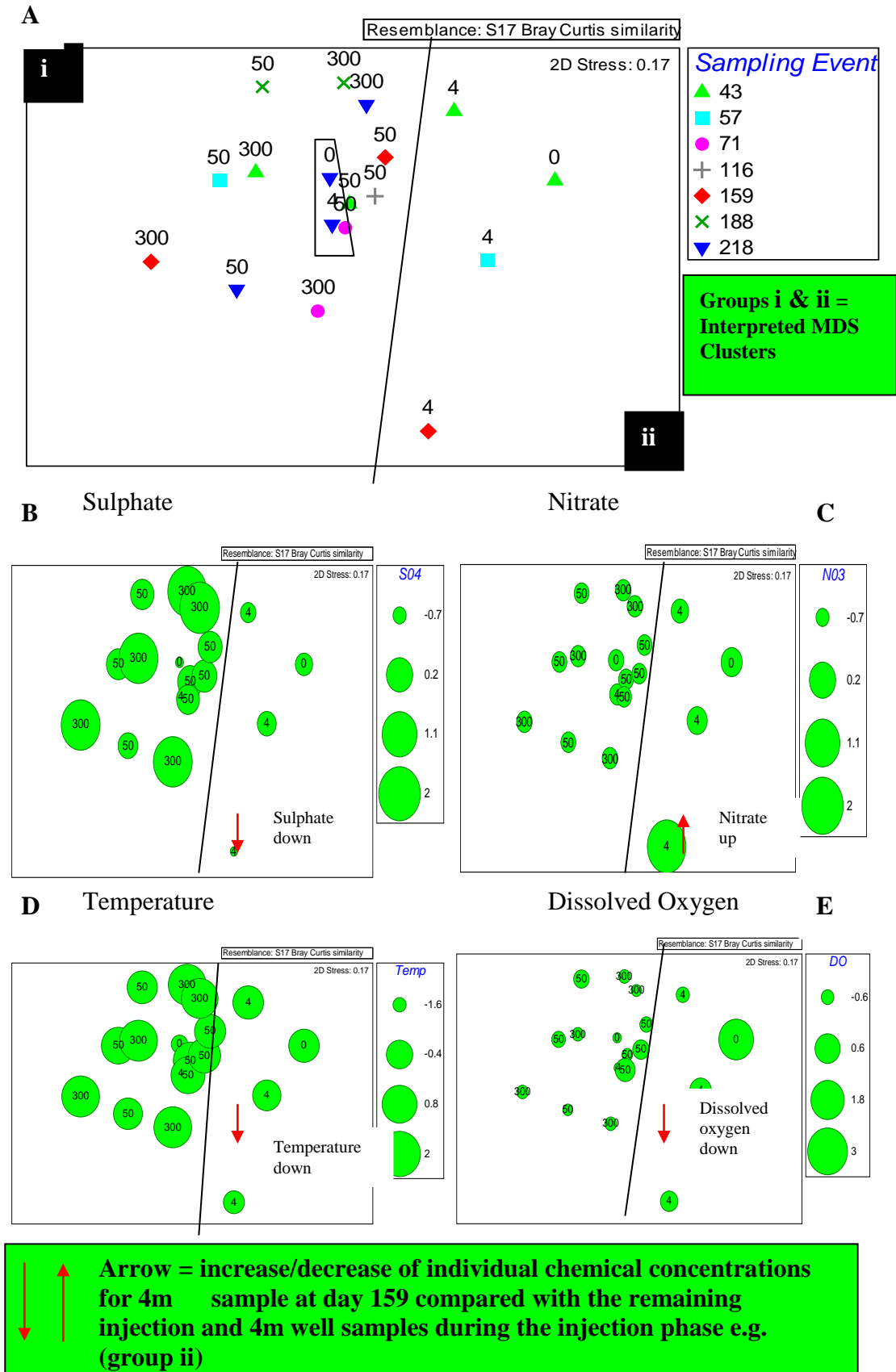
**Figure 4-21** DGGE of bacteria rDNA/PCR banding patterns and associated binary matrix table for band matching data for all Sulphate-reducing cultures from Adelaide ASR ‘water-only’ over time.





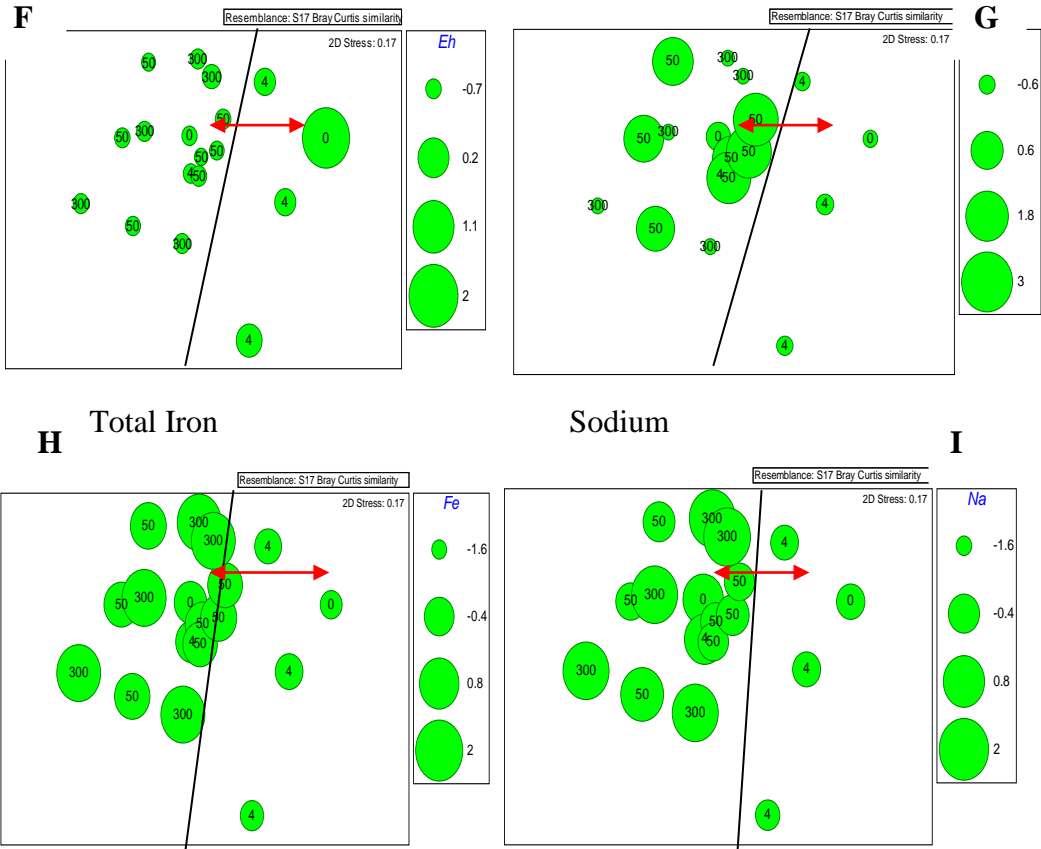
**Figure 4-22** 2D MDS Plot for amplified Microbial rDNA/DGGE banding patterns for (a) all Injection well, 4m, 50m and 300m Sulphate-Reducing Cultures from 'water-only' samples with overlaid chemical data for (b) S04, (c) N03, (d) Temp, (e) DO, (f) Eh, (g) NH3 (h) total Fe, (i) Na+

Injection, 4m, 50, and 300m sulphate reducing 'water-only' populations



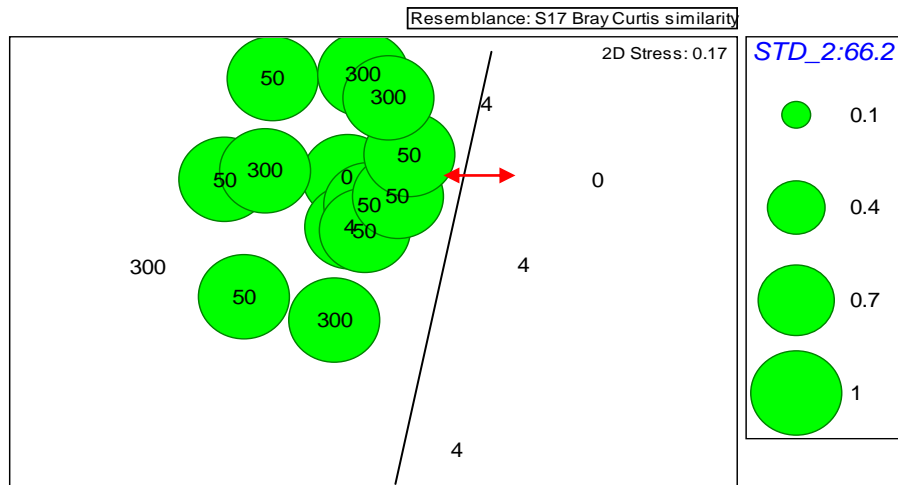
Redox

Ammonia



**Double arrow** ←→  
= highlighting difference in individual chemical concentrations for the injection and 4m samples during the injection phase compared with the injection and 4m sample during the recovery phase.

**Figure 4-23** 2D MDS Plot for amplified Microbial rDNA/DGGE banding patterns for (a) all Injection, 4m, 50m and 300m Sulphate-Reducing Cultures from ‘water-only’ with overlaid DGGE Class Type 66.2



**4.4.2** Adelaide ASR Microbial Results for Sulphate-Reducing Cultures ‘Water-Sediment’ Samples

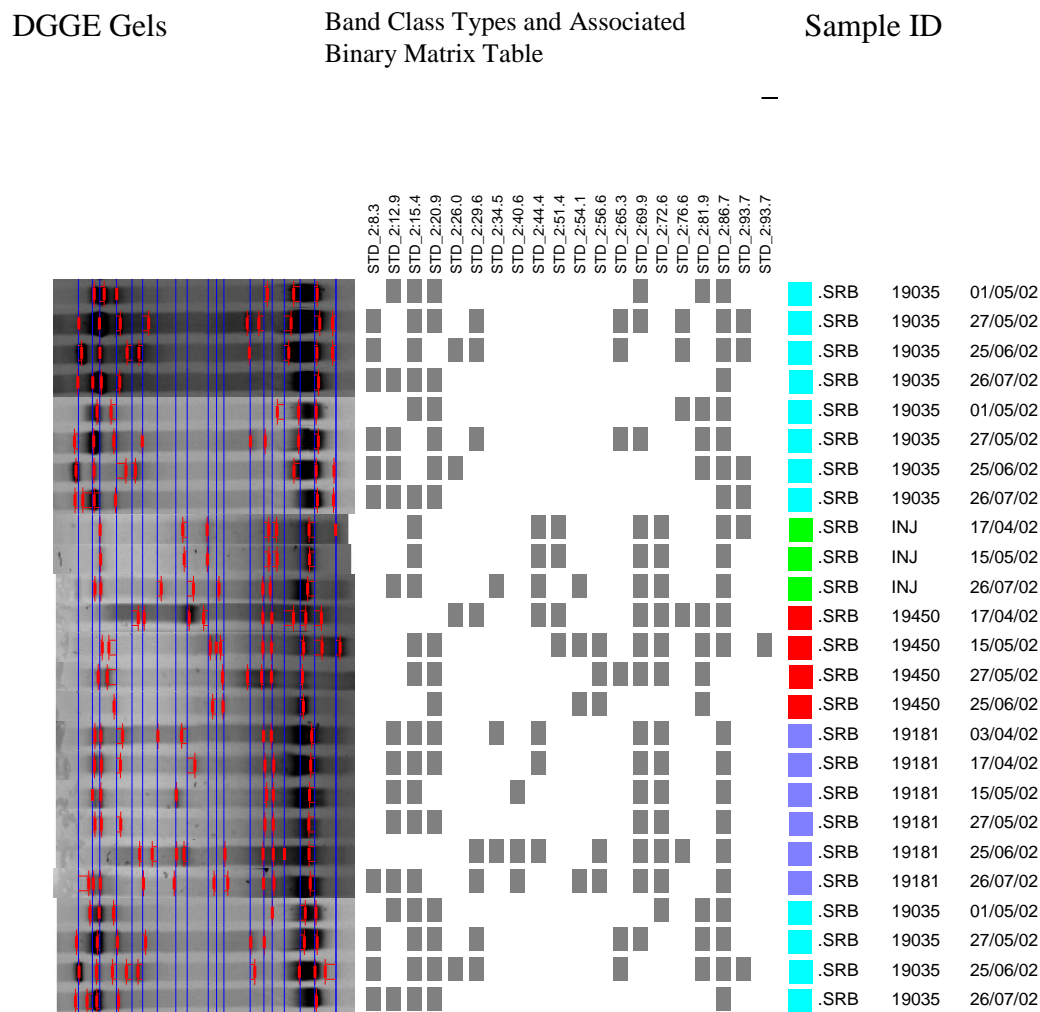
Figure 4-24 shows the DGGE DNA/PCR banding patterns for all sulphate-reducing cultures from Adelaide ‘water-sediment’ samples over time. This Figure illustrates that there were twenty band class types ranging from (STD\_2):8.3 to (STD\_2):93.7. The majority of the samples containing these band class types were clearly visible but the background (300m) bore showed greater band intensity. A MDS was undertaken on the binary matrix data to ascertain similarities and differences in sulphate reducing ‘water-sediment’ population structures between samples.

**Figure 4-25 (a) clearly the 50m and 300m well sulphate reducing bacterial community structures from ‘water-sediment’ samples, defined as population groups i and ii. The majority of injection and 4m well sulphate reducing bacterial populations were separated from groups i and ii forming a defined population group iii. Exceptions include 4m at sampling event 57 and the injection well recovery sulphate reducing bacterial populations from ‘water-sediment’ samples which are clustered with the 50m well populations in group ii.**

Figure 4-25 (b,c,d,e,f,g,h,i,j) show that these two injection and 4m well samples which were clustered with the 50 and 300m well populations, differed in their chemical concentrations at these observation wells, compared with group iii injection and 4m well samples.

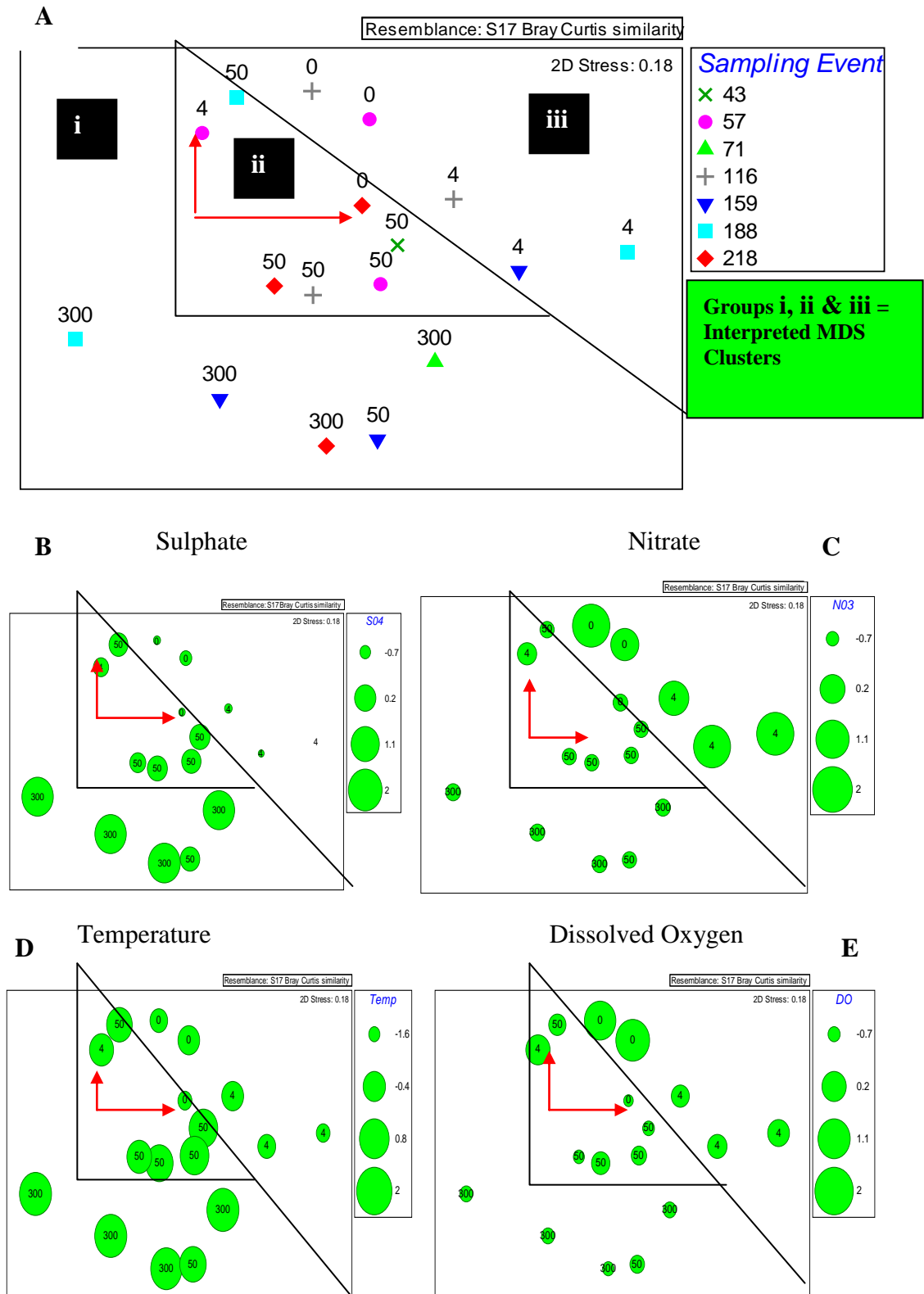


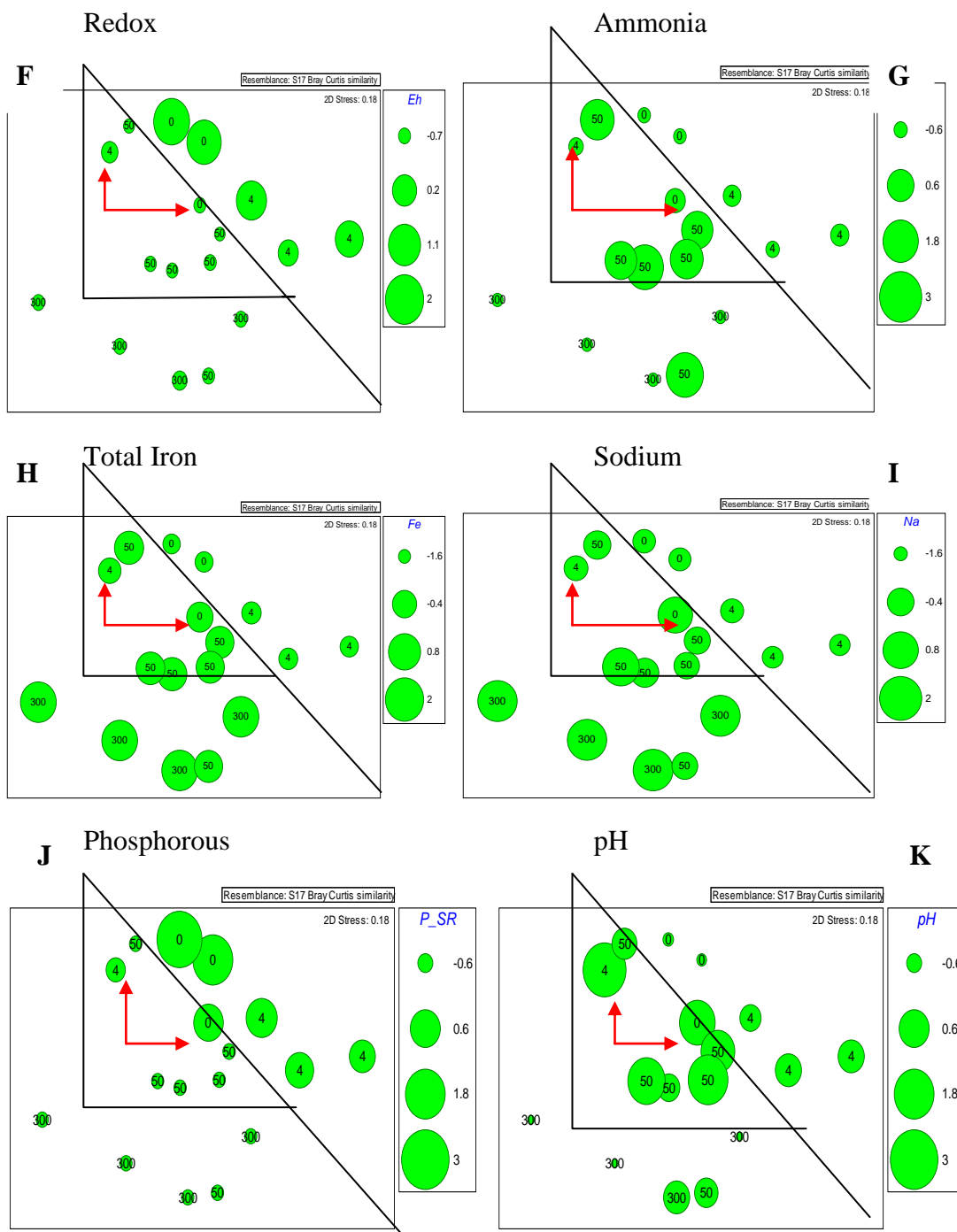
**Figure 4-24** DGGE of bacteria rDNA/PCR banding patterns and associated binary matrix table for band matching data for all Sulphate-reducing cultures from 'water-sediment' Adelaide ASR samples




**Figure 4-25** 2D MDS Plot for Microbial rDNA/DGGE banding patterns for (a) all Injection, 4m, 50m and 300m Sulphate-Reducing Cultures from ‘water-sediment’ with overlaid chemical data for (b)  $\text{SO}_4$ , (c)  $\text{NO}_3$ , (d) Temp, (e) DO, (f) Eh, (g)  $\text{NH}_3$  (h) total Fe, (i)  $\text{Na}^+$ , (j) P-SR and (k) pH

Injection, 4m, 50m and 300m sulphate reducing ‘water-sediment’ populations





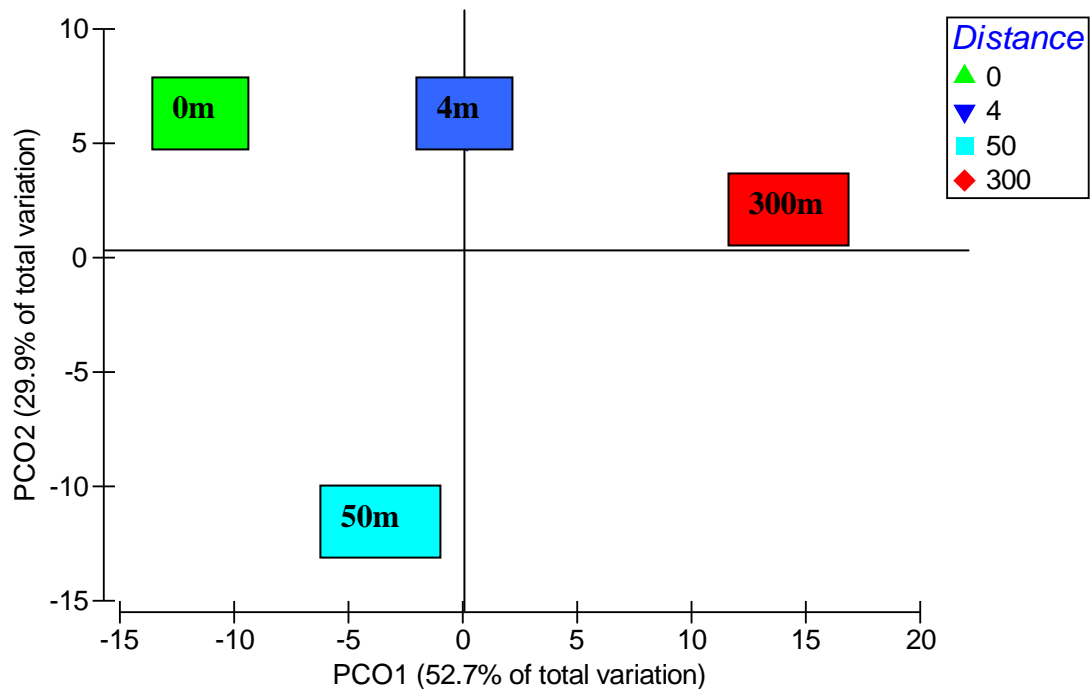
  
**Arrows indicate  
 4m well sample at  
 sampling event 57  
 and Injection well  
 sample at sampling  
 event 218**

#### 4.4.3 Adelaide ASR Combined Microbial Results for Sulphate-Reducing Cultures 'Water-only' AND 'Water-Sediment' Samples

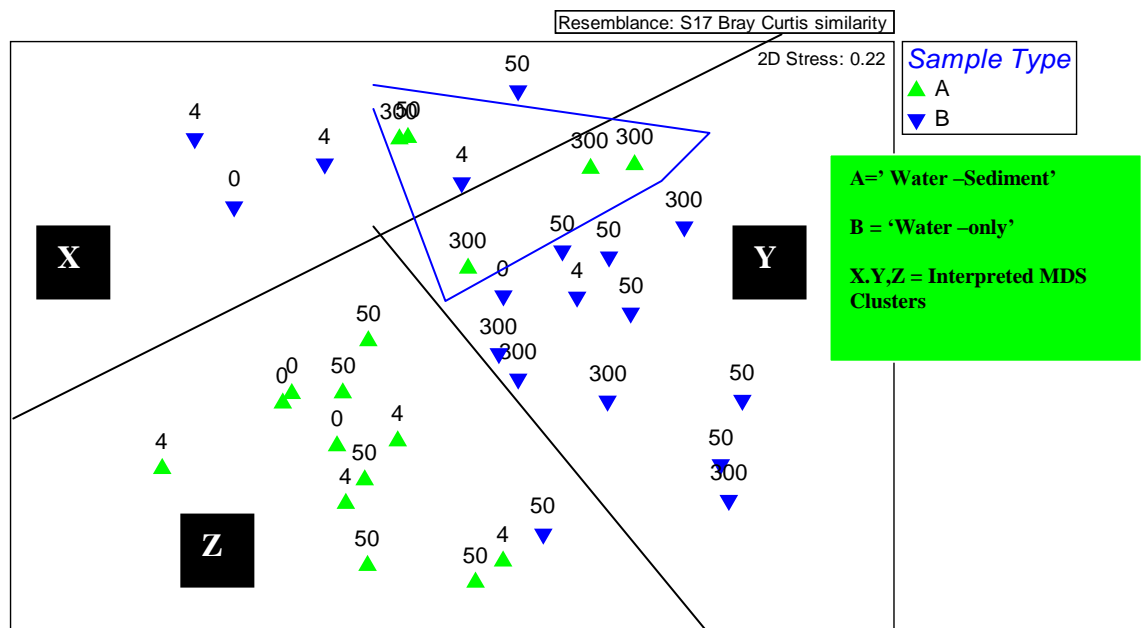
Figure 4-26 represent 82.6% of the total variation which separates the sulphate-reducing community structures over distance and time. Figure 4-26 shows that the injection and 4m well sulphate reducing bacterial populations are more similar in microbial community structure than compared with the 50m and 300m well populations.

The results from Figure 4-27, describes the differences between the sulphate reducing bacterial populations for samples A (water-sediment) and B (water-only) over time. The MDS plot clearly shows that the sulphate reducing bacterial populations from the 'water-only' samples (B) are more dynamic over time defined as groups X and Y. Group X predominately consists of 4m well sulphate reducing bacteria whereas population group Y predominantly consists of 50m and 300m well populations. In contrast the 'water-sediment' sulphate reducing bacterial populations are predominantly clustered in one group which includes the injection, 4m and 50m well communities defined as group Z. Exceptions include the 300m well sulphate reducing community structures which are highlighted in blue. These background sulphate reducing bacterial populations are different to all other 'sediment-water' samples. Therefore, apart from the 300m well populations, the sulphate reducing bacterial populations cultured from 'water-sediment' samples were relatively stable over time.

**Figure 4-26** 2D PCO using the reduced similarity matrix based on distance for the Injection, 4m, 50m and 300m well Sulphate-reducing culture samples



**Figure 4-27** 2D MDS Plot for amplified Microbial rDNA/DGGE banding patterns for all Injection, 4m, 50m and 300m Sulphate-reducing Cultures from (A) water-sediment and (B) water-only samples

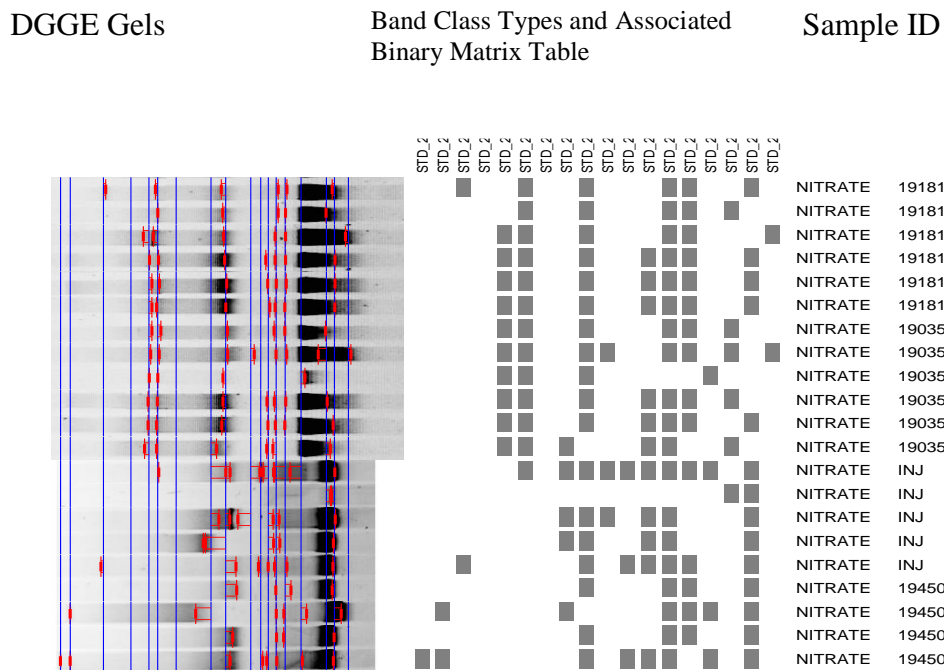


## 4.5 Adelaide ASR Nitrate-Reducing Culture RESULTS for Water-only and Water-Sediment Samples

### 4.5.1 Adelaide ASR Microbial Results for Nitrate-Reducing Cultures 'Water-only' Samples

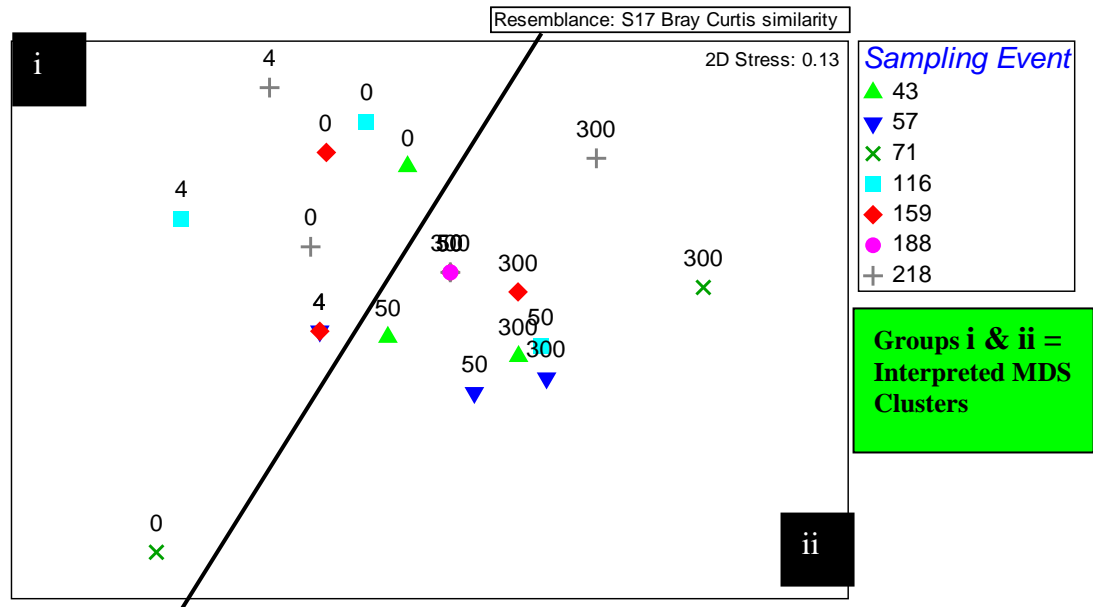
Figure 4-28 shows the DGGE DNA/PCR banding patterns for all nitrate-reducing cultures from Adelaide 'water-only' samples over time. This Figure illustrates that there were twenty one band class types ranging from (STD\_2):2.6 to (STD\_2):84.3. The band class types varied in band intensity within and between samples but some band class types were also consistently weak or dominant between samples. There was clearly a consistent dominant band for all samples (STD\_2:80.3). A MDS was undertaken on the binary matrix data to ascertain similarities and differences in 'water-only' nitrate reducing bacterial population structures between samples. Figure 4-29 (a) separated the injection and 4m well samples defined as group i from the 50m and 300m well samples defined as group ii. Despite the limited supply of nitrate during the recovery phase (Figure 4-29c), the injection and 4m well nitrate reducing bacterial populations did not considerably change in community structure during this cycle.

**Figure 4-28** DGGE of bacteria rDNA/PCR banding patterns and associated binary matrix table for band matching data for all Nitrate-reducing cultures from Adelaide ASR 'water-only' samples over time.

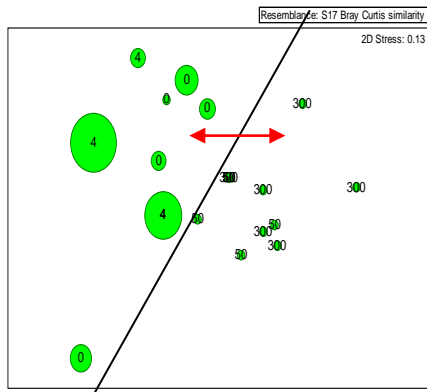


**Figure 4-29** 2D MDS Plot for amplified Microbial rDNA/DGGE banding patterns for (a) all Injection, 4m, 50m and 300m Nitrate-Reducing Cultures from ‘Water-Only’ Samples with overlaid chemical data for (b)  $\text{NO}_2$ , (c)  $\text{NO}_3$ , (d)  $\text{NH}_4$ , (e)  $\text{SO}_4$ , (f) Temp, (g) DO (h) Eh (i) total Fe, (j)  $\text{Na}^+$ , (k) P-S

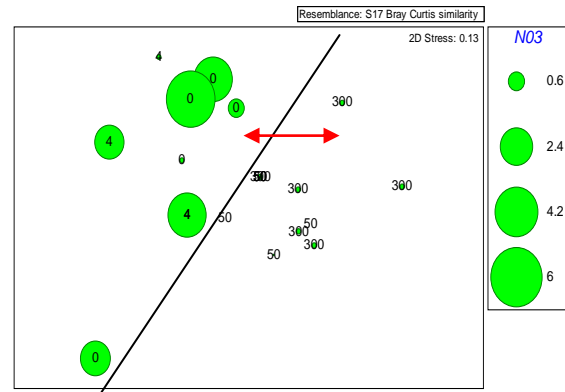
**A** Injection, 4m, 50m and 300m well nitrate reducing ‘water-only’ populations



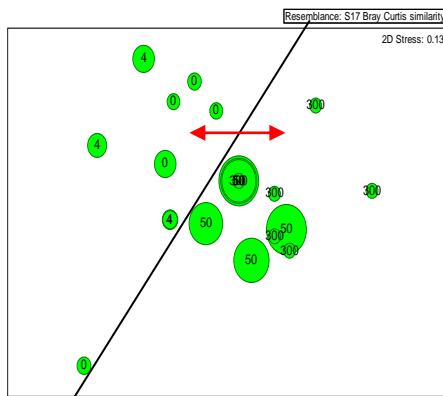
**B** Nitrite



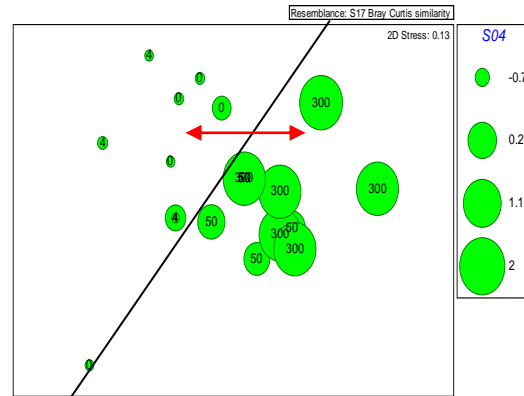
Nitrate



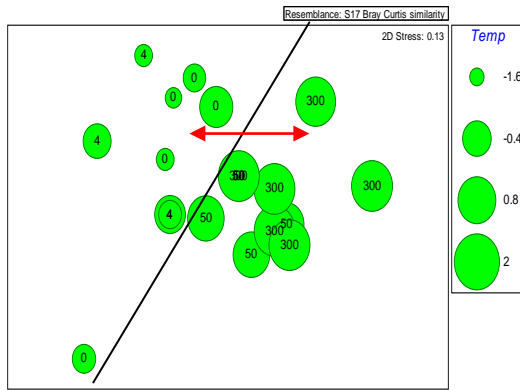
**D** Ammonia



Sulphate

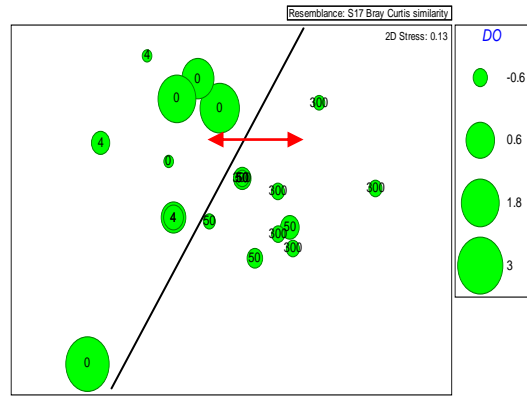


**F** Temperature

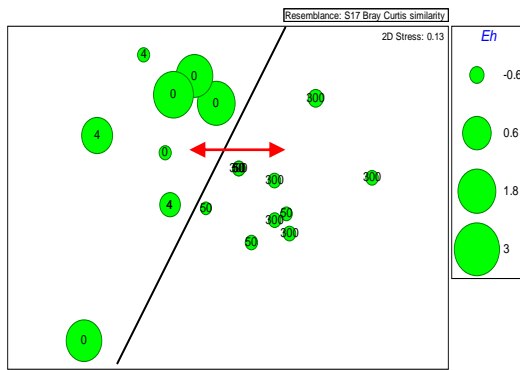


Dissolved Oxygen

**G**

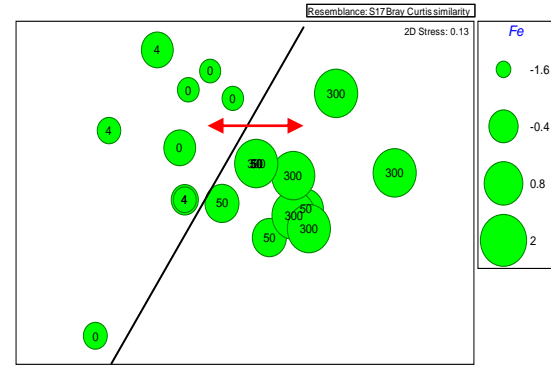


**H** Redox

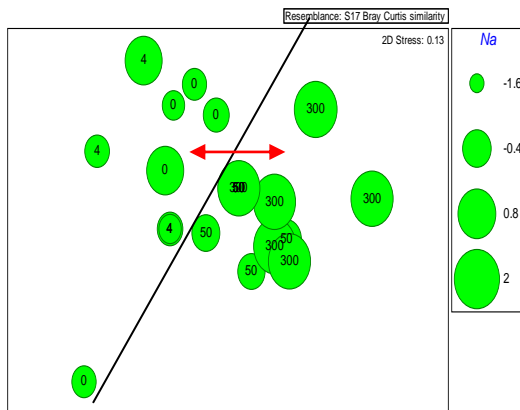


Total Iron

**I**

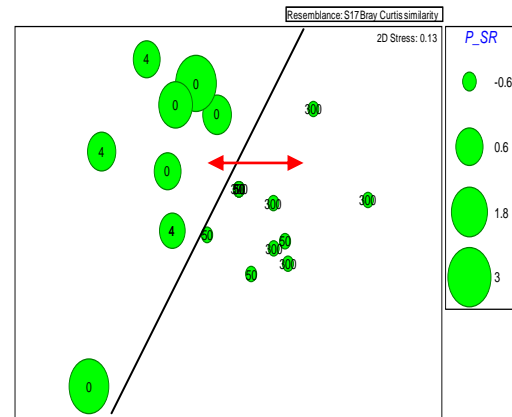


**J** Sodium



Phosphorous

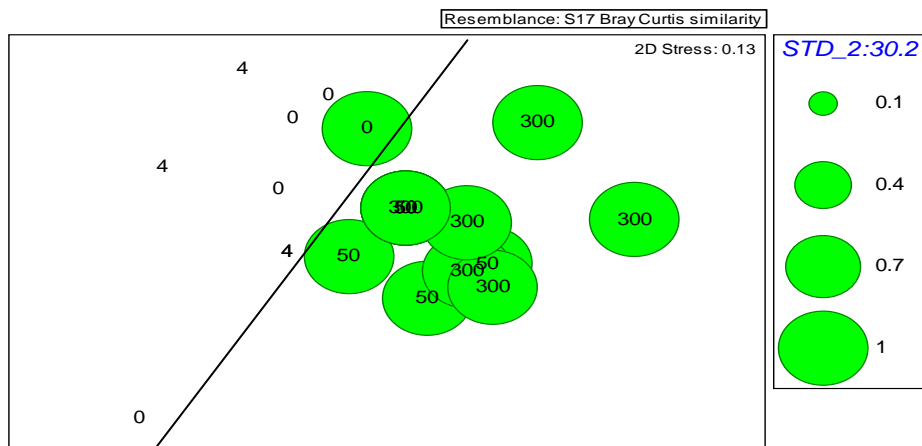
**K**



**↔ Double arrow = highlighting difference in individual chemical concentrations for interpreted MDS groups i and ii.**



**Figure 4-30** 2D MDS Plot for amplified Microbial rDNA/DGGE banding patterns for (a) all Injection, 4m, 50m and 300m Nitrate-Reducing Cultures from ‘water-only’ samples with overlaid DGGE band class type 30.2



4.5.2 Adelaide ASR Microbial Results for Nitrate-Reducing Cultures ‘Water-Sediment’ Samples

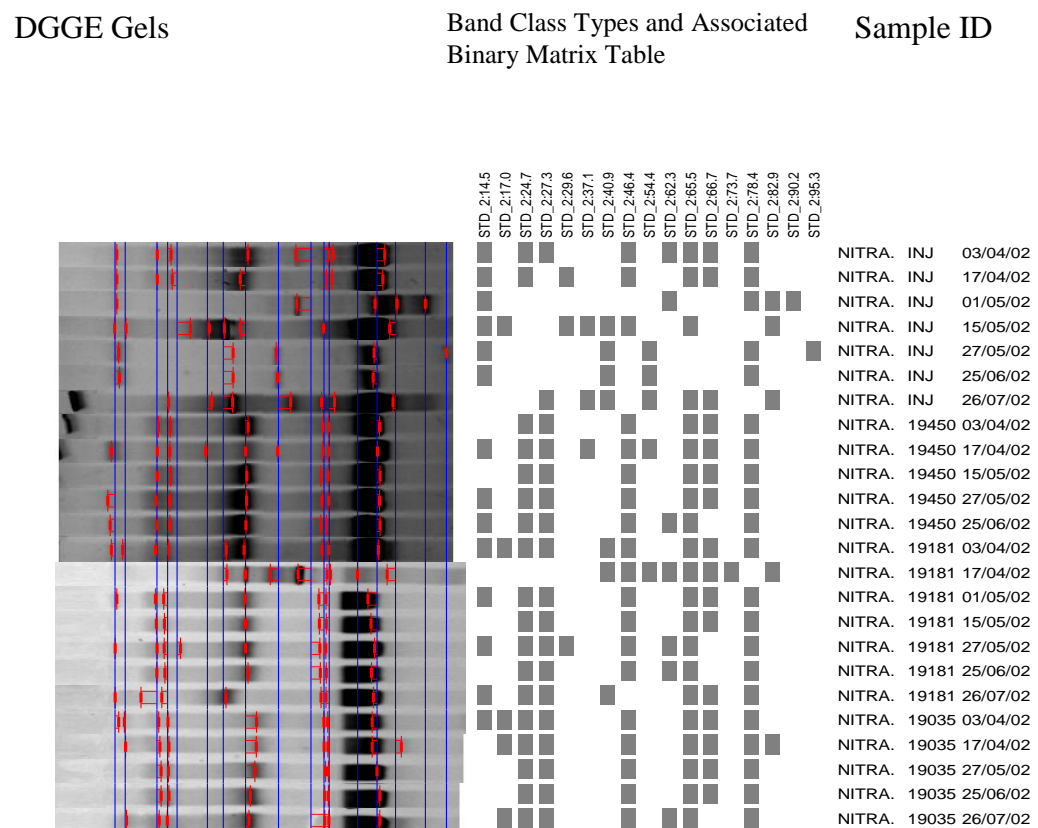
Figure 4-31 shows the DGGE DNA/PCR banding patterns for all nitrate-reducing cultures from Adelaide ‘sediment-water’ samples over time. This Figure illustrates that there were seventeen band class types ranging from (STD\_2):14.5 to (STD\_2):95.3. The band class types showed greater band intensity consistently for the 4m well (19181) and for over half of the injection well samples. There was clearly a consistent dominant band for all samples (STD\_2:78.4). A MDS was undertaken on the binary matrix data to ascertain similarities and differences in nitrate reducing population community structures between samples.

Figure 4-32 (a) clearly shows that the nitrate reducing bacterial population structure in the injection well is very different to nitrate-reducing community structures identified from all other distances. These results are in contrast with the ‘water-only’ nitrate reducing bacterial populations where the injection well community structures were also clustered with the 4m bacterial populations. Comparison of DGGE gels (Figure 4-28& Figure 4-31) clearly show that the injection and 4m well nitrate-reducing bacteria isolated from ‘water-sediment’ resulted in more intense banding in most cases compared with ‘water-only’ nitrate reducers. These results suggest that the addition of sediment at the injection well creates a favourable environment for a shift in nitrate reducing population structure which was different to the community structure at all other observation wells. Despite containing an overall nitrate reducing bacterial population structure which was very different to those present at all other observation

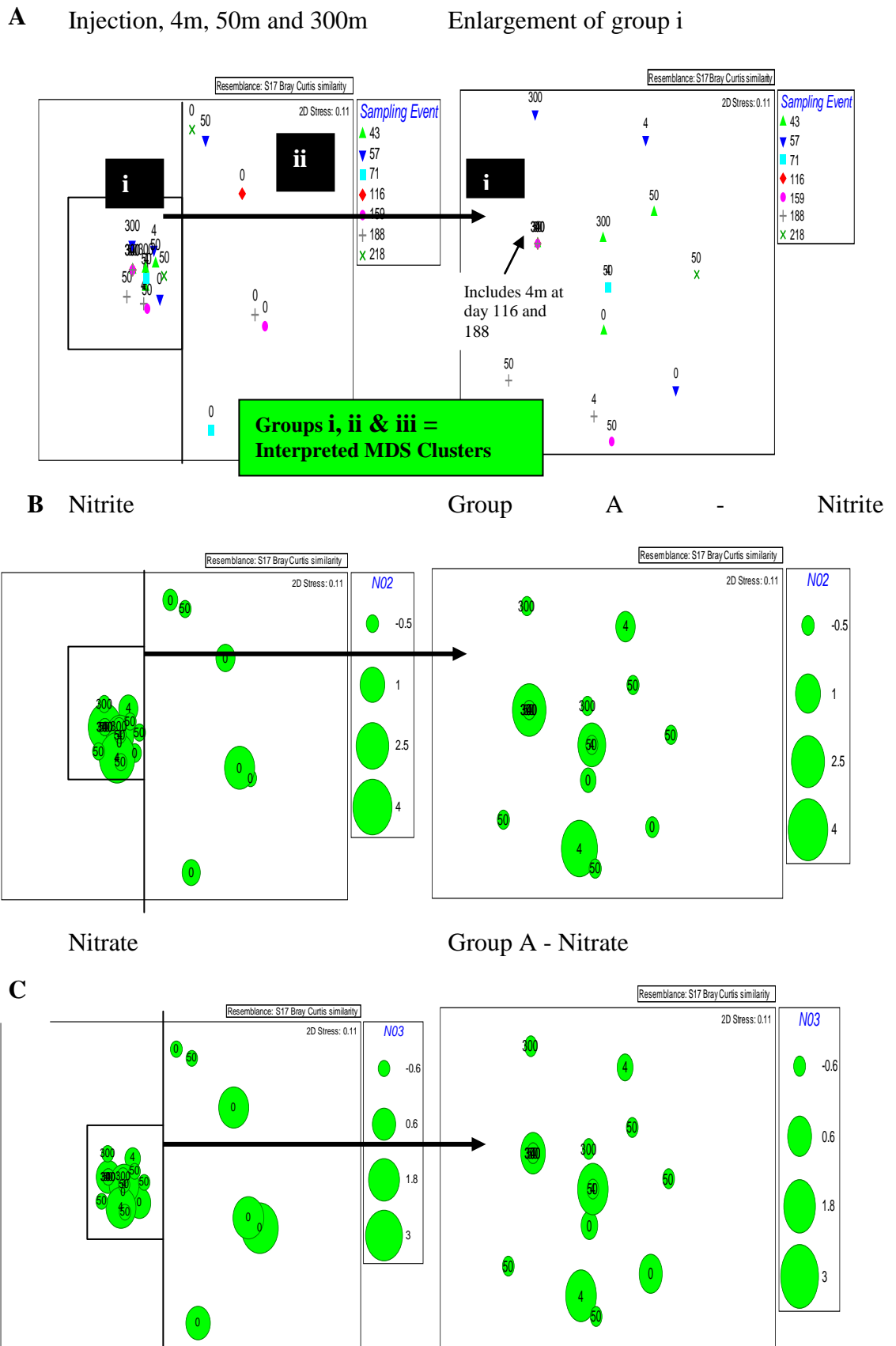
wells, visual inspection of the injection well DGGE profiles clearly showed much greater variability in banding pattern over time. All other nitrate reducing bacterial populations from distances other than the injection well showed relative spatial consistency. Due to the tight clustering of nitrate reducing bacterial populations from 4m, 50m and 300m an enlargement of group i from Figure 4-32 (a) was undertaken to visualise the identification of each sample as shown in Figure 4-32 (b).

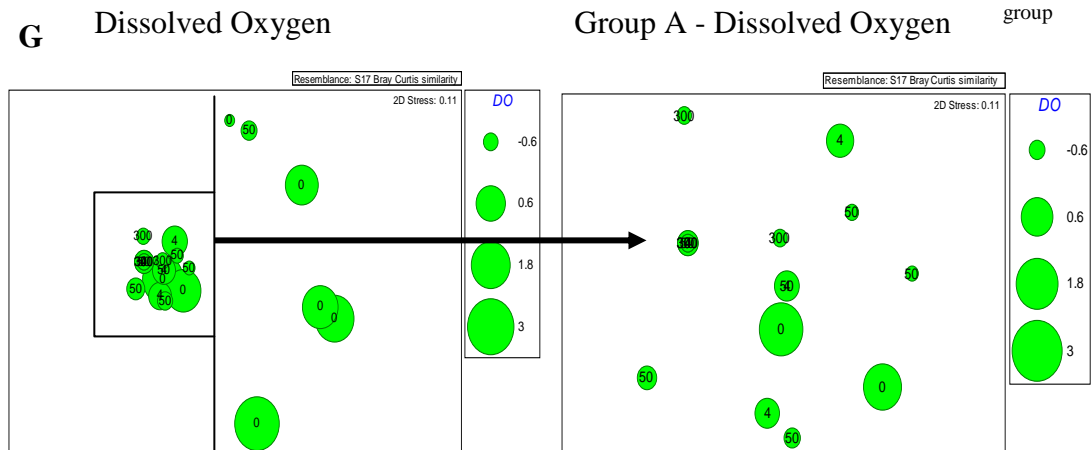
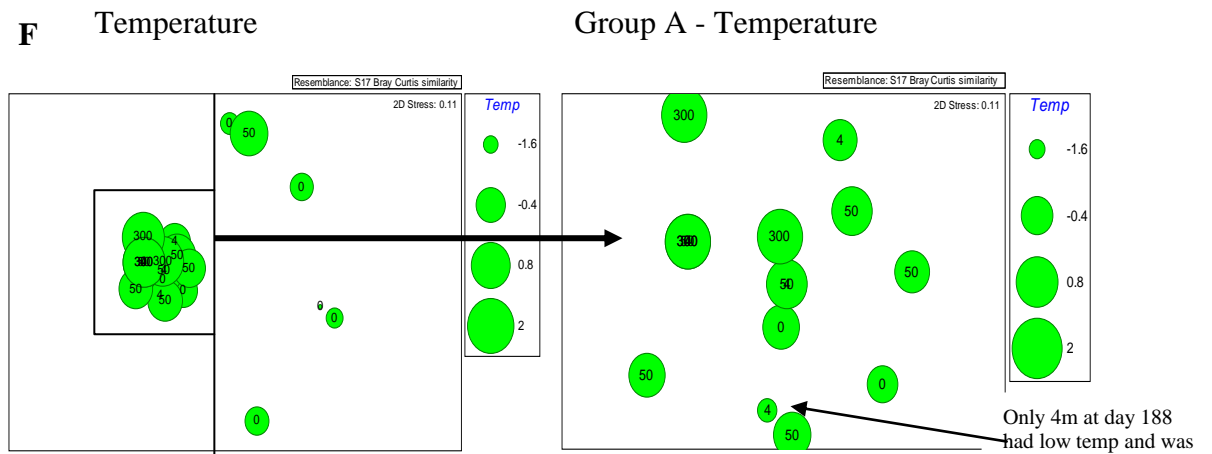
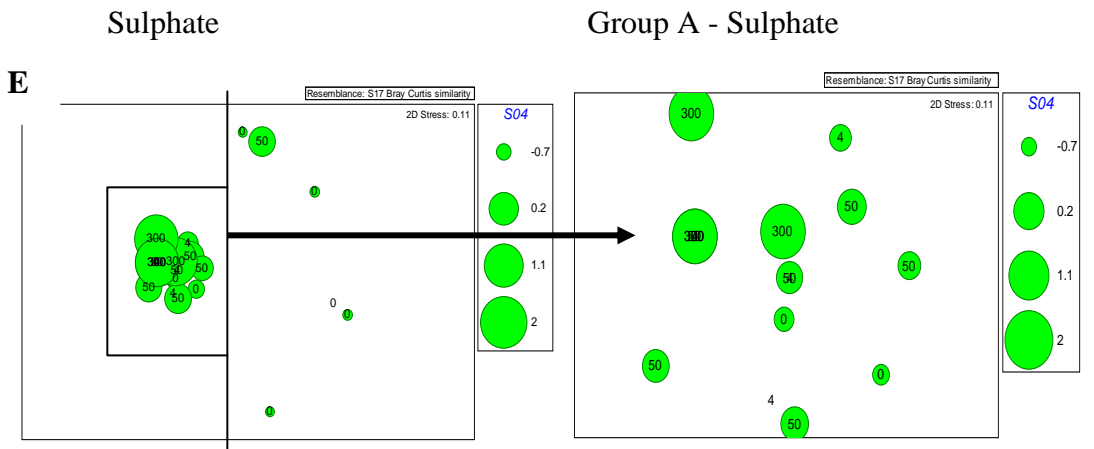
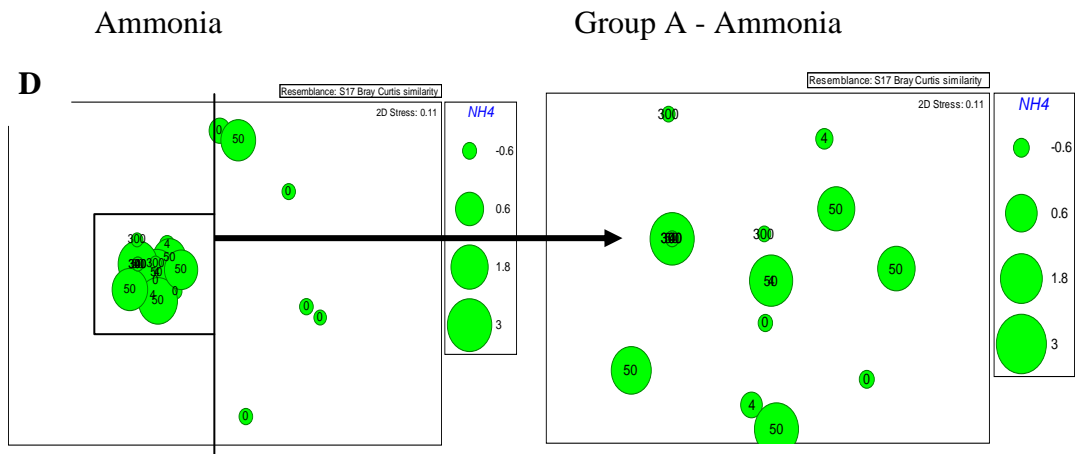
Because the injection well nitrate reducing populations were not clustered with the 4m well community structures which had similar chemistry there was a greater discrepancy between chemical parameters explaining the differences in MDS separation of bacterial communities (Figure 4-32b-i). Figure 4-33 (a) shows the change in nitrate reducing bacterial dynamics at the injection well which illustrates changes over time. Figure 4-33 (b) shows that this change in population dynamics appears to be associated with the concentration of electrical conductivity.

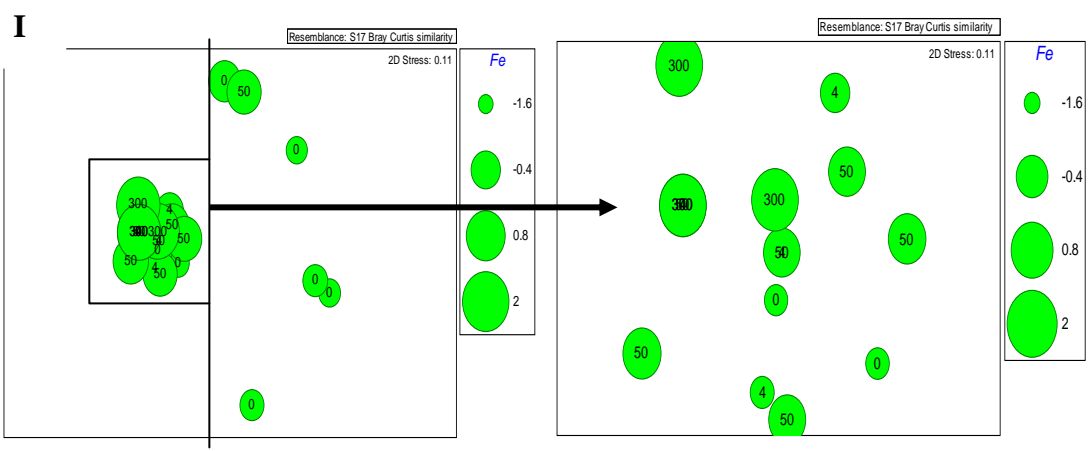
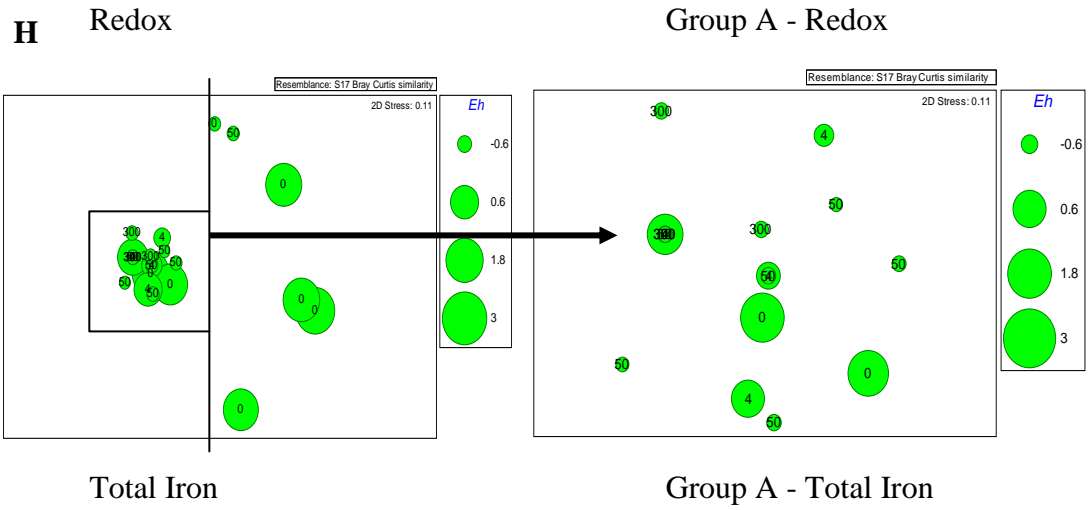
**Figure 4-31** DGGE of bacteria rDNA/PCR banding patterns and associated binary matrix table for band matching data for all Nitrate-reducing cultures from Adelaide ASR ‘water-sediment’ samples over time.



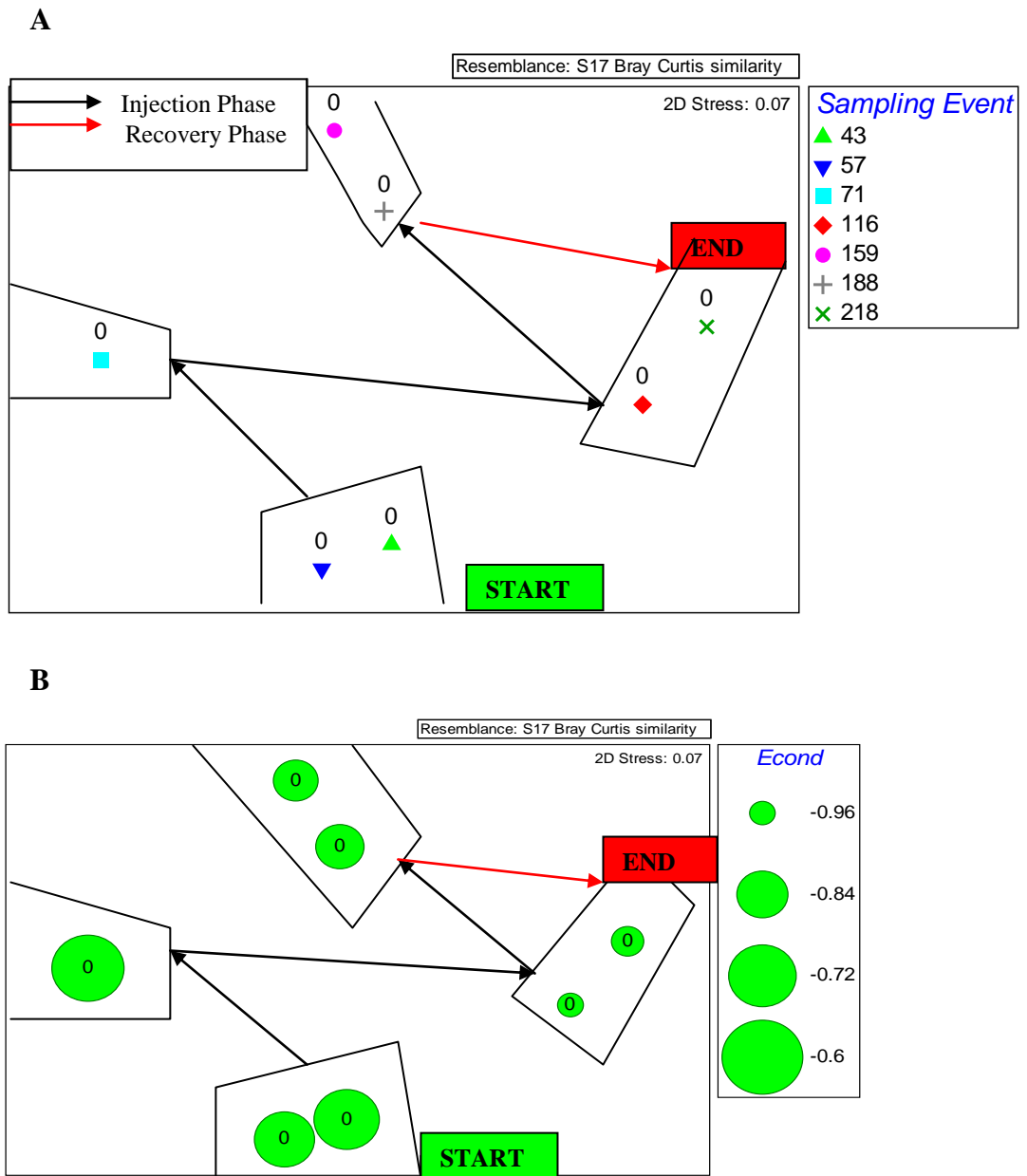
**Figure 4-32** 2D MDS Plot for amplified Microbial rDNA/DGGE banding patterns for (a) all Injection, 4m, 50m and 300m Nitrate-Reducing Cultures from 'water-sediment' samples with overlaid chemical data for (b)  $\text{NO}_2^-$ , (c)  $\text{NO}_3^-$ , (d)  $\text{NH}_4^+$ , (e)  $\text{SO}_4^{2-}$ , (f) Temp, (g) DO (h) Eh and (i) total Fe







**Figure 4-33** 2D MDS Plot for amplified Microbial rDNA/DGGE banding patterns for (a) injection well samples only for Nitrate-Reducing Cultures from 'water-sediment' samples and (b) overlaid chemical data for electrical conductivity.

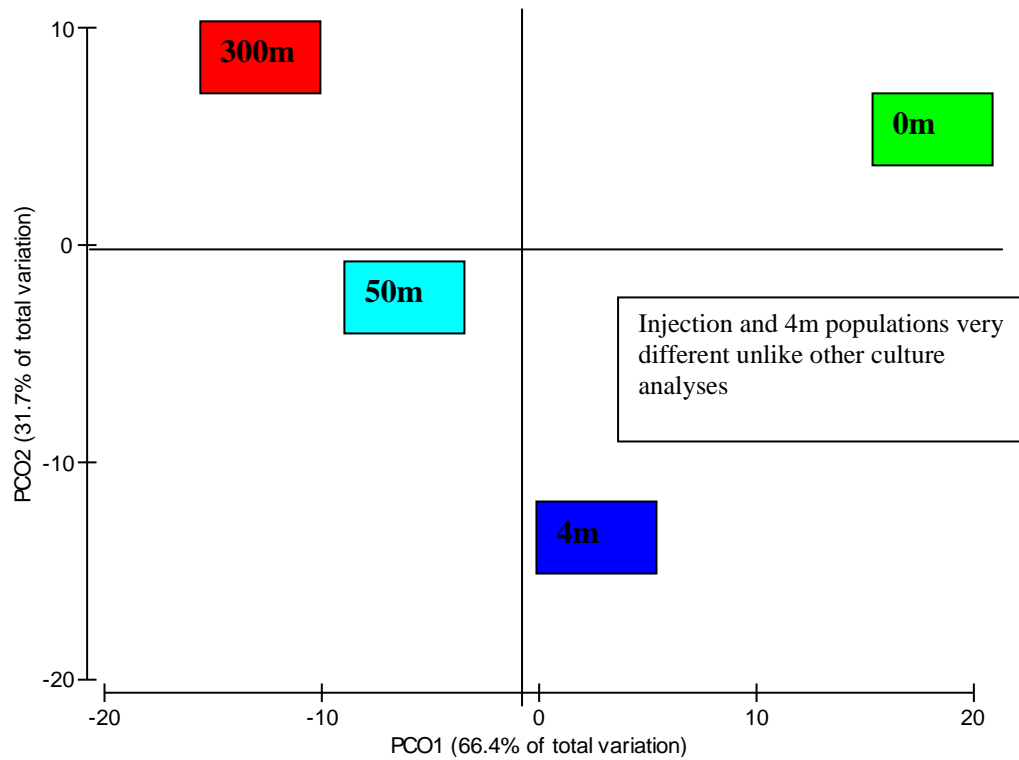


#### 4.5.3 Adelaide ASR Combined Microbial Results for Nitrate-Reducing Cultures 'Water-only' AND 'Water-Sediment' Samples

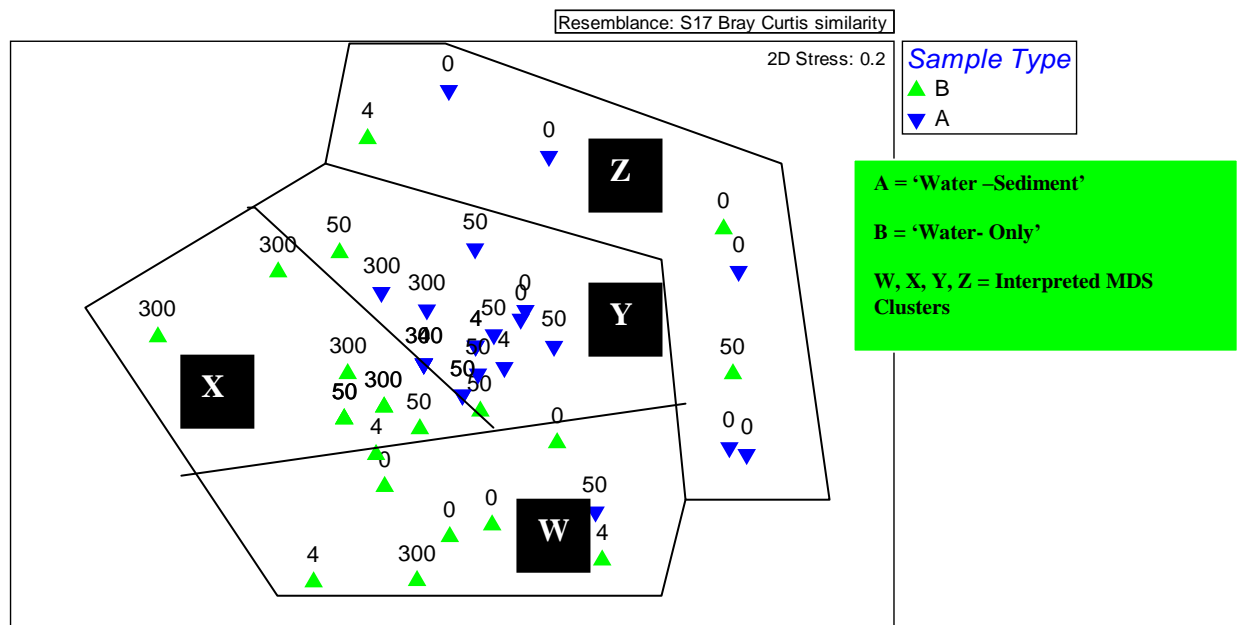
Figure 4-34 clearly shows a distinction between the injection and 4m well nitrate reducing bacterial populations. In contrast the nitrate-reducing community structure from the 50m and 300m wells were more similar in population structure.

Figure 4-35 appears to cluster samples predominantly according to distance but also the majority of samples also clustered according ecological niche. Groups W and X largely consist of 'water-only' samples whereas group W contain mainly injection and 4m well samples and group X contains 50m and 300m well samples. The 50m well samples appear to be more random and are placed in groups X, Y and Z. The 'water-sediment' samples from 50m and 300m are predominantly clustered in group Y with only one 50m well sample placed in group W. Group Z predominantly consists of injection well samples from 'water-sediment' samples.

**Figure 4-34** 2D PCO using the centroid reduced similarity matrix based on distance for the Injection, 4m, 50m and 300m well Nitrate-reducing culture samples



**Figure 4-35** 2D MDS Plot for amplified Microbial rDNA/DGGE banding patterns for all Injection, 4m, 50m and 300m Nitrate-reducing Cultures from (A) 'water-sediment' and (B) 'water-only'





## 4.6 Adelaide ASR Microbial Population Dynamics and Biogeochemical DISCUSSION

### 4.6.1 *DGGE profiles for all cultures at Adelaide ASR Site*

**Fermentative Bacteria** - The DGGE banding patterns shown in Figure 4-10 for fermentative bacteria cultured from 'water-only' groundwater samples predominantly showed strong band intensity between samples. This suggested that fermentative bacteria were easily cultured from all samples. A small number also displayed moderate band intensity. These differences in band intensity suggested there were dominant and weak fermenting populations that could be cultured from ASR samples. The DGGE banding pattern (Figure 4-14) for 'water-sediment' samples displayed similar variation in band intensity between samples to that described for 'water-only' fermentative bacteria. These results suggest that the fermentative bacteria were somewhat ubiquitous in groundwater samples for both 'water-only' and 'water-sediment' samples at all distances over time. These results therefore suggest that fermentative bacteria were able to inhabit groundwater with a diverse range of environmental conditions (

Figure 4-9 a-k).

**Sulphate-Reducing Bacteria** - The DGGE banding pattern shown in Figure 4-22 for 'water-only' sulphate-reducing cultures demonstrated that there was variation in band intensity between samples. The results clearly isolated the background bore as being a favourable environment for sulphate-reducing bacteria due to the enhanced band intensity for all samples from this distance. Sulphate-reducers occurred in only a limited number of samples from the injection well but did include the recovery sample at sampling event 218. A greater number of sampling events cultured sulphate-reducers from the 4m well compared with the injection well but the greatest number occurred for 50m well samples. Sulphate-reducing growth occurred for all sampling events at 50m.

The DGGE banding patterns shown in Figure 4-24 for 'water-sediment' sulphate reducing cultures also demonstrated that these samples had variation in band intensity between samples. The background bore also displayed elevated band intensity across all samples. Thus as also shown for the 'water-only' samples the background bore appeared to be a more favourable environment for sulphate-reducing bacteria. Analogous to the 'water-only' samples, the 50m well 'water-sediment' samples also resulted in the greatest number of sampling events which cultured sulphate-reducing bacteria. In addition similar to the 'water-only' samples the injection well also resulted in the least number of sampling events that cultured sulphate reducing bacteria. Again

sulphate reducing bacteria were cultured from the injection well during the recovery phase.

Overall these results indicate that sulphate-reducing bacteria were clearly more dominant from the background well. Although the intensity of bands from the 50m well were relatively weak in comparison, sulphate-reducing bacteria were consistently cultured from most sampling events. The sulphate-reducing population at the injection and 4m well were less easily cultured suggesting that their actual numbers fluctuated at these distances. These results suggest that groundwater environmental conditions at the injection and 4m wells were less favourable for sulphate-reducing growth during the injection phase. In contrast they were cultured from both 'water-only' and 'water-sediment' samples during the recovery phase suggesting that environmental conditions had become more favourable. These results thus indicate that sulphate-reducers migrated from greater distances e.g. 50m during the recovery phase. Alternatively the results perhaps indicate that sulphate reducers were present throughout the injection phase but were present at undetectable numbers due to unfavourable conditions. Additionally sulphate reducers may have been successful within micro-niches only and therefore were not detected during sampling.

**Nitrate-Reducing Bacteria** – The DGGE banding pattern shown in Figure 4-28 for 'water-only' samples demonstrated that two dominant bands occurred in most samples which varied in intensity. Additional random bands were also apparent between samples which were predominantly of weak intensity. The DGGE banding pattern shown in Figure 4-31 for 'water-sediment' samples was similar to the 'water-only' samples shown in Figure 4-28. There were two dominant bands which were found in most samples some of which varied in intensity. The particularly obvious visual dominance of a few nitrate-reducing DGGE bands were in contrast to the fermentative (Figure 4-10 and Figure 4-14) and sulphate-reducing bacteria (Figure 4-21 and Figure 4-24).

The 4m well nitrate-reducing cultures predominantly contained bands of greater intensity suggesting that nitrate-reducing bacteria were abundant at the 4m well as they were cultured with great success. It is clear from the DGGE gels that the injection well samples displayed greater temporal diversity as the banding patterns substantially

varied over time. In contrast the DGGE banding patterns for nitrate-reducing bacteria at the 4m, 50m and 300m wells showed temporal stability.

#### 4.6.2 *Multidimensional Scaling (MDS) overview of the Injection and 4m well cultures verses 50m and 300m well cultures.*

**Fermentative Bacteria - Figure 4-11 (a) and Figure 4-12 (b) demonstrates that the fermentative cultures from 50m and 300m ‘water only’ samples were very diverse over time. These samples did not form distinct distance-based clusters unlike ‘water-sediment’ fermentative cultures (Figure 4-15), sulphate-reducing (Figure 4-22a;**

**Figure 4-25a) and nitrate-reducing (Figure 4-29a; Figure 4-32a) cultures.**

There were notable differences between the fermentative bacteria cultured during the injection and recovery phase for ‘water only’ samples. The injection and 4m well fermentative cultures for ‘water only’ (Figure 4-11a; Figure 4-12a) formed a distinct cluster. These fermentative bacterial populations were very stable during injection of treated effluent. Injection ceased and a storage period followed with a subsequent recovery phase. The fermentative bacterial populations were notably different during the recovery phase compared with the injection phase (Figure 4-12a).

The ‘water-sediment’ fermentative cultures demonstrated a slightly different temporal pattern than described for the ‘water only’ cultures. The injection and 4m well cultures from ‘water-sediment’ samples predominantly formed a distinct cluster (Figure 4-15). Despite being distinct they contrasted with ‘water only’ fermentative cultures (Figure 4-11a) as they were more dynamic during the injection phase. Similar to the ‘water only’ cultures the 4m recovery phase ‘water-sediment’ fermentative cultures were clearly distinct from the injection phase isolates. The ‘water-sediment’ contrasted with ‘water only’ in that the recovery phase 4m fermentative cultures were also clustered with injection well samples from sampling events 43 and 57.

**Sulphate-Reducing Bacteria – Figure 4-22a clearly separated the injection and 4m sulphate-reducing ‘water only’ samples (group ii) from the 50m and 300m well samples (group i). Exceptions included the recovery phase samples at day 218 which were positioned in group (i).**

**Figure 4-25a also clearly separated the injection and 4m well ‘water-sediment’ samples (group iii) from the 50m and 300 well samples (groups i & ii). Exceptions again include the recovery phase samples at day 218 which were positioned in group (ii). In contrast with ‘water-only’ samples there were a greater number of sulphate-reducers recovered from the groundwater at 4m during the injection phase. Spatial and temporal differences between the ‘water-only’ and ‘water-**

**sediment' samples included the 300m well samples (group i) which formed a distinct group from the 50m well 'water-sediment' samples (group ii) as shown in Figure 4-25a. The MDS plot (**

Figure 4-25a) also indicated that the background bore at 300m was most dissimilar in microbial population structure to the injection and 4m well microbial community.

**Nitrate-Reducing Bacteria – Figure 4-29a clearly separated all injection and 4m well 'water-only' samples from the 50m and 300m well samples. Figure 4-32a contrasts with all previous above analyses for fermentative cultures (Figure 4-11a ; Figure 4-15a), sulphate-reducing (Figure 4-22a ;**

Figure 4-25a) and nitrate-reducing (Figure 4-29a) cultures. The injection well 'water-sediment' samples from day 71 and throughout the remaining injection cycle and subsequent recovery period formed a very distinct population. These results clearly separated the injection well samples as injection continued from the 4m, 50m and 300m well samples which was not apparent in any other analyses. These results therefore suggest there was a relationship between the sediment environment and the nitrate-reducing bacteria which particularly encouraged the formation of a different temporal community structure at the injection well.

#### 4.6.2.1 Summary of MDS Overview of Injection and 4m well cultures verses 50m and 300m well cultures

In summary the results suggest a general trend in that the injection and 4m well cultures from each biogeochemical group were clustered together on the MDS plots. In contrast the 50m and 300m well cultures were clustered together on the MDS plots. These two MDS groups formed distinct clusters separating the injection and 4m well cultures away from the 50m and 300m samples. Therefore two distinct MDS microbial population structures occurred in many cases. The results suggested that the injection and 4m well consisted of a similar microbial population structure which differed from the 50m and 300m well microbial population structure.

In addition it was apparent that the injection and 4m well recovery phase microbial samples were very different to the injection cycle. The recovery phase samples for the injection and 4m well clustered away with from all other samples at these distances. Thus these microbial results are similar to the 'chemical signature' PCA analyses where it was shown that the recovered water was very different to the injectant (Figure 4-4). These results therefore indicate a relationship between the change in environmental conditions at the injection and 4m well and the microbial population structures which developed at these distances (fully described in 4.6.3).

The 'chemical signature' PCA analyses suggested that the injection and 4m well samples were similar to each other (Figure 4-4) and the microbial analyses have also suggested that the injection and 4m well microbial population structure were very similar. In addition the 50m well 'chemical signature' analyses suggested that this distance was very different to that described for the injection and 4m well (Figure 4-4). Again the microbial analyses separated the 50m well cultures from the injection and 4m well samples in most instances. The differences in microbial community structure between distances are more fully described in 4.6.4.

#### 4.6.3 Recovery versus Injection Cycle Microbial Populations at the Injection and 4m well

Microbial evaluation of groundwater at the injection, 4m and 50m were not analysed prior to the injection cycle. Thus the background bore at 300m was taken as the ambient groundwater microbial population of the aquifer.

**Fermentative Bacteria** - Figure 4-11a illustrates the clear separation in microbial community structure for all sampling events between the injection and recovery phase for the injection and 4m well 'water-only' samples. Figure 4-15 also separated 'water-sediment' samples from the injection well between the injection and recovery cycles. An exception included sampling event 43. These results suggest that as injection progressed the fermentative bacterial population that could be cultured changed in microbial community structure but eventually returned to its 'starting population' during the recovery phase. Figure 4-11 (b-i) and Figure 4-16 (b-j) clearly demonstrated the difference in chemistry between the injection cycle and recovery phase for individual environmental parameters. These results therefore mimic trends described for the changes in chemistry between the injection and recovery phase where groundwater eventually returned to ambient conditions (Figure 4-4).

**Sulphate-Reducing Bacteria** – **Figure 4-22a illustrates the clear separation in microbial community structure for all sampling events between the injection and recovery phase for the injection and 4m well samples. Figure 4-25a also clearly separated 'water-sediment' samples at the injection and 4m well between the injection and recovery cycle except for sampling event 57 at the 4m well. These results therefore replicate the fermentative bacterial results. As injection progressed the fermentative and sulphate-reducing populations that could be cultured changed in microbial community structure but eventually returned to its 'starting population' during the recovery cycle. Figure 4-22 (b-j) and**

Figure 4-25 (b-k) demonstrated the clear difference in chemistry between the injection cycle and the recovery phase for individual environmental parameters. These results therefore again mimic trends described for the changes in chemistry between the injection and recovery phase where groundwater eventually returned to ambient conditions (Figure 4-4).

**Nitrate-Reducing Bacteria – Figure 4-29a, and Figure 4-32a for nitrate-reducing bacterial MDS analyses contrasted with fermentative (Figure 4-11a ; Figure 4-15) and sulphate-reducing (Figure 4-22a ;**

Figure 4-25a) MDS plots. The nitrate-reducing bacterial populations cultured from both ‘water-only’ and ‘water-sediment’ samples showed no distinction between the injection and recovery cycles. Figure 4-29 (b-l) and Figure 4-32 (b-r) demonstrates the substantial difference in chemistry between the injection cycle versus the recovery phase. Despite these chemical differences there was no distinction in nitrate-reducing bacterial population structure between the injection and recovery cycle.

**These results also suggest that despite potentially unfavourably conditions for nitrate-reducing bacteria to proliferate during the storage period (low nitrate concentrations,**

Figure 4-9c), these bacteria were present in sufficient number for successful culturing. It is suggested that that a longer storage period may have resulted in persistently low concentrations of nitrate, thus causing a significant reduction in nitrate reducing bacteria. It is postulated that the nitrate reducing bacteria had not decreased in sufficient number within a one month storage period despite unfavourable conditions. One exception includes nitrate-reducing bacteria that could not be cultured at 4m during the recovery phase for ‘water-sediment’ samples. Therefore these results indicate that nitrate-reducing bacteria were beginning to substantially decrease in number.

#### 4.6.3.1 Overview of Injection versus recovery Cycle at Injection and 4m well

The chemical analyses showed a clear increase in nitrate, phosphorous and oxygen and a decrease in temperature at the injection well during the injection cycle (

Figure 4-9 c,h,d,i). Many of the injected chemicals migrated to the 4m well as demonstrated by the similarity in chemical signature between the injection and 4m well

(Figure 4-6). The contrasting chemical difference between the injection and recovery phase was that during storage nitrate, phosphorous and oxygen were consumed which considerably reduced redox levels. Injection ceased during storage and thus there was no supply of nitrate, phosphorous and oxygen from the injectant. Therefore, there were very contrasting environmental conditions between the injection and recovery phase for biological growth to occur. As a result the microbial populations during the recovery phase (excluding nitrate-reducing bacteria) clustered away from the injection cycle populations. The recovery cycle microbial populations clustered more closely with 50m and 300m well samples suggesting that microbial populations returned to ambient conditions during recovery. This was also observed for the chemical profile. Therefore these results suggest that microbial populations clustered according to the chemical gradient.

It was therefore of interest to analyse further, which distances contained microbial populations which were most similar to each other. The centroid PCO analysis averages microbial community structure for each distance over time for each microbial/biogeochemical group in order to evaluate the similarity in microbial community structure between distances.

#### 4.6.4 Average Similarity/Dissimilarity in Microbial Population Structure over time between different Distances through the aquifer –the centroid Principal Coordinate Analysis (PCO)

**Fermentative and Sulphate-Reducing Bacteria** - The PCO centroid analysis combines both ‘water-only’ and ‘water-sediment’ DNA profiles in order to obtain sufficient data for each microbial group tested. The PCO centroid analysis averages the microbial diversity over time to obtain a single position on a PCO plot. The analysis combines all temporal DNA profiles at each distance for each microbial group producing a centroid similarity matrix based on distance.

Figure 4-19 and Figure 4-26 demonstrated that both fermentative and sulphate-reducing injection and 4m well bacterial populations were more similar to each other than 50m and 300m populations. The centroid PCO analyses therefore supports MDS evidence for similarity in microbial population structures between the injection and 4m wells verses 50m and 300m wells (4.6.2). The results determined that the average microbial

population structure was separated according to distance from the injected treated effluent.

**Injection created a chemical migrating plume where the chemical status at the injection and 4m well was similar (Figure 4-4). The chemical plume eventually migrated to the 50m well (determined by a conservative chloride tracer – Martin and Dillon, 2005). Despite the injectant fully penetrating groundwater at the 50m well, the overall chemical signature was much different to that observed at the injection and 4m well (**

Figure 4-9 a-k). Therefore the chemical composition of the tertiary effluent changed considerably during its migration to 50m distance. An additional ‘mixing/displacement’ with ambient groundwater would also occur creating a dilution affect.

**Nitrate-Reducing Bacteria** - In contrast to the similarity between the injection and 4m well microbial population structure for fermentative (Figure 4-19) and sulphate-reducing (Figure 4-26) bacteria the nitrate-reducing bacteria (Figure 4-34) were considerably more dissimilar between the injection and 4m distances. Figure 4-32a showed that the ‘water-sediment’ nitrate reducing bacteria at the injection well were very different. It is therefore likely that this ‘difference’ contributed to the greater dissimilarity between the nitrate reducing bacteria between the injection and 4m well. Therefore these results suggest the sediment environment created a larger divergence between the microbial populations that occurred at the injection and 4m well (4.6.6).

#### 4.6.4.1 Summary of Similarity in Microbial Population Structure between Distances

**The injection and 4m wells had similar chemical signatures when injection ceased at day 188 (Figure 4-6). The 4m well was more directly impacted from the treated effluent chemical plume compared with the 50m well (Figure 4-2 and**

**Figure 4-9 a-k). Therefore due to a greater similarity in nutrients at the injection and 4m well they contained similar microbial populations. The clustering of 50m samples with 300m samples, despite breakthrough of chemical plume at 50m is postulated as being a function of the actual chemistry of this breakthrough. During chemical plume migration many nutrients were utilised by the groundwater microbes (**

Figure 4-9 a-k) and recycled via microbial metabolism thus markedly changing the type and availability of nutrients at the 50m well. Thus despite full penetration of tertiary



effluent to 50m, the overall aquifer chemistry at this distance showed greater similarity to ambient groundwater. Thus the 50m well was less impacted by the migrating plume than the injection and 4m wells. These chemical differences and similarities between distances were also reflected in the microbial analyses. The microbial community structures mimicked trends observed for groundwater chemistry in response to the migrating chemical plume. The microbial and chemical populations between the injection and 4m wells were more similar to each other than the 50m and 300m well populations.

#### 4.6.5 Two Different Ecological Niches – ‘Water-Sediment’ (Sample Type A) versus ‘Water-Only’ (Sample Type B) Microbial Populations

**Fermentative Bacteria** – Although band intensity between ‘water-only’ and ‘water-sediment’ samples were similar, their overall respective microbial population structure were different as shown in Figure 4-20. The fermentative bacteria cultured from ‘water-only’ were more dynamic over time (defined as group Y) compared with fermentative bacteria from ‘water-sediment’ (defined as group X). The non-sediment ecological niche therefore created a more diverse population of culturable fermentative bacteria. In contrast the ‘water-sediment’ fermentative bacterial samples showed considerable temporal stability. Stability in temporal populations indicates that these dominant fermentative species were able to withstand competition over time.

Biofilms may form on particulates and thus the addition of aquifer material to the chambers may have concentrated additional nutrients from the groundwater. This may therefore have provided an elevation in the availability of electron acceptors and donators. In addition the aquifer material may have provided additional environmental micro-niches between and within sediment particles. Despite the apparent advantages for the addition of aquifer material it did not transcend into greater diversity for culturable fermentative bacteria. Free living microbes *in situ* created a more diverse range of culturable fermentative bacteria. The results suggest that the aquifer material resulted in a specialised niche which favoured opportunistic species which subsequently dominated and prevented any significant change in microbial diversity over time. There was no obvious separation of fermentative bacteria based on distance from the injection well.

Sulphate-Reducing Bacteria - **In contrast to the fermentative bacteria the sulphate-reducing bacteria were clearly separated based predominantly by ecological niche. Figure 4-27 primarily separated the ‘water-sediment’ samples defined as group Z from the ‘water-only’ samples defined as group Y. Exceptions included the 300m well ‘water-sediment’ samples Figure 4-27 - highlighted in blue). The 300m groundwater ecosystem represented a nutrient starved adverse environment (**

**Figure 4-9 a-j). High concentrations of sulphate (200 mg/L) (**

**Figure 4-9b) within the environment may indicate that sulphate-reduction was limited by available carbon resources and other nutrients required for microbial metabolism (as suggested by the chemical data**

Figure 4-9 a&c). Thus any increase in the concentration of nutrients at an interface that has the potential to develop into a biofilm may provide a competitively advantageous environment for microbial growth. Therefore perhaps biofilm formation and associated nutrient concentration at the interface created by the aquifer material resulted in a different sulphate-reducing bacterial population in ambient groundwater.

The addition of aquifer material at the 300m well may have had additional benefits compared with fermentative and nitrate-reducing bacteria in that sulphate reducing bacteria favour environments with lower redox conditions (Table 2.1). Thus the aquifer material may have provided microenvironments which encapsulated lower redox levels that sulphate reducing bacteria were able to successfully colonise. Additional nutrients via biofilm formation and lower redox levels within microenvironments already rich in sulphate perhaps created advantageous conditions for the proliferation sulphate reducing bacteria.

In addition a third cluster defined as group X was apparent which predominantly contained a small number of injection and 4m well sulphate-reducing bacteria from the ‘water-only’ samples. If the 300m ‘water-sediment’ samples are excluded, overall the ‘water-only’ sulphate-reducing cultures displayed greater temporal diversity as they were spread across both groups X and Y. These results are therefore supporting evidence described for fermentative bacteria in that ‘free-living’ bacteria are more dynamic over time.

**Nitrate-Reducing Bacteria** - There was clearly a greater separation of samples based on distance and ecological niche for nitrate-reducing bacteria (Figure 4-35) than described for the fermentative (Figure 4-20) and sulphate-reducing bacteria (Figure 4-27). The injection well samples for 'water-sediment' samples were very different defined as group Z (Figure 4-32a). The remaining 'water-sediment' samples were predominantly tightly clustered in group Y. Fermentative bacteria cultured from 'water-only', overall showed greater temporal diversity as samples were spread across more of the defined groups, in particular the 50m well samples were placed in clusters X, Y and Z. Therefore similar to the fermentative and sulphate reducing bacteria the 'free living' bacteria were more dynamic in microbial population structures over time.

#### 4.6.5.1 Summary for Two Different Ecological Niches – Water-Sediment (Sample Type A) versus Water-Only (Sample TypeB) Microbial Populations

The assessment of chambers with and without added aquifer material indicated that the aquifer material was potentially important at the 300m well for sulphate-reducing reducing bacteria. In contrast aquifer material does not produce clearly distinct clusters for fermentative bacteria which displayed temporal population stability at all distances. Similarly the nitrate-reducing bacterial also displayed temporal population stability with added aquifer material except for injection well samples which were very distinct. These results indicate that aquifer material may have contributed towards the development of stable microbial populations for fermentative and nitrate-reducing bacteria.

The chambers which received aquifer material were inoculated with native groundwater from the 300m observation well prior to placing the chambers at each distance (sampling station). Each chamber therefore, initially started with a 'background' microbial population which had previously been unaffected by the chemical plume created by injection of treated effluent. Additionally, these bacteria were collected from groundwater and thus these background microbial samples were not associated with aquifer material at the time of collection. Consequently the introduction of these microbes into a chamber containing aquifer material immediately created contrasting environmental conditions. It appears this change favoured a stable microbial population structure over time. This was perhaps due to biofilm formation creating a more advantageous environment for the proliferation of dominant microbial species that were consistently able to competitively exclude other microbial species.

Overall, bacteria cultured from ‘water-only’ samples for fermentative, sulphate-reducing and nitrate-reducing media all resulted in greater temporal diversity. These results suggest that the addition of aquifer material resulted in increased population stability over time despite the potential for additional micro-niches between and within sediment particles. A very noticeable difference to this norm between ‘water-sediment’ versus ‘water-only’ was observed for the nitrate-reducing bacteria. The ‘water-sediment’ injection well samples were very distinct.

#### 4.6.6 A unique nitrate-reducing population structure associated with aquifer material at the Injection Well and its potential links to Clogging

Whilst analysing the differences in microbial population structures between ‘water-only’ and ‘water-sediment’ samples it was noted that there was a unique bacterial community. Nitrate-reducing bacteria associated with aquifer material for injection well samples formed a very distinct MDS cluster (Figure 4-32a). Despite being distinct from all other nitrate-reducing bacteria from all other distances, these nitrate reducers were very very diverse over time (Figure 4-33). Clogging of the injection well is an issue with ASR which can dramatically reduce the hydraulic efficiency and thus economic viability of this water-reuse option. Clogging can be derived from biological growth associated with biofilm and polysaccharide formation. These results indicate that aquifer material which has the potential to form a biofilm produced a very unique nitrate-reducing microbial community structure over time at the injection well as shown in Figure 4-32a.

This unique nitrate reducing bacterial community at the injection well associated with aquifer material was evaluated. Band class types from these nitrate reducing bacteria were overlaid onto the original MDS to ascertain if unique DGGE bands were associated with this community (data not shown). These analyses did not identify a single band class type(s) that occurred in all injection well samples that did not also occur in the 4m, 50m and 300m well samples. Therefore the nitrate-reducing bacterial populations at the injection well could not solely be attributed to a single microbial species. These results may suggest that it is the actual conditions associated with the injection well that created different combinations of nitrate-reducing bacteria which perhaps favoured greater polysaccharide production. These results may therefore have important implications for the control of clogging at MAR sites.

#### 4.6.7 Chemical and Microbial Dynamics during the Injection Cycle

The groundwater chemistry was very different at the injection and 4m well compared with 50m and 300m wells across sampling events (

Figure 4-9 a-k). Nitrate predominantly increased over time at the injection and 4m well during the injection cycle (

**Figure 4-9c). Nitrate-reducers were cultured from the injection and 4m well from both ‘water-only’ and ‘water-sediment’ niches (**

Figure 4-25a ; Figure 4-29a). ‘Sediment-water’ nitrate-reducing cultures displayed greater band intensity obtained from DGGE (Figure 4-28 ; Figure 4-31). These results suggested that the addition of aquifer material created a more favourable environment for nitrate-reducing bacteria to proliferate.

**Dissolved organic carbon (DOC) consistently decreased over time at the injection well (**

**Figure 4-9a). At the 4m well the overall DOC did decrease over time but also displayed small temporal fluctuations in peaks and troughs of concentrations. Additionally there was a slight increase in DOC at the 50m well over time but the concentrations were considerably less than shown for the injection and 4m well samples. Therefore much of the carbon was consumed within a 4m radius of the injection well. It is postulated that the labile fraction of available groundwater carbon was quickly consumed in conjunction with nitrate and phosphorous at the injection and 4m well. Oxygen concentrations were very different between the injection and 4m well (**

Figure 4-9d) indicating that oxygen was quickly consumed in the near vicinity of the injection well thus indicating elevated levels of microbial metabolic activity.

It has previously been reported that organic carbon in ambient groundwater (300m) was more refractory in nature (Skjemstad et al., 2002). Thus the ‘effort’ required to metabolise DOC at 300m is expected to be greater than the ‘effort’ required to

metabolise labile DOC from injected treated effluent. Therefore injection of treated effluent did not only increase the nutrient concentration of the groundwater from oligotrophic to eutrophic but also changed the types of nutrients available for microbial metabolism which were different to ambient groundwater.

#### 4.6.8 Biogeochemistry of Nitrate and Sulphate at the Injection Well

The injection of treated effluent during the injection cycle at the injection well favoured nitrate-reducers for a number of reasons. As the injection cycle began the injected treated effluent created a constant supply of nitrate during the injection cycle at the injection well and additionally at the 4m well due to plume migration. Nitrate-reducers also had a constant supply of phosphorous and although total iron levels were low at the injection and 4m well this mineral was available for metabolic growth. Iron has often been cited as being a limiting factor for metabolic growth despite the abundance of electron acceptors and donators in the marine environment (Pakulski *et al.*, 1996; Hutchins *et al.*, 1998; Hutchins *et al.*, 1999). Small concentrations of iron are required by most bacterial groups for metabolic growth. Therefore nitrate-reducers had a constant source of nitrate, phosphorous and in addition small concentrations of total iron. Nitrate reducers favour redox zones that contain a maximum of 1mg/L of oxygen for nitrate reduction to occur. Table 2.1 indicated the potential standard free energy for various TEAPs. It is suggested that the constant supply of oxygen via injection of treated effluent maintained higher potential free energy values thus nitrate reduction out-competed sulphate-reduction. The higher levels were maintained despite metabolic activity due to replacement of consumed oxygen via injection. Many researchers (e.g., Lovely and Goodwin, 1988; Cozzarelli *et al.*, 2000) have described the formation of redox zones in groundwater from point of recharge. In unpolluted groundwater the point of recharge results in elevated oxygen levels which favours nitrate-reduction in the anoxic subsurface. Therefore it is sensible to suggest that the redox level at the injection well favoured nitrate-reducers at the expense of sulphate reducing bacteria and methanogens. The results indeed suggest that nitrate-reducing bacteria were

favoured at the injection well as these bacteria were consistently cultured from select media in contrast to sulphate-reducing bacteria.

**It is clear that conditions for sulphate-reducers were not favoured at the injection well during the injection cycle as these microbes were difficult to culture from the injection and 4m well (Figure 4-22a and Figure 4-25a). The injection well resulted in the least number of sampling events which recovered culturable sulphate-reducing bacteria compared with nitrate-reducing (Figure 4-29a and Figure 4-32a) and fermentative bacteria (Figure 4-11a and Figure 4-15a). The inhibition of sulphate-reduction at the injection well (Figure 4-22a) is likely to have occurred as a consequence of elevated oxygen and nitrate concentrations during the injection phase (**

Figure 4-9 c&d). Despite unfavourable conditions sulphate-reducers were cultured on day 43.

**Groundwater from the 300m well was used to monitor changes in the ambient microbial population structure in response to ASR at each distance. The chambers consisted of microbial populations from the 300m well as the membrane in the chambers prevented the movement of microbes larger than 0.45  $\mu\text{m}$ . Culturable sulphate reducing bacteria were recovered from the background well and all other distances, therefore sulphate-reducing bacteria were present in the ambient groundwater and were thus present in all chambers. The conditions at the injection well at sampling event 43 were considered very unfavourable for sulphate-reduction to occur e.g. high dissolved oxygen and redox (**

Figure 4-9 d&e). The initial recovery of sulphate-reducing cultures from these unfavourable conditions may thus be a consequence of high numbers of sulphate reducing bacteria present in the original ambient groundwater which were transferred to the injection well via the chambers.

**It was clear that sulphate-reduction was a more favourable process at the injection well after a one month storage period during the recovery phase (Figure 4-22a & Figure 4-25a). Despite being unable to culture sulphate-reducers as the injection cycle progressed, it is evident they were present as they were able to be recovered during the recovery cycle. Perhaps they were present in low numbers, in favourable micro niches or in a dormant state such as endospores, waiting for more favourable conditions to return. During recovery the redox decreased as the groundwater was not buffered by incoming oxygen from the injectant as occurred during the injection cycle. Additionally microbial utilisation of electron acceptors and donators were not replaced. Therefore**

there was a decline in nutrients, and as a result there was a much reduced redox environment. Despite a decline in many nutrients, sulphate was still abundant as due to unfavourable conditions for sulphate-reduction during the injection cycle. Thus due to higher levels of DOC than compared with background samples, the abundant supply of sulphate and the increasingly reduced conditions at the injection and 4m well resulted in a favourable environment for sulphate reducing bacteria. It is therefore suggested that sulphate-reducers became active during the storage phase hence their detection during the recovery cycle. In addition the enhanced reduced conditions during storage and declining nitrate and phosphorous concentrations resulted in the chemistry at the injection and 4m well being more similar to that described for ambient groundwater (Figure 4-4). Therefore as chemistry returned to ambient conditions the type of sulphate-reducers recovered from the injection and 4m well also returned to populations which were more similar to the background populations than the injection and 4m well populations than was detected during the injection cycle (Figure 4-11a).

Overall these results suggest that nitrate-reducers were predominantly favoured at the injection well during the injection phase. It was very evident during the injection cycle that sulphate-reducers were not favoured in either ecological niche (water-only or water-sediment) despite the potential for chambers containing aquifer material to produce lower-redox levels within micro-environments. The inhibition of sulphate-reduction was anticipated to occur from excessively high redox levels and abundance of nitrate due to injected wastewater creating very favourable conditions for nitrate-reduction to competitively out-compete many alternative forms of microbial metabolism. The change in chemistry during storage resulted in more favourable conditions for sulphate-reduction to occur during the recovery cycle.

It is assumed that fermenting bacteria were observed to be ubiquitous at all distances due to the intense and diverse banding patterns obtained for all distances (Figure 4-10 and Figure 4-14) and thus their contribution to specific biogeochemical cycles at specific distances along the chemical gradient will not be discussed. In addition total iron was negligible at the injection well and will only be discussed at the 4m well.

#### 4.6.9 Biogeochemistry of Nitrate and Sulphate at the 4m Well



**Dissolved oxygen and redox levels at the 4m well were greatly reduced compared with the injection well but were much higher than the concentrations shown for the 50m and 300m distances**

**Figure 4-9. Thus the chemistry at the 4m well was different to the injection well and the redox concentration at 4m varied over time (**

Figure 4-9 d&e). The possible microbial processes actively occurring *in situ* at the 4m well will be discussed.

**The recovery of sulphate reducing bacteria from ‘water-only’ samples during the injection cycle at the 4m well did not occur after sampling event 159 (Figure 4-22). The results from Figure 4-22 (b,c,d,e) show that as the injection cycle progressed to sampling event 159, sulphate concentrations declined. These results suggest that environmental conditions favoured sulphate reduction during the initial injection phase at the 4m well. Despite a reduction in sulphate, the actual decrease was relatively small (**

Figure 4-9b) but notably the decline coincided with an increase in nitrate concentration (Figure 4-22c).

**A decrease in sulphate concentration and an increase in nitrate concentration coincided with a decline in redox (**

**Figure 4-9e). These results indicate that sulphate-reducing bacteria were perhaps out-competing nitrate-reducing bacteria for this time period at 4m distance. Sulphate-reducing bacteria were only cultured on sampling events 42, 57 and 159 at the 4m well during the injection cycle. These sampling events coincided with the three large declines in redox at this distance (**

Figure 4-9e). These results indicate that sulphate-reduction was occurring at the 4m well periodically during the injection cycle when redox was observed to decline.

It is postulated that the decline in redox was caused by the proliferation of nitrate-reducing bacteria rapidly utilising resources due to favourable conditions. From sampling event 71, nitrate progressively increased at 4m. It is suggested that the continual renewal of nitrate by incoming injectant may have resulted in groundwater nitrate input exceeding removal by nitrate reduction. Therefore in this scenario an increase in nitrate concentration does not translate into a non-nitrate reducing zone.

The peaks in redox before each of the three redox declines during the injection cycles coincided with the non-recovery of sulphate-reducing bacteria. Thus it is suggested that these time periods coincided with population explosions of nitrate reducing bacteria and a decline in sulphate reducing bacteria. A substantial increase in nitrate reducing bacteria may have lowered the *in situ* redox at the 4m well resulting in more favourable conditions for sulphate reduction. The abundance of sulphate and periodic lowering of the redox levels may have given the sulphate-reducing bacteria a competitive edge on an episodic basis. Thus they were able to competitively exclude other bacterial groups such as nitrate-reducing bacteria despite availability of nitrate.

The temperature at the 4m well continually decreased during the injection cycle consistent with the decrease in the temperature of the injectant. A decrease in temperature indicates substantial mixing/groundwater displacement with the injectant (Martin and Dillon, 2005). The results suggest that as injection continued there was a progressive chemical equilibrium between the injection and 4m well which changed the chemistry at 4m. At sampling event (day 188) both the injection well and 4m well were at chemical equilibrium suggesting complete breakthrough of injectant (Figure 4-6).

The constant supply of injectant thus changed aquifer geochemistry such as the redox balance and nitrate concentrations. The continual change in aquifer geochemistry due to plume migration thus also contributed to the competitive interactions between sulphate-reduction to nitrate-reduction. In response to changes in aquifer geochemistry there were competitive interactions between nitrate-reducing and sulphate-reducing bacteria. These microbial interactions directly changed groundwater chemistry through their metabolic activities. Thus there was an interaction between abiotic and biotic changes that occurred.

The results also suggest that at times of favourable nitrate-reduction, sulphate-reducers were unable to be cultured suggesting that nitrate reducers had substantially increased in number. These results suggest that nitrate-reducing bacteria out-competed sulphate reducing bacteria which resulted in a substantial decline in sulphate reducers to the extent that there were insufficient cells for culturing. These results are therefore clearly showing how chemical parameters can explain changes in microbial populations.

**Sulphate-reducers from ‘water-sediment’ samples were also cultured during the latter part of the injection cycle in contrast with ‘water-only’ samples. These results suggest that the higher redox levels from day 71 at 4m (**

Figure 4-9e) resulted in unfavourable conditions to support growth of sulphate-reducers in 'water-only' samples. The observed growth from 'water-sediment' samples may be a function of a lower redox condition that was enclosed within micro-environment niches between and within sediment grains. Micro-environments have been reported in sediment investigations where the environmental conditions may be markedly different to overall sediment chemical concentrations (Jorgensen, 1977). Therefore perhaps the sediment facilitated lower redox conditions which facilitated the growth of sulphate-reducing bacteria in aquifer material that could not occur in 'water-only samples.

#### 4.6.9.1 Summary of Biogeochemistry of Nitrate and Sulphate at the 4m Well

It is suggested that the episodic proliferation of sulphate reducing bacteria occurred due to a lowering of the redox as a direct result of nitrate-reducing population explosions. It is suggested that the proliferation of sulphate-reducing bacteria was short-lived as environmental parameters quickly shifted back to favourable conditions for nitrate-reduction to occur. It is suggested that redox increased during sulphate-reducing metabolic activity due to the constant supply of oxygen-rich injectant. Additionally redox may have increased due to a decrease in metabolic activity by sulphate reducers compared with nitrate-reducing bacteria. Nitrate reduction is a more energy favourable reaction (Table 2.1) and may therefore have resulted in greater metabolic activity. It is suggested that there was a competitive interaction in the form of a feedback loop between nitrate and sulphate reducing bacteria where nitrate reduction was predominantly favoured. Sulphate reduction only occurred when nitrate reducing bacteria adversely changed the environmental conditions due to their metabolic activities. The constant supply of oxygen and nitrogen fed into the loop as the injection of oxygen and nitrogen swung favourable conditions back to that preferred by nitrate reducing bacteria. A recent publication has detailed competitive interactions between nitrate-reducing and sulphate-reducing bacteria for a oil reservoir injected with nitrate (Hubert and Voordouw, 2007).

#### 4.6.10 *Biogeochemistry of Groundwater Iron at the 4m well*

**The next favourable electron acceptor after nitrate is iron according to groundwater redox zonation (Table 2.1). Therefore perhaps at the 4m well the variation in temporal redox conditions also resulted in competitive exchanges between nitrate-reducing and iron-reducing bacteria. The total iron concentration in the ambient groundwater was relatively high approximating 1.4mg/L (**

Figure 4-9g). The injected treated effluent was low in iron and only reached a maximum of 0.2 mg/L during the course of the injection cycle. The eventual migration of the chemical plume to 4m perhaps contributed to a decrease in iron concentrations from sampling events 71 to 188 due to the injectant displacing the groundwater which coincided with the increase in redox.

Despite iron being the next favourable electron acceptor after nitrate, it is suggested that the lower concentrations of iron and increased redox at the 4m well presented unfavourable conditions. Thus it is improbable that iron-reducing bacteria were able to competitively exclude other microbial groups. Iron reducing bacteria were recovered from the 4m well, but it was found to be difficult to obtain DNA from iron-reducing bacteria. Therefore DGGE banding patterns were not obtained for this microbial group (data not included). Despite conclusive molecular results it is suggested that the lower concentrations of iron compared with the higher levels of nitrate and sulphate indicated that iron-reduction by iron-reducing bacteria was not a dominant microbial processes at the 4m well.

#### 4.6.11 An Overview of Biogeochemical Similarities Between the 50m and 300m well during the Injection Cycle.

Chemical Overview of 50m and 300m Well - Although the ASR plume migrated to 50m distance the overall groundwater chemistry at this distance was very different to that observed for the injection and 4m well (Figure 4-4). Additionally, in contrast with the injection and 4m well samples, the concentration of various chemical parameters were relatively stable at 50m over time as shown in Figure 4-13 (a,b,c,d).

**The 50m and 300m well samples were geochemically very similar in many ways. Both the 50m well and the 300m well contained negligible concentrations of nitrate and phosphorous which are required for metabolic growth (**

**Figure 4-9 c&h). An additional similarity between the 50m and 300m samples is that they maintained low redox levels (**

**Figure 4-9e) over time (<100 mV). In contrast total iron concentrations were highest (0.3 mg/L) in the background groundwater (**

Figure 4-9g). Despite lower iron concentrations at 50m distance it was unlikely that total iron did not limit microbial growth during the injection cycle (0.4-0.8 mg/L).

**Overall aquifer chemistry suggests that groundwater at 50m and 300m predominantly consisted of refractory carbon (Skjemstad *et al.*, 2002), limited nitrate and phosphorous concentrations and non-limiting iron and sulphate concentrations all encapsulated within an anoxic environment. The main difference between the 50m and 300m distances chemically was that the 50m well contained much higher concentrations of ammonia (**

Figure 4-9f). Elevated concentrations of ammonia at 50m distance may have possibly originated from the migrating plume. Ammonia concentrations were comparatively low in the injection and 4m wells. The results indicated that nitrate reduction was the dominant microbial process at the injection and 4m well. It is therefore suggested that nitrate was reduced to ammonia which migrated along the flow path to 50m distance. The anoxic conditions would have prevented nitrification of ammonia to nitrate and thus ammonia accumulated *in situ*.

**Biogeochemical cycles at the 50m and 300m well -** The possible microbial processes that occurred *in situ* at 50m and 300m despite nutrient restraints will be considered. Groundwater nitrate concentrations were negligible (

Figure 4-9c) and therefore it is unlikely that nitrate reducing bacteria were very prolific at the 50m and 300m wells. Groundwater sulphate levels were not microbiologically limiting at the 50m and 300m wells but sulphate concentrations were relatively stable over time (

Figure 4-9b). These results suggest that sulphate-reduction was inhibited despite low redox levels *in situ* and the recovery of culturable sulphate-reducers from these distances. Thus it is suggested that low levels of DOC and/or other necessary environmental parameters e.g. nitrate and phosphorous were absent *in situ* thus inhibiting sulphate-reducing bacteria at 50m and 300m distance.

**Sulphate-reducing bacteria were very successful once inoculated from samples into select media for all 50m and 300m sampling events. These results suggest that sulphate reducing bacteria were consistently present *in situ* in sufficient numbers for culturing. Therefore these results support the evidence for prevention of sulphate-reducing bacterial growth at 50m and 300m due to nutrient limitations. These results therefore suggest that sulphate reducing bacteria were present in a dormant state *in situ*. Despite this observation it was noted during sampling that the chambers from the 50m well had turned black from iron sulphides suggesting active sulphate reduction. Thus a geochemical process may have been buffering the sulphate concentrations despite microbial utilisation as there were only minute fluctuations in sulphate at 50m (**

Figure 4-9b) over time.

**Iron concentrations at the 50m well were consistently much higher than shown for the injection and 4m well. It is suggested that because the 50m distance was less affected by the migrating plume, total iron concentrations were less diluted than shown for the injection and 4m well. Total iron was naturally higher in ambient background (300m) groundwater (**

**Figure 4-9g) relative to all other sampling stations. Thus a substantial decrease at the injection and 4m well may be attributed to the injectant displacing ambient groundwater. Thus the greater concentration of total iron at 50m was most likely as a direct result of the lag period for the plume to migrate to 50m distance. Therefore there was a dilution delay from the injectant displacing the 50m groundwater. Additionally there was a greater opportunity for injectant hydrogeochemical changes to occur during migration. During recovery, iron concentrations increased as injection ceased and redox declined (**

Figure 4-9 g&e) resulting in favourable conditions for Fe III to Fe II reduction to occur via iron-reducing bacteria. Because total iron only was measured, differentiation between iron species was not possible. It is proposed that the more reduced conditions during storage resulted in mobilisation of some iron which thus increased the total iron recorded. Iron-reducing bacteria were cultured from groundwater at 50m and 300m distance but as DNA data is not available, significant differences in iron-reducing bacteria between distances are unknown.

#### **4.6.12 Indigenous Groundwater Microbes Adaptation to the Subsurface**

The oligotrophic nature of groundwater at 300m and 50m could possibly have limited microbial growth due to low concentrations of DOC, nitrate and phosphorous (Figures 4-9 a,c,h). Although the 50m well contained higher levels of DOC, refractory carbon as mentioned requires greater effort (Vallino *et al.*, 1996) to metabolise.

Transient fluctuations in water chemistry have been cited for the marine environment (Jordan and Joint, 1998; Archer *et al.*, 1997). Many aquatic environments represent low nutrient ecosystems and thus increases in nutrients are quickly utilised in microbial survival and growth. Therefore chemical concentrations can quickly return to low levels and subsequently maintained at low levels due to rapid and constant utilisation. The chemical levels recorded at 300m were arguably at sufficient concentrations to support active and diverse microbial populations. Many studies have indicated that aquatic bacteria, because they are predominantly starved exist in a dormant state in order to save precious energy. Thus it has been suggested that they only become active in favourable conditions such as an elevation in nutrient concentrations (Roszak and Colwell, 1987). The transfer of native groundwater microbes from 50m and 300m into a nutrient rich environment created by selective media would certainly reactivate dormant bacteria into an active metabolic growth.

Additional studies have indicated that bacteria isolated from adverse nutrient poor environments are very efficient at utilising small increases in nutrients (Montague and Dawes, 1974). Results from this study indicated that small changes in chemical concentrations were associated with changes in the fermentative community structure at 50m distance (Figure 4-18 a,b,c,d,e,f,g). Therefore it is possible that minor variations in chemical concentrations may result in a significant shift in microbial population structure. Efficient nutrient uptake by microbes has been cited as being analogous to the  $v_{MAX}$  efficiency of enzymes binding to their substrates (Griffiths and Haight, 1973). Thus more efficient nutrient uptake occurs at a lower  $v_{MAX}$ . It is therefore suggested that bacteria adapted to a nutrient starved environment can competitively bind to nutrients at a lower threshold than non-adapted bacteria (Montague and Dawes, 1974). Thus microbes adapted to oligotrophic environments may have a greater affinity for nutrient uptake. Therefore because unpolluted groundwater is generally nutrient poor any small changes in nutrient concentrations, has the potential to cause substantial changes in microbial population dynamics.

It was evident that groundwater from 50m and 300m were predominantly able to culture bacteria from a greater number of sampling events than for the injection and 4m wells. In addition growth often resulted in greater band intensity compared with the injection and 4m well samples. These results suggested that the bacteria cultured from

50m and 300m distances proliferated within the culture media. It is therefore proposed that native groundwater microbial populations at 50m and 300m distance were adapted to respond to small changes in nutrients and thus proliferated in the nutrient rich environment created by media. In contrast the microbial populations from the injection and 4m well had perhaps already changed in response to nutrients from the chemical gradient.

The microbial MDS analyses showed clear differences between the injection and 4m wells versus the 50m and 300m wells. These results therefore clearly show that bacteria which inhabit nutrient depleted groundwater at 50m and 300m distance, whether in an active or dormant state *in situ*, were very successful once transferred to nutrient rich selective media. These results also suggest that breakthrough of the chemical plume at 50m did not significantly affect microbial population dynamics as these samples behaved in a similar fashion to that described for background populations which did not receive nutrients from the chemical plume.

#### 4.6.13 Microbial Population Studies using Molecular Techniques on Microbes Cultured from Environmental Samples

Many combined studies describing microbial population dynamics and environmental parameters have concentrated on extracting DNA from the 'raw sample' (Fahy *et al.*, 2005 : Haack *et al.*, 2004). Therefore population studies principally evaluated all DNA extracted within the gene pool of a given sample. In order to relate changes in microbial population dynamics associated with target chemical parameters such as sulphate reduction, the DNA bands required further hybridization and/or sequencing for bacterial identification. This study in contrast selectively cultured bacterial groups targeted at groundwater biogeochemical processes. For example, variation in nitrate and sulphate concentrations indicated that nitrate and sulphate reducing bacteria were present *in situ*. Thus the 'raw sample' was used to inoculate specific media types and positive growth indicated the presence of specific microbial groups within the original samples.

Culturing is often not used for population studies due to the bias of culturing and the advancement of molecular techniques. This study had demonstrated the advantages of using cultures to understand biogeochemical processes occurring *in situ*. Cultures targeted at specific biogeochemical processes can be related to actual chemical changes



which occurred *in situ*. Thus microbial processes can be related to biogeochemical cycles without time consuming sequencing and hybridizations. It is suggested however that a random selection of isolated colonies from each microbial group should be tested for positive identification. An assumption of this study is that it is assumed that the DNA recovered from sulphate-reducing media belongs to sulphate-reducing bacteria. Positive identification by molecular techniques could therefore be used for quality control and assurance purposes (QAQC).

It was the intention of this study to undertake both culture and non-culture analyses in order to fully dissect aquifer biogeochemistry in response to ASR. It was learnt from this Adelaide ASR study site that the volume of groundwater collected needs to be optimised for sufficient DNA recovery for molecular analyses. This methodological problem was rectified and optimised in the following Perth MAR study.

#### 4.6.14 Conclusions

- 1) Injection of treated effluent into the aquifer only affected microbial populations in closest proximity to the injection well e.g. the injection and 4m wells. It is suggested that this was due to elevated concentrations of labile carbon, nitrate, phosphorous and oxygen associated with the injectant. These metabolites were quickly consumed resulting in uncharacteristic microbial populations within groundwater. Decreased biodiversity was not apparent as the number of bands recovered from all distances was visibly similar for each microbial group between all distances. Although there was not a decrease in biodiversity a different microbial community structure developed in response to the injectant. Thus the microbial community

structures were different between impacted and unimpacted groundwater in response to ASR.

- 2) The eventual chemical plume breakthrough at 50m did not significantly change the indigenous microbial population structure in groundwater. The bacterial populations at this distance showed similar temporal variation to that described for the background well at 300m which was unaffected by ASR. It is suggested that the chemistry of the migrating plume had sufficiently acclimated to ambient groundwater conditions due to residence time and biogeochemical changes during its migration.
- 3) The fermentative and sulphate-reducing microbial populations mimicked changes in groundwater chemistry at the injection and 4m well. It was shown that biotic and abiotic changes occurred during the injection cycle and storage period. These changes returned the quality of reclaimed water to that which was characteristic of ambient groundwater.
- 4) Nitrate-reducing bacteria did not return to their original 'starting' population at the injection and 4m well after a one month storage period. It is suggested that the sustained change in biodiversity was due to elevated and sustained nitrate reducing conditions that occurred during the injection cycle. The environmental conditions during the injection cycle resulted in vast numbers of nitrate reducing bacteria which were normally uncharacteristic in groundwater due to low nitrate concentrations. Thus it may be prudent to increase the storage period so that nitrate-reducing bacterial populations can return to their original 'starting populations' due to sustained low nitrate concentrations within groundwater.
- 5) The addition of sediment to chambers resulted in increased population stability over time despite the potential for additional micro-niches between and within sediment particles. A very noticeable difference between 'water-sediment' compared with 'water-only' samples was observed for the nitrate-reducing bacteria.
- 6) Microbial populations could be linked to the chemical gradient from ASR for the fermentative and sulphate reducing bacteria. PCO analyses clearly

showed that the injection and 4m well microbial populations were more similar to each other than the 50m and 300m well microbial populations.

## **SECTION 5 - Perth MAR RESULTS and DISCUSSION**

The Perth MAR site based in Western Australia consisted of a shallow unconfined freshwater aquifer within 6 to 8 meters of sand overlying limestone. Infiltration galleries received 50 KL/day of secondary treated sewage effluent which percolated through the unsaturated sand to the underlying limestone. The pumping bore extracted 250 KL/day of water and thus five times the volume of infiltrated treated effluent was extracted from the aquifer. Within the zone of treatment in the aquifer, extending from the infiltration galleries to the recovery bore, 15 monitoring bores were placed at different distances and depth from the infiltration gallery to study groundwater biogeochemical changes in response to MAR. Commissioning of the site occurred on

the 29<sup>th</sup> November 2005 and the observations for this study analysed groundwater samples from 22<sup>nd</sup> November 2005 to 3<sup>rd</sup> April 2006.

Statistics used to evaluate aquifer biogeochemistry include: principal component analysis (PCA) to determine overall changes in aquifer geochemistry. Multidimensional scaling (MDS) and centroid principal coordinate analysis (PCO) are used to determine the overall changes for both non-culture and culture bacterial populations. Bubble plots of individual chemical parameters are overlaid onto the microbial culture MDS plots in order to evaluate biogeochemical interactions. PERMANOVA was used to assess significant differences between observed clustering of bacterial populations detailed in MDS analyses. PERMDISP was used to assess significant differences in homogeneity of dispersion between samples.

The format of the Perth MAR section consists of the: aquifer geochemical results followed by a discussion. The microbial results are then analysed which are subsequently discussed in conjunction with the outcomes of aquifer geochemistry. Table 5.1 details the observation well specifics which can be used as a reference for geochemical and microbial Figures. The exact dates of the sampling events detailed in all geochemical and microbial Figures are shown in Table 3.5.

**Table 5.1** Sample information to be used for all graphical legends. Distance refers to distance from the Infiltration Gallery

Distance from Infiltration Galleries (m)		Sample ID		Depth below ground (m)
*-177.0	=	FLB_BG	Background Bore	15
*-10.0	=	FLB_05		10
0.0	=	FLB_GW & FLB_GE	Infiltration Galleries	0.5 to 1
2.5	=	FLB_01 & FLB_02		11 & 9
5.0	=	FLB_06		12
7.0	=	FLB_07		13
8.0	=	FLB_08 & FLB_09		15 & 14
15.0	=	FLB_10 & FLB_11		14 & 19
32.0	=	FLB_16		18
50.0	=	FLB_17	Extraction Bore	24
<b>*Negative value for distance indicates that groundwater samples are hydraulically up-gradient from the field site.</b>				

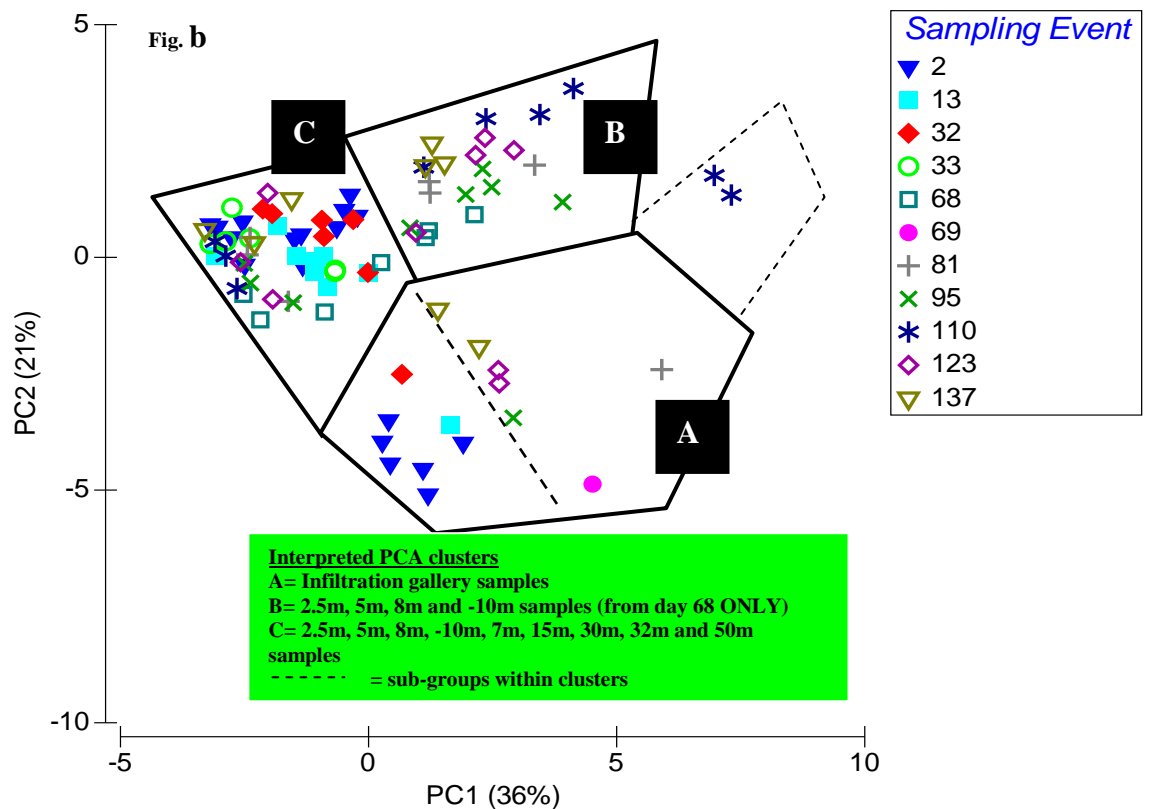
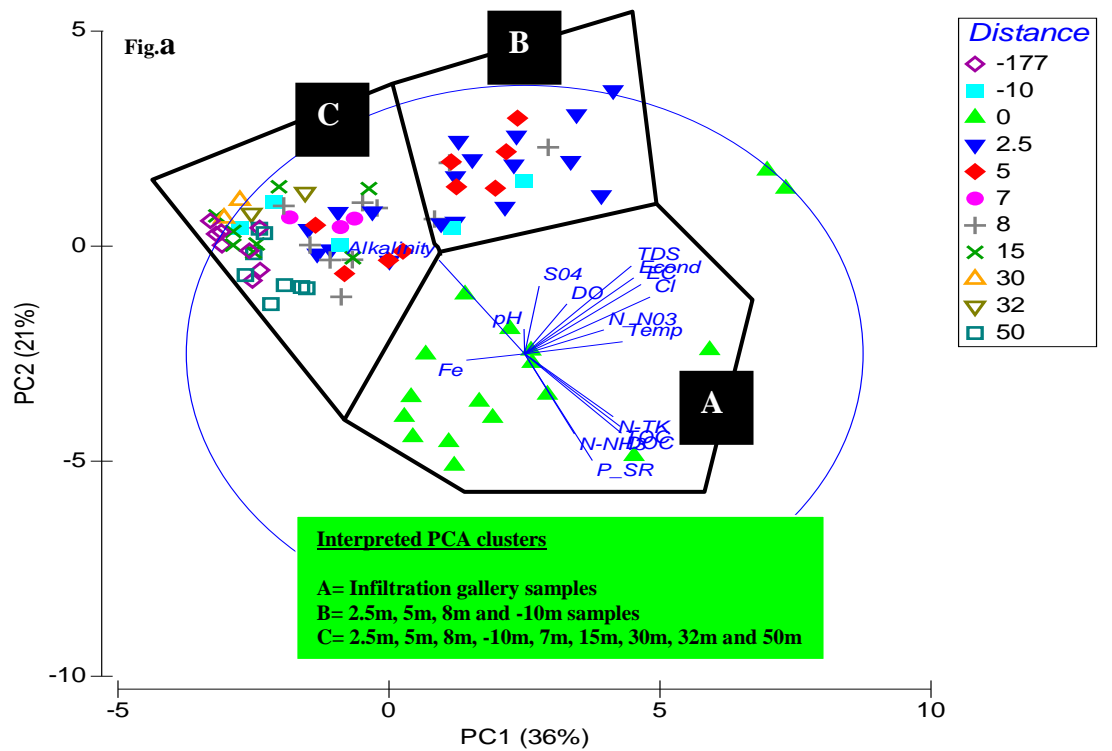
## 5.1 Perth MAR Geochemical RESULTS and DISCUSSION

### 5.1.1 *Perth MAR Geochemical Results*

In order to assess the large amount of chemical data over time for all samples (Appendix 21) a Principal Component Analysis (PCA) was undertaken. The data for all chemical analytes shown in Table 3.4 for all sampling events shown in Figure 3-5 were normalised so that data could be compared statistically on a 'like-with-like' basis. Figure 5-1 (B) demonstrates that there is a distance based nutrient gradient from the galleries over time. The gallery samples are predominantly centred by the chemical analytes (chemical vectors) being used in this analysis that form a distinct cluster defined as group A. Group B consists of samples from 2.5m, 5m, 8m and -10m only. The remaining samples defined as group C also consist of samples from 2.5m, 5m, 8m and -10m but additionally contain samples from 7m, 15m 30m, 32m and 50m. Therefore the PCA analysis separates the samples into three distinct clusters.

Figure 5-1 (b) is a replicate of Figure 5-1 (a) except the 'sampling event' is detailed in order to highlight sample differences over time. The analysis indicates that there is a slight difference in chemical composition over time for the gallery samples defined as group A. The PCA plot shows that the initial gallery samples from day 2 to 32 form a distinct cluster to that of all other gallery samples. In addition gallery samples for day 110 are placed furthest away from this cluster being in closer proximity to samples at -10m, 2.5m, 5m and 8m for sampling days 68 to 123 defined as group B. The remaining group of samples defined as group C consists of samples from group B but from earlier sampling events namely day 2 – 33. Group C consists of all remaining samples but importantly contains the background well at -177m and the extraction well at 50m for all sampling events and samples at greater distances from the infiltration gallery than shown for group B.

**Figure 5-1 PCA Plot of all Chemical Data from Perth MAR Water Samples demonstrating (a) Distance (m) from Infiltration Gallery and (b) – PCA clusters based on sampling event (days)**



In order to evaluate aquifer chemistry more effectively, the infiltration gallery information was removed because the high concentration of nutrients in these samples was dominating the analysis. The PCA plot shown in Figure 5-2 (a) illustrates the extent to which samples from 2.5 to 8m in group A are positively associated to

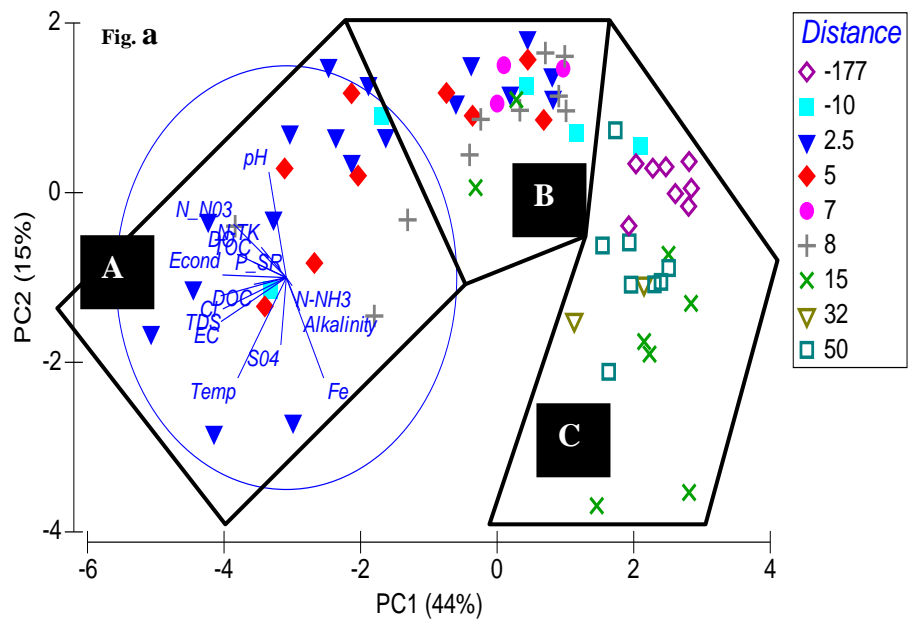
chemicals such as TOC, DOC, nitrate and phosphorous. Figure 5-2(b) excludes the chemical variables so that samples can be clearly identified. Samples from 2.5 to 8m which are clustered in group A consist of later sampling events only (day 68-123). Earlier sampling events (day 2-33) for these samples are placed in group B. Group B is located in the opposite direction of the chemical pointers for TOC, DOC, nitrate and phosphorous. A third group defined as group C contained samples at greater distances from the infiltration gallery such as the background bore at -177m, the extraction bore at 50m and samples at 15m, 30m and 32m.

An additional analysis was undertaken to assess the background chemical signature of samples from 2.5m to 8m. The results shown in Figure 5-3(a) and (b) demonstrate that background chemical signature for samples from 2.5m to 8m prior to MAR commissioning were predominantly clustered in Group C. Therefore the extraction well, background bore, all samples from 15m and 32m and samples prior to MAR commissioning from 2.5m to 8m were clustered in Group C. In addition samples prior to MAR commissioning for 5m (sampling events -2 and -3) were slightly different in their overall chemical signature than all other samples placed in Group C. These samples were not tightly clustered with all other samples from this group.

The correlation between the chemicals monitored was evaluated and full results are shown in appendix 6. Prominent positive and negative correlations are shown in Table 5.2

**Figure 5-2 - PCA Plot of Chemical Data (excluding GW) from Perth MAR Water Samples demonstrating (a) Distance (m) from Infiltration Gallery and (b) PCA cluster based on sampling event (days)**

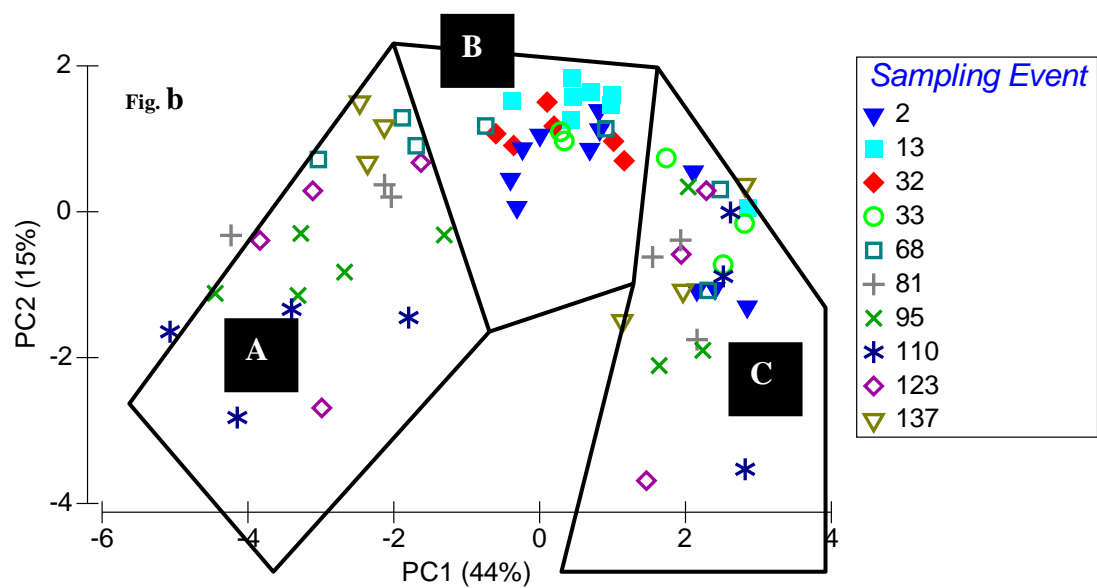




**Interpreted PCA Clusters**

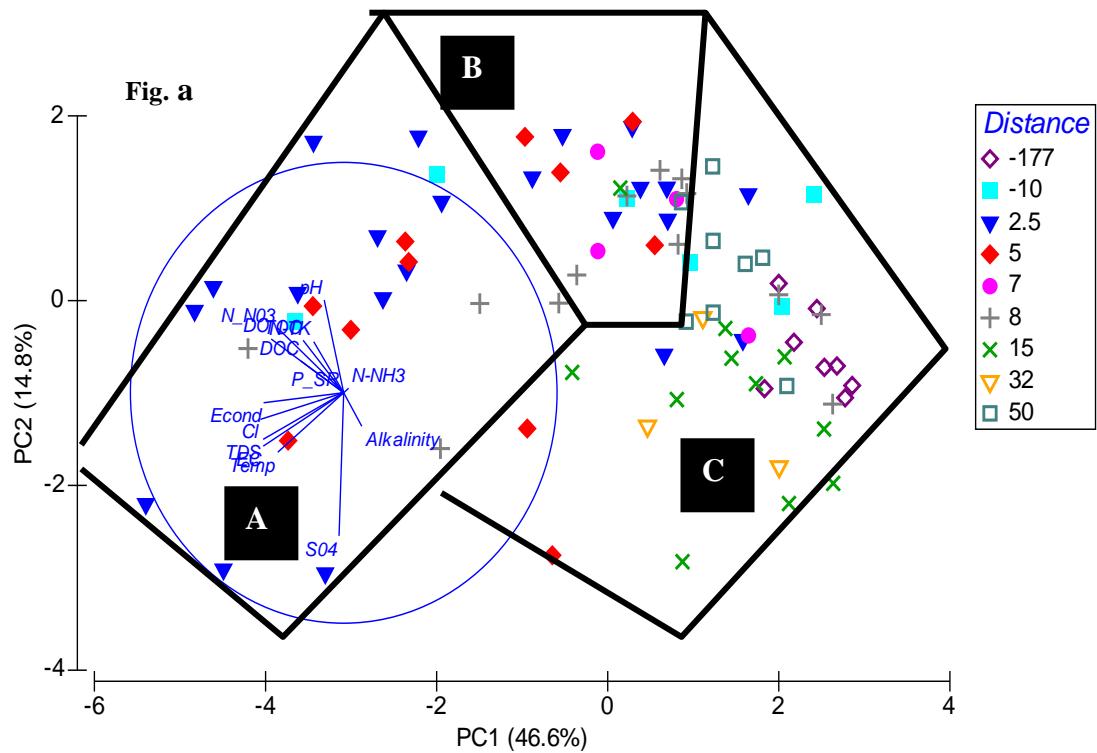
A= 2.5m, 5m, 8m and -10m samples (from day 68 ONLY)

B= 2.5m, 5m, 8m and -10m and 7m (from day 2 to 68 ONLY)



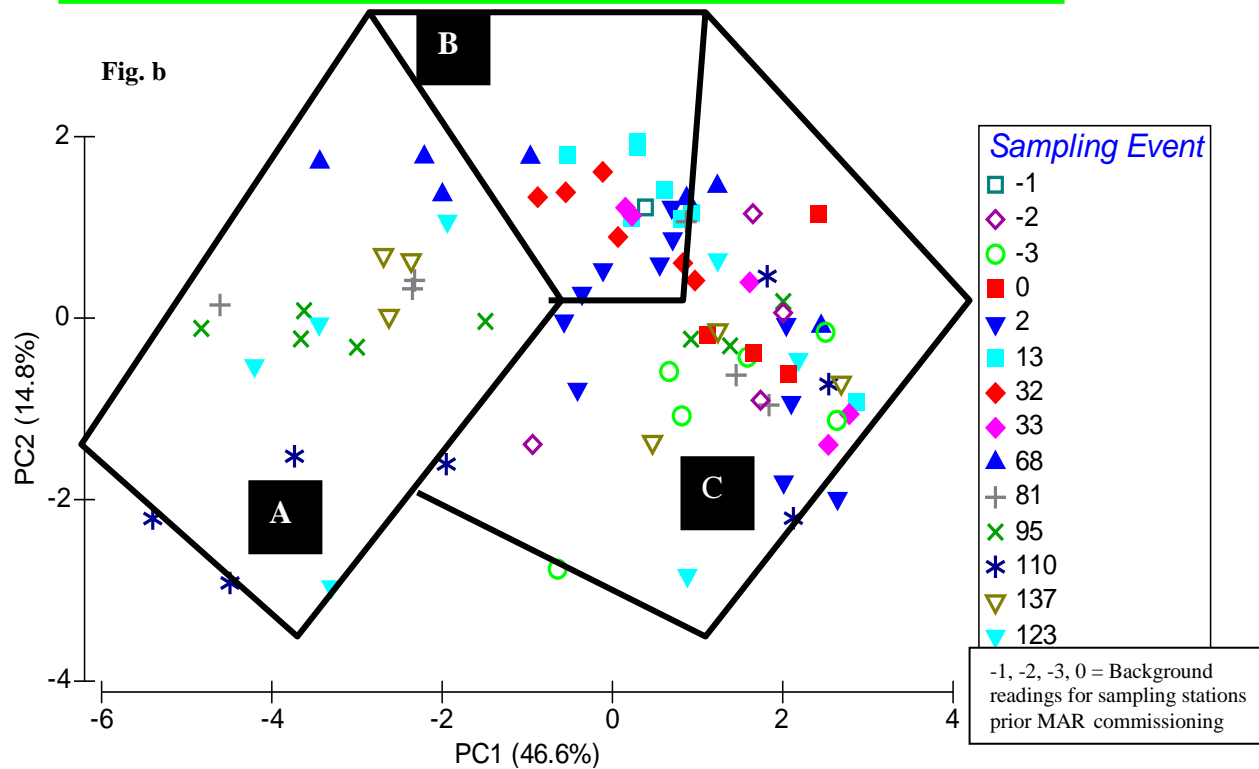
**Figure 5-3 - PCA Plot of Chemical Data (excluding GW) from Perth MAR Water Samples demonstrating (a) Distance (m) from Infiltration Gallery and (b) PCA cluster**

based on sampling event (days) – Including background readings for all sampling stations prior to MAR commissioning



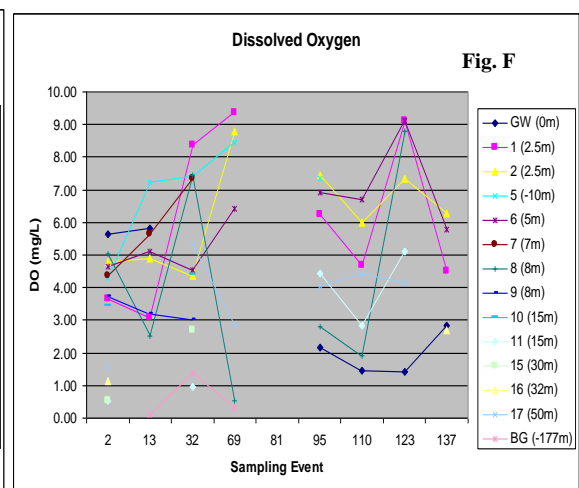
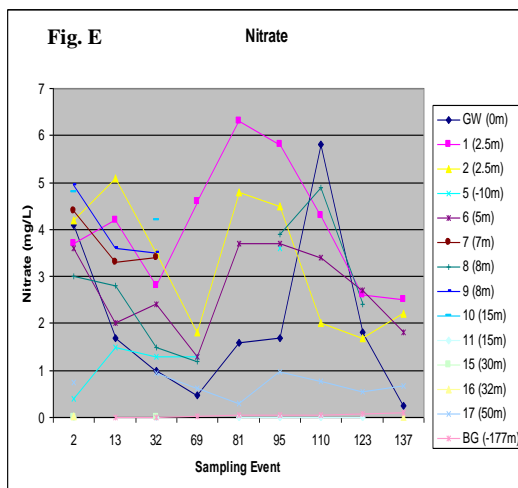
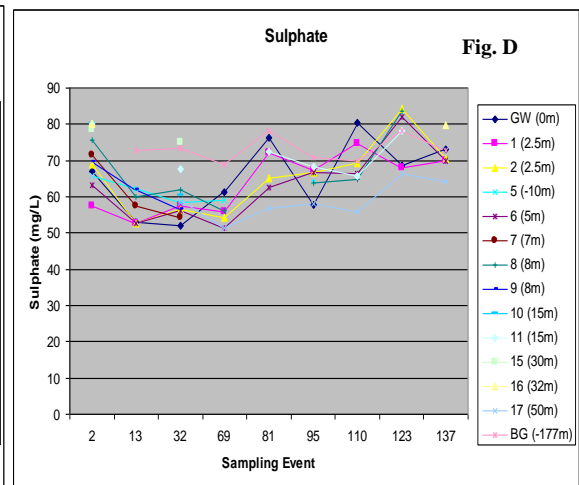
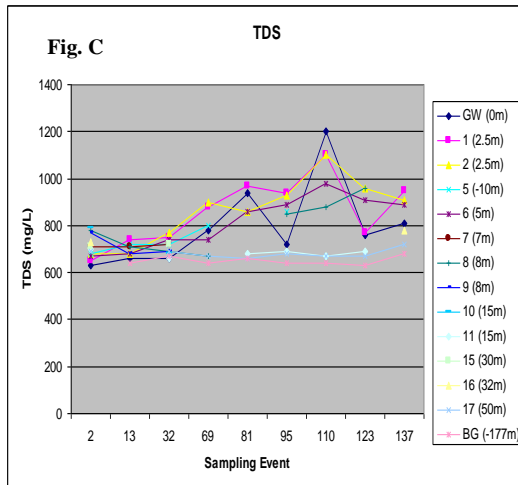
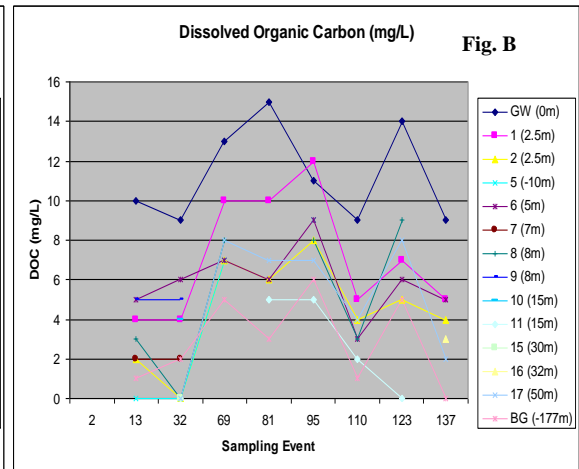
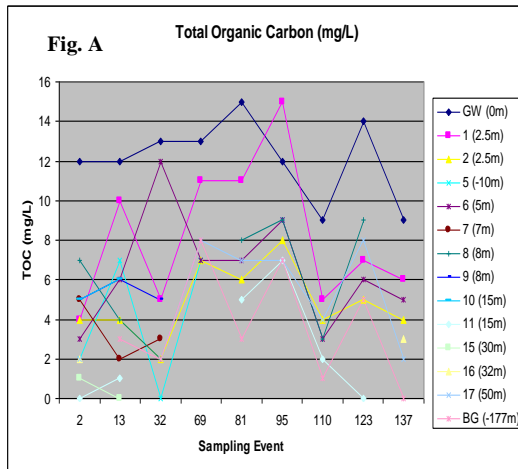
**Interpreted PCA Clusters**

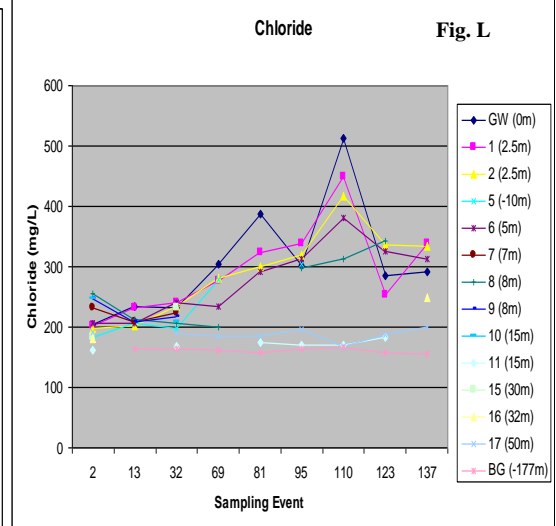
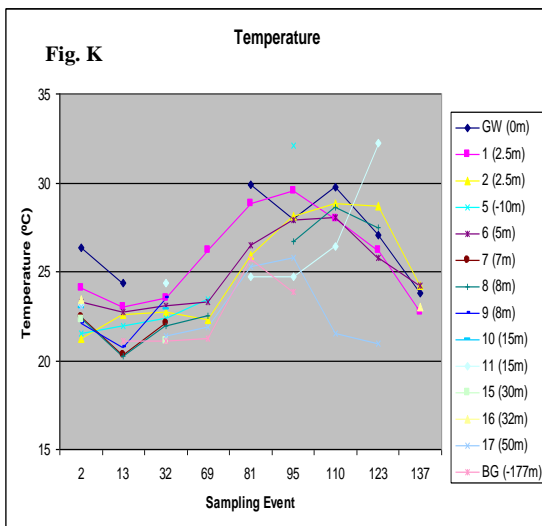
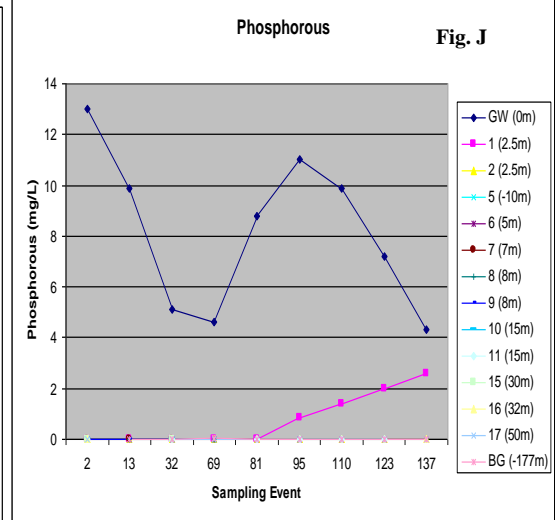
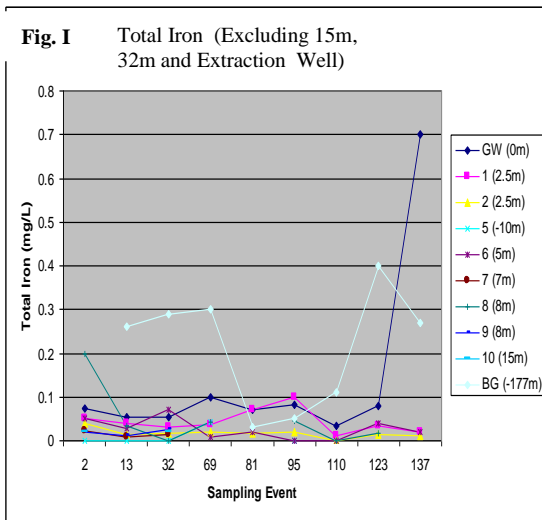
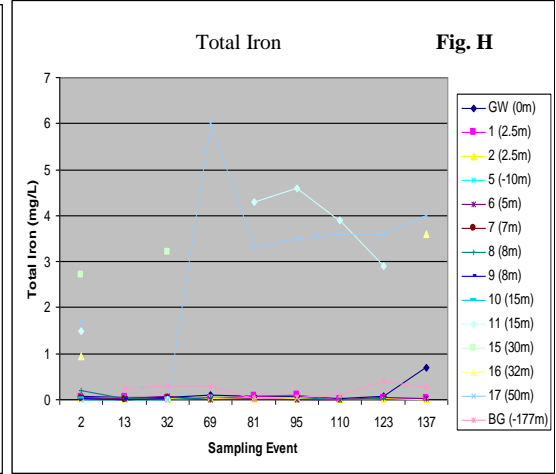
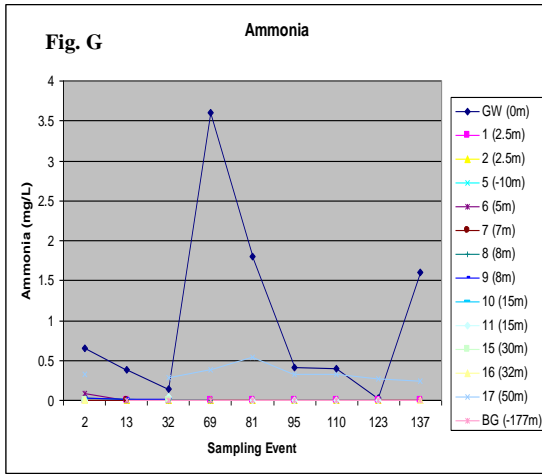
A= 2.5m, 5m, 8m and -10m samples (from day 68 ONLY)  
 B= 2.5m, 5m, 8m and -10m and 7m (from day 2 to 68 ONLY)  
 C= 15m, 30m, 32m and 50m samples AND background readings for all sampling stations prior to MAR commissioning

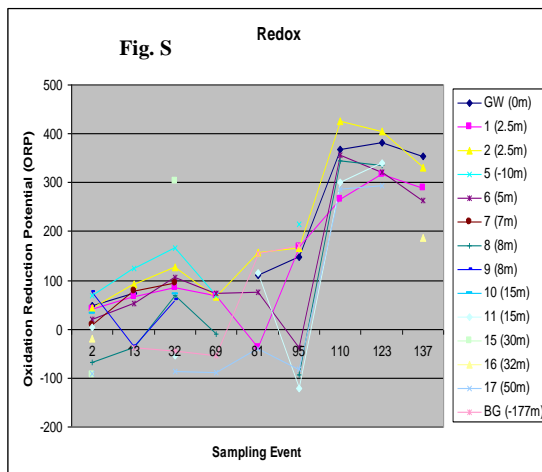
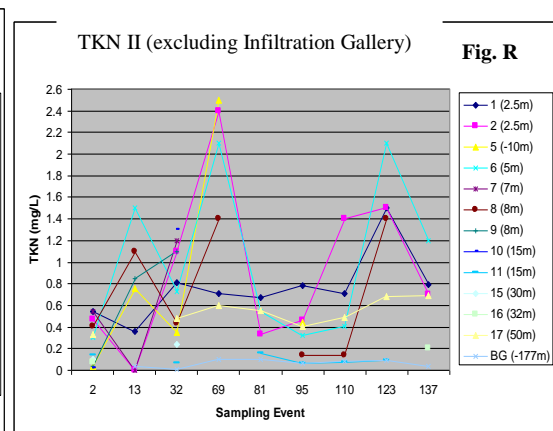
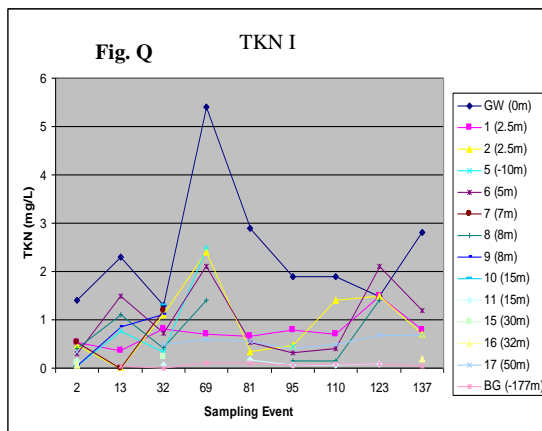
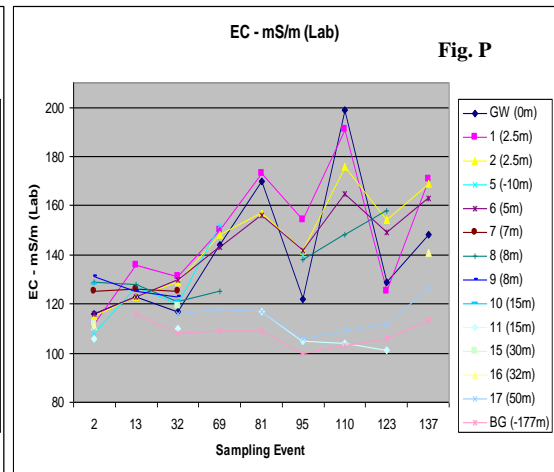
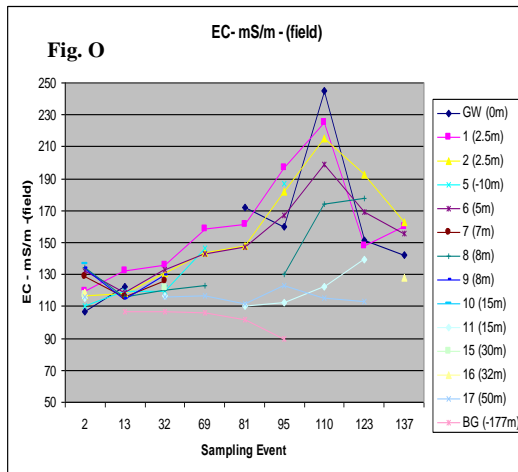
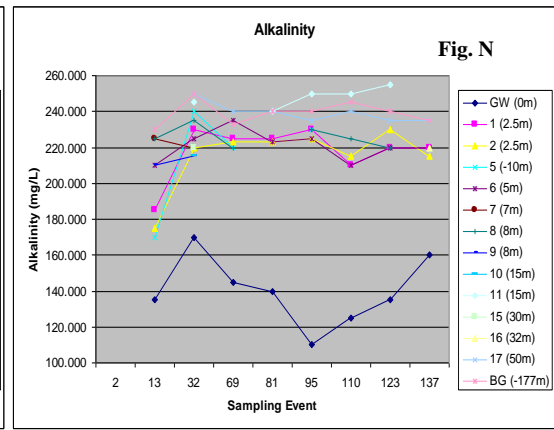
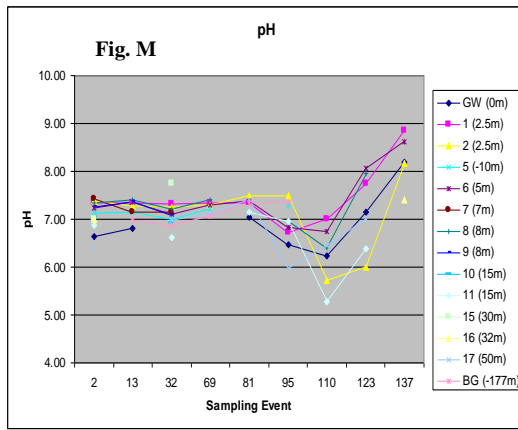


**Figure 5-4** Line graphs for Individual Chemical parameters: (A) TOC, (B) DOC, (C) TDS, (D) S04, (E) N03, (F) DO, (G) NH3, (H) Total Fe, (I) Total Iron, (J) P-SR, (K)

Temp., (L) Cl-, (M) pH, (N) Alkalinity, (O) EC-field, (P) EC-Lab, (Q) TKN-I, (R) TKN-II and (S) ORP







**Table 5.2** Prominent results from appendix 8 - Correlation values for Perth MAR Environmental Chemicals derived from all measurements over time for all sampling stations.

Group A	Group B	Group C	Group D
Chloride vs. +0.93 Econd	Alkalinity -0.67 vs. DOC	DOC vs. N- +0.69 TK	N-NH3 +0.70 vs. N-TK
Chloride vs. +0.93 TDS	Alkalinity -0.82 vs. P_SR	DOC vs. +0.71 P_SR	
Econd vs. +0.93 TDS	Alkalinity -0.72 vs. TOC	TOC vs. = +0.68 P_SR DOC vs. +0.86 TOC	

- A) Chloride was positively correlated to electrical conductivity. These two analytes were also strongly associated to total dissolved solids (TDS).
- B) The results show that dissolved organic carbon, total organic carbon and phosphorous were negatively associated to alkalinity
- C) The results show that TOC and DOC are highly correlated. In addition phosphorus is associated with both TOC and DOC. In contrast total nitrogen is 0.69 correlated with DOC whereas TOC is only 0.57 correlated to nitrogen (appendix 8).
- D) Ammonia is correlated to total nitrogen although no other nitrogen variables are correlated.

### 5.1.2 *Perth MAR Geochemical Discussion*

The principal component analyses (Figure 5-1 and Figure 5-2) incorporated all chemicals described in Table 3.4 which were used to track changes in chemical signature over time (Figure 3-5). The PCA analyses suggested a spatial and temporal chemical gradient from the infiltration gallery. Primarily, the infiltration gallery samples displayed a very different chemical signature to all other distance-based samples (Figure 5-1a). Samples from the infiltration gallery were collected from the gallery distribution sumps. The collected wastewater samples therefore had not interacted with any potential hydrogeochemical diluents such as ambient groundwater, soil or limestone at this MAR site before collection. The infiltration gallery samples for this primary reason contrasted to all other sampling stations. All other sampling stations were located several metres below and at varying distances from the infiltration gallery in the underlying limestone at the water table depth.

PCA analysis of the infiltration gallery indicated temporal variability in chemical signature (Figure 5-1b). The chemical constituents of treated effluent from wastewater treatment plants can show random variability. Diurnal variability related to consumer demand and seasonal weather patterns have been implicated. The results shown in Figure 5-1 (b) in contrast suggested systematic temporal variation in chemical signature from the infiltration gallery. Sampling events 2 to 33 were clustered together (Figure 5-1b) in a subgroup that was different to all other infiltration gallery samples from later sampling events. As time progressed, the chemical signature of the infiltration gallery changed from its initial clustering at day 2 to 33 but stabilised again from day 68 to 95. Stabilisation in chemical signature may indicate the establishment and stabilisation of a microbial community within the infiltration gallery thus influencing wastewater chemistry. An additional subgroup from the infiltration gallery occurred at day 110 (Figure 5-1b). It can be shown from Figure 5-4 Figures 5 (L,P,S) that chloride, electrical conductivity and redox peaked at this sampling event. In contrast Figure 5-4 (A,B,J,M) show that TOC, DOC, phosphorous and pH declined. These results suggest that organic carbon was being removed at the infiltration gallery. Removal of organic carbon via microbial metabolism also leads to the depletion of electron acceptors thus resulting in chemical changes over time e.g. nitrate Figure 5-4 (e). These results suggest that the chemical signature of treated effluent sampled from the infiltration gallery changed over time. The chemical signature did not appear to change randomly but in a progressive manner.

Plume migration from the infiltration gallery was fully analysed in Figure 5-2(a&b). The PCA plots clearly separated samples into three distinct groups. Group A predominately consisted of samples from 2.5m and 5m from sampling events 68 to 137, whereas group B also contained these samples but from sampling events day 2 to 33 only.

These results suggest the chemical plume from the infiltrated wastewater influenced aquifer chemistry at 2.5m and 5m somewhere between day 33 and 68. The implication of plume migration at this time period was based upon the shift in chemical signature from group B to group A. Figure 5-2a indicated that many of the samples from group A were positively associated with chemical variables such as DOC, nitrate, phosphorous and temperature. This result indicates that samples within group A, contained greater concentrations of these chemicals compared with Group B samples. Therefore these results suggest that as time progressed, the bores closest to the infiltration gallery (2.5 and 5m) received an elevation in nutrients. Samples from 8m were also placed in Group A and B. In contrast with samples at 2.5m and 5m, 8m samples did not migrate to group A until sampling event 95. These results suggest a greater lag period in plume migration in groundwater at greater distances from the infiltration gallery.

Group C contrasted with Groups A and B as this group predominantly did not contain samples from any of the distances placed in groups A and B. Samples within Group C did not appear to be affected by plume migration as these samples did not vary from group to group over time. These results suggest stability in chemical concentrations over time. In addition Group C was placed in the opposite direction of the chemical variables detailed in the PCA plot. Clustering away from these chemical variables suggests a negative association. These results therefore indicate that samples placed within Group C, contained a lower concentration of most chemicals (except for iron and sulphate) compared with Groups A and B.

Group C contained samples from the background and extraction bore and samples from 32m and 15m. The background bore was located 177m northeast and hydraulically up-gradient from the infiltration gallery. The background bore was therefore geographically located at greatest distance away from the infiltration gallery. The extraction bore was the next distance to be located at greatest distance from the



infiltration gallery followed by 32m and 15m samples. All these samples at greatest distance from the infiltration gallery were thus placed in Group C. The stability of temporal chemical signature for this group indicates that the background bore, extraction well and samples from 32m and 15m were unaffected by the migrating nutrient plume. These results therefore suggest that samples at greatest distance from the infiltration gallery were least unaffected by the migrating chemical plume in the sampling period analysed.

The extraction well was placed in group C with the background samples. These results suggest that the recovered water had sufficiently improved in water quality as the extraction bore was clustered with the background samples. The extraction well was particularly being monitored for its ability to extract water which was suitable for irrigation purposes. The improved water quality changes of recovered water may have occurred for a number of reasons. The infiltration galleries received 50 KL/day of treated effluent and the pumping bore extracted 250 KL/day. Therefore a greater volume of water was recovered than entered the aquifer in the form of treated effluent. The increased hydraulic gradient created by pumping out 250 KL/day would have the affect of drawing groundwater into the extraction bore. It is suggested that groundwater would enter the extraction well from different directions around and at varying depths determined for the extraction bore. Therefore extraction bore samples did not consist of groundwater from the actual defined depth and distance of the extraction well. Extracted water was a combination of recovered treated effluent and groundwater. Despite containing an element of treated effluent the recovered water clustered with background bore samples when analysing the overall chemical signature. These results thus indicate that the element of treated effluent within recovered water had improved in water quality from its migration for the infiltration gallery.

Group C also contained samples from observation bores at 15m distance, 20m depth (FLB\_11) and 32m distance, 18m depth (FLB\_16) suggesting that breakthrough of the nutrient plume did not intersect these depths at these particular distances. Therefore sampling stations at greater distance and depth from the infiltration gallery showed temporal chemical stability. Non-variability in groundwater chemistry suggested these sampling stations, similar to the background and extraction bore were also unaffected by the nutrient plume. These results may therefore indicate preferential flow paths within the limestone aquifer so that the migrating plume either bypassed or had not yet

migrated to these distances and depths. Group C is therefore indicative of samples unaffected by plume migration. Samples in group B ranged from 2.5m to 8m distance from the infiltration gallery. These sampling stations subsequently migrated to group A at sampling event 68 suggesting plume migration. The conclusion that Group C was indicative of ambient groundwater conditions suggests that samples from 2.5m to 8m, which were clustered in Group B, had already received plume penetration at sampling event day 02.

The MAR site was commissioned on 29<sup>th</sup> September 2005. Microbial and chemical analysis for this particular investigation did not begin until 22<sup>nd</sup> November 2005. Therefore treated effluent infiltrated for fifty five days prior to microbial and chemical analysis. Treated effluent had already had the potential to migrate from the infiltration gallery prior to sampling event 02 for this study. Some chemical information was available for a selection of sampling stations prior to commissioning and therefore this data was analysed in conjunction with post-commissioning data as shown in Figure 5-3 (a) and (b). These results predominantly show that samples prior to MAR commissioning (sampling events -1, -2, -3 and 0) from 2.5m to 8m were clustered in Group C. The pre-commissioning samples therefore clustered with the background, extraction, FLB\_11 and FLB\_16 sampling stations that have already been shown to display temporal chemical stability. Thus the pre-commissioning samples at these distances represent ambient groundwater for sampling stations 2.5m to 8m. The results therefore suggest that the plume migration had already occurred at sampling event 02, a time point from which microbial analysis was undertaken. Despite potential breakthrough of nutrients at sampling event 02, the results suggest that only partial breakthrough of the plume had occurred, as these sampling stations subsequently migrated to Group A from sampling event 69. Figure 5-3(a) and (b) therefore details a progressive association to nutrients over time for sampling stations at 2.5m to 8m distance from the infiltration gallery. In addition Figure 5-3(a) and (b) suggest the background chemical signatures from 5m (sampling events -2 and -3) were slightly different to that of all other samples in Group C. They were considered to be slightly different as they were not tightly clustered with all other samples in Group C. Despite a slightly different starting chemical signature the 5m samples behaved similarly to samples from 2.5m and 8m once partial breakthrough of plume migration was detected in Group B.

In summary, overall the chemical analyses clearly demonstrated spatial and temporal plume development from the infiltration gallery. Importantly the results suggested that the background well and extraction well were unaffected by plume migration, with the extraction well being unaffected due to the large dilution effect of pumping 5x the infiltration rate. Samples from 15m distance, 20m depth (FLB\_11) and 32m distance, 18m depth (FLB\_16) were also placed in group C. Samples defined as Group C overall showed temporal chemical stability suggesting these samples were unaffected by plume migration over time. The infiltration gallery which contained undiluted treated effluent direct from the wastewater plant was clearly very different chemically to all other samples. Breakthrough of treated effluent into groundwater was first detected at observation wells which were closest to the infiltration gallery e.g. 2.5m and 5m. Initially the analysis suggested that breakthrough did not occur until after day 33. Analysis of background chemical data demonstrated that partial breakthrough of the chemical plume had already occurred at sampling event 02. There was a progressive move to a more positive association to nutrients over time for sampling stations in close proximity to the infiltration gallery. In addition there was a greater lag phase in breakthrough of plume migration for observation wells at greater distance from the infiltration gallery. For example the chemical signature at 8m did not increase in chemical concentrations until after day 68 as opposed to day 33 for sampling stations 2.5m and 5m.

## 5.2 Perth MAR Microbial non-Culture RESULTS

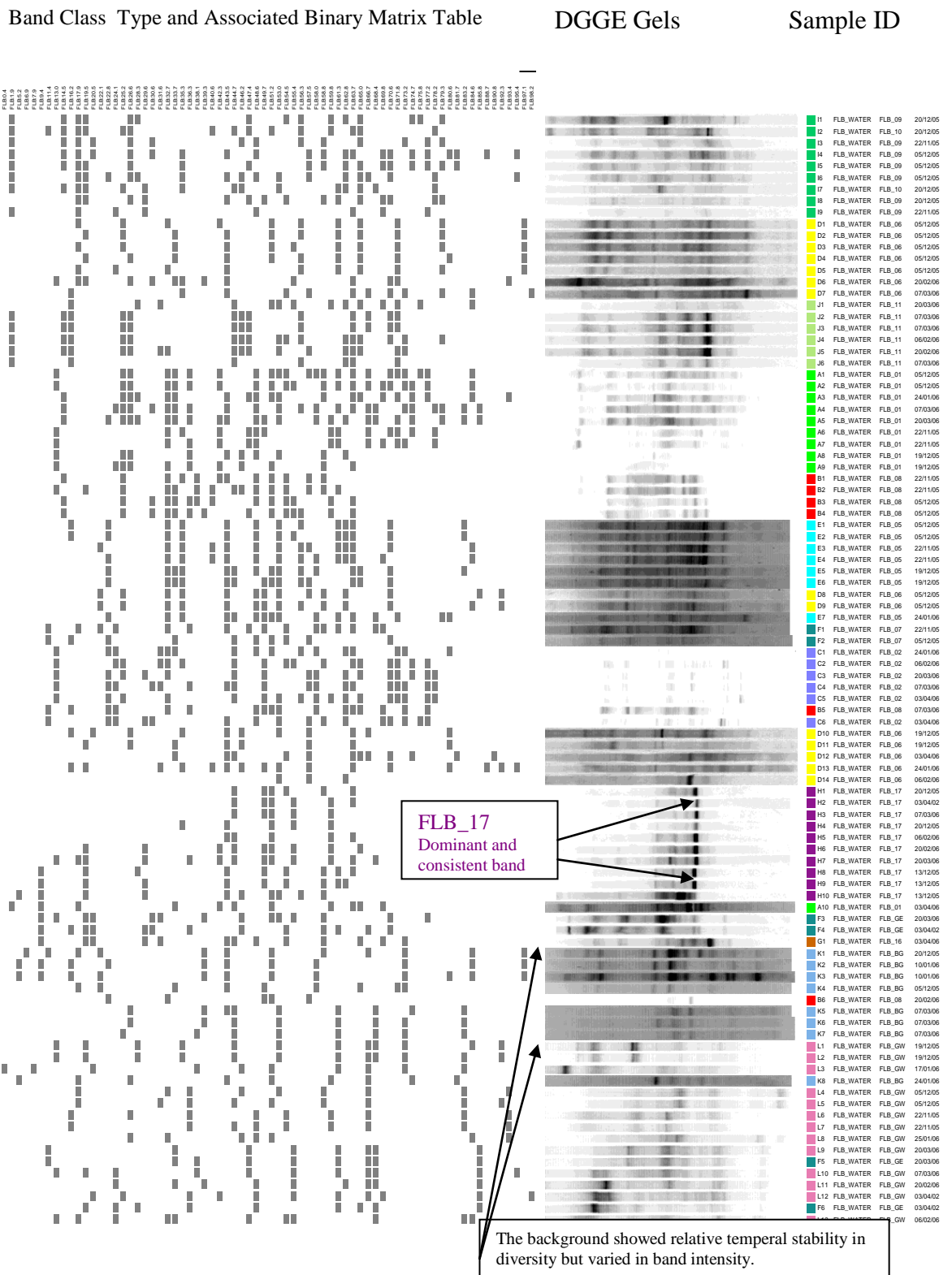
### 5.2.1 Microbial Diversity of Perth MAR Infiltration Site for non-culture Water Samples via DGGE DNA banding patterns

Figure 5-5 shows the DGGE DNA banding patterns for all non-culture water samples from the Perth MAR site over time. The associated binary matrix table is also shown. The binary matrix table was created by matching bands for all samples for presence/absence of an assigned band group. The band class groups range from FLB: 0.4 to 98.2. There are seventy two band class types in total detected over the full range of samples over time. Figure 5-5 also illustrates that the extraction bore (FLB\_17) is clearly different to that of all other samples as this sampling station contains a consistent and dominant band over time. In contrast all other samples vary in band intensity and diversity of band class types. Thus unique band class types that occur over time between sampling stations at different distances are not clearly visible.

### 5.2.2 Multidimensional Scaling analysis of microbial DGGE rDNA banding patterns from Perth MAR non-culture water samples over time

The two dimensional MDS plot shown in Figure 5-6 indicates potential clustering of distance based groupings of distinct microbial populations. These microbial populations are indicated as groups A, B, C and D namely the infiltration gallery, extraction well, background bore and a miscellaneous group which incorporates all other remaining populations. The background bore and extraction well are placed on the most opposite furthest side to the gallery samples. The remaining samples denoted as D consist of bacterial populations from 2.5 to 32m from the infiltration gallery.

**Figure 5-5** DGGE of bacterial rDNA/PCR banding patterns and associated binary matrix table for band matching data for all non-culture water samples from the Perth infiltration site over time.



5.2.3 Principal Coordinate Analysis (PCO) of amplified Microbial rDNA/DGGE banding patterns for all Perth MAR non-culture Water Samples over time

**Table 5.3 illustrates that 72% of microbial variation as assessed by DGGE DNA banding patterns can only be explained over five axes, namely principal coordinates 1, 2, 3, 4 & 5. This diverse variation between microbial populations over distance and time can be shown when different combinations of PCO's are plotted.**

Figure 5-7(a) illustrates the microbial DGGE/DNA variation explained by PCO's 1,3 and 4. This PCO plot clearly distinguishes a selection of extraction well microbial populations which form a very distinct group. Notably these bacterial populations represent microbial non-culture water samples which were taken at the earliest stage of sampling at 13 and 26 days respectively. Thus this plot shows that after the 13<sup>th</sup> December 2005 the microbial population from the extraction well appear to have changed.

**Figure 5-7(b) illustrates microbial variation as assessed by DGGE DNA banding patterns between non-culture water samples for PCO1, PCO2 and PCO3. As also illustrated for the MDS plot (Figure 5-6) the extraction well (17) and background bore (BG) bacterial populations appear to be very distinct from the infiltration gallery communities (GW).**

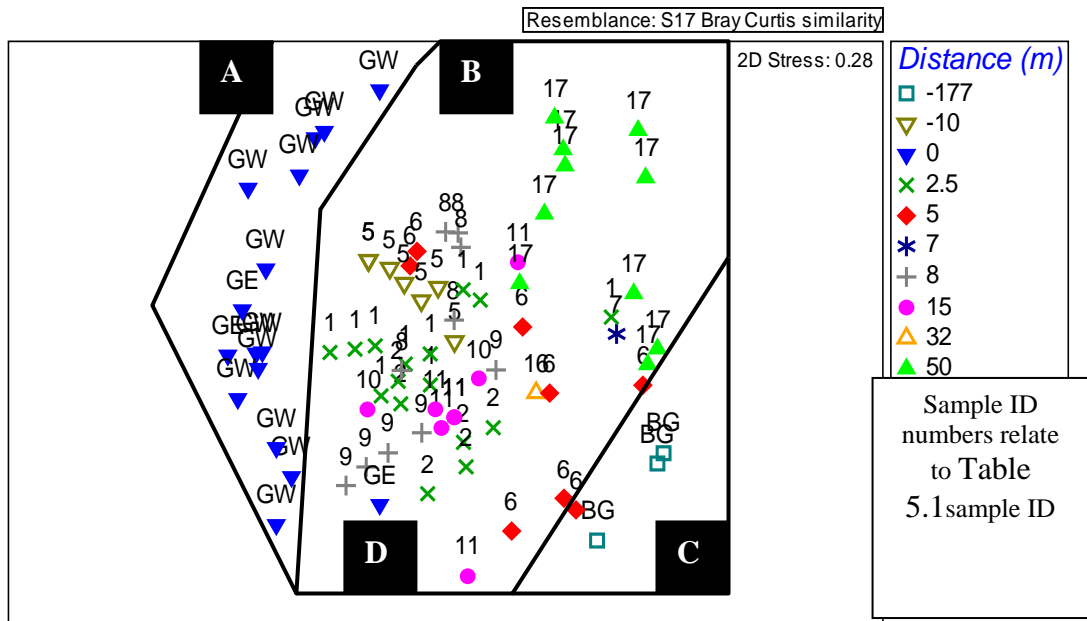
Figure 5-7(c) maximises the microbial variation between PCO's 2, 3 & 5. The microbial populations which are now the most clearly distinguishable are the initial samples taken from the infiltration gallery (day 2 – 32).

Summary of MDS and PCO – MDS and PCO analyses suggested the occurrence of distance-based population groups based on microbial DGGE/DNA data over time. The various Figures indicated that the microbial populations between distances and at different sampling events were dynamic.

**Table 5.3** Percentage of variation explained by individual PCO axes obtained for PCO analysis on microbial rDNA/DGGE banding patterns for all Perth MAR non-culture Water Samples over time

Principal Coordinate Axes (PCO's – 1-5)	Percentage of Variation explained by each axis	Total Cumulative Variation explained by PCO axes
1	18	18
2	17	35
3	15	50
4	12	62
5	10	72

**Figure 5-6** 2D MDS Plot for amplified Microbial rDNA/DGGE banding patterns for all Perth MAR Water Samples over time

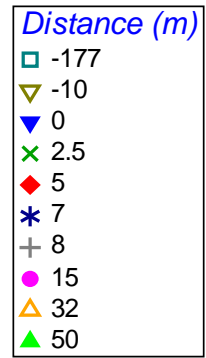
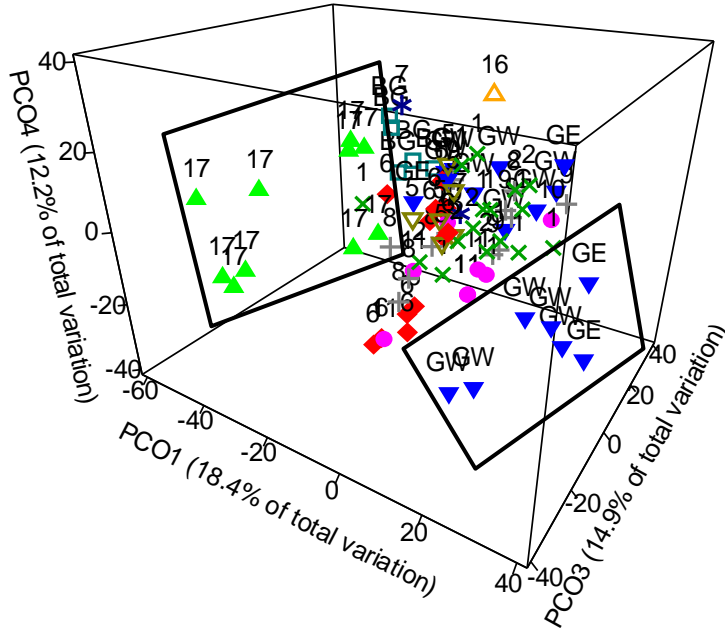


**Figure 5-7** 3D PCO plot of amplified Microbial rDNA/DGGE banding patterns for all Perth MAR Water Samples over time using (a) PCO's 1, 2 and 3. (b) PCO's 1, 3 and 4 (c) PCO's 2, 3 and 5.



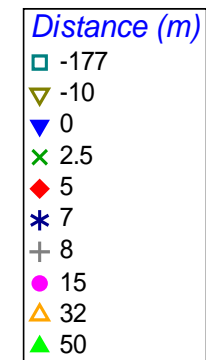
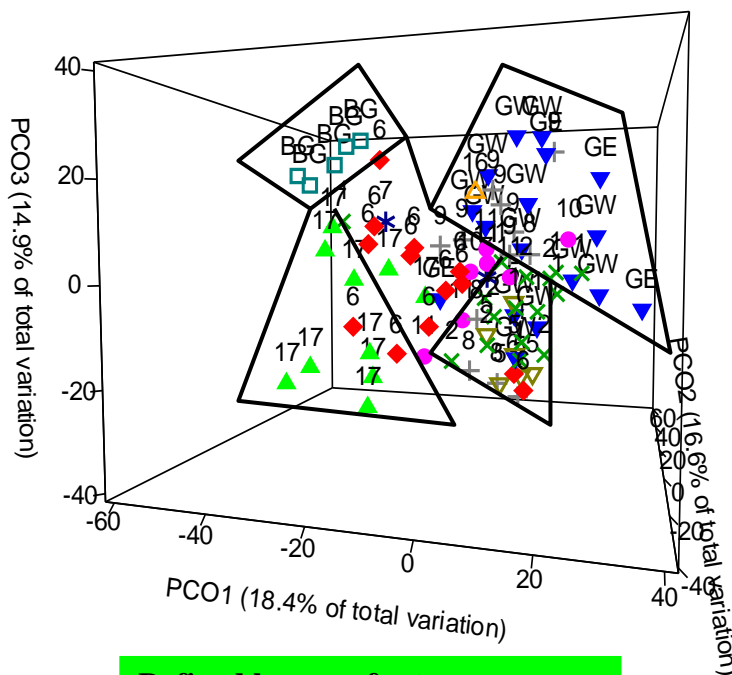
**Defined boxes refer to interpreted PCO clustering**

**Fig. A**



Sample ID numbers relate to Table 5.1 sample ID

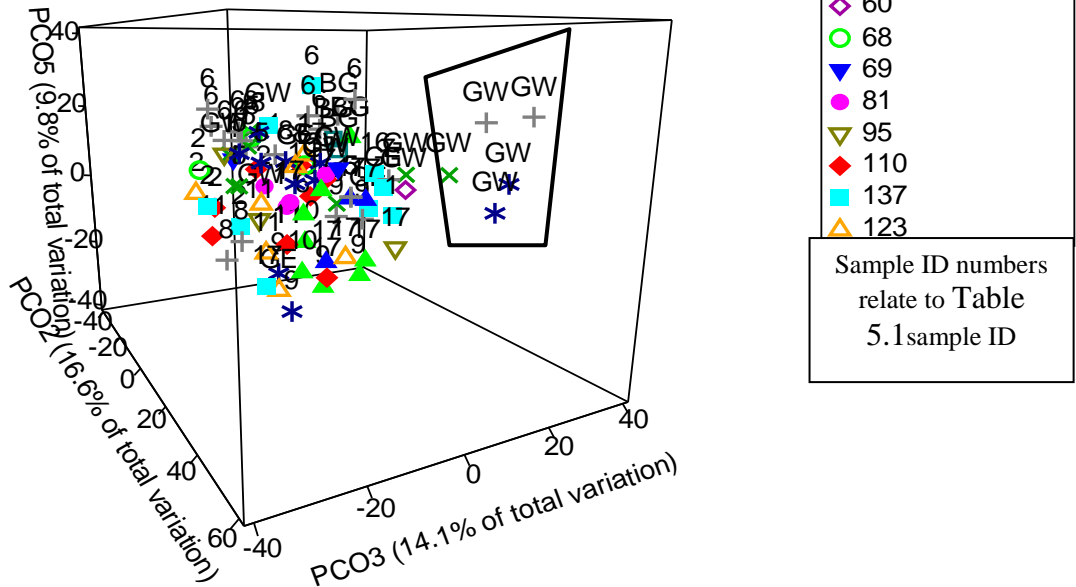
**Fig. B**



Sample ID numbers relate to Table 5.1 sample ID

**Defined boxes refer to interpreted PCO clustering**

Fig. C



5.2.4 PERMANOVA – Statistical significance test for assessing microbial variation of Perth MAR non-culture water samples DGGE/rDNA banding patterns for all distances over time.

The PERMANOVA tested whether there were significant differences between the variation in microbial community structures over distance and time.

Table 5.4 shows a PERMANOVA Type 1 analysis and Table 5.5 shows a PERMANOVA Type 3 analysis. Although the actual numerical values within the two Tables vary slightly, the outcome of both Tables are the same. Microbial populations significantly varied due to an interaction of distance and time ( $p < 0.05$ ). The results also suggest that there was a significant affect from time and distance independently ( $p < 0.05$ ). The F statistic also indicates that distance was the main factor contributing to significant difference in microbial population structures ( $F = > 7.00$ ).

Table 5.6 describes the statistical value of each pair-wise test undertaken for the three amalgamated sampling events used to calculate the PERMANOVA shown in Table 5.5. The T statistic is significant ( $p < 0.05$ ) for each comparison. Notably the comparison with the greatest T value and lowest P value (greatest statistical significance) occurred

between data for the initial microbial populations (Sampling Period 1) and the microbial structure for the final time period (Sampling Period 3). Therefore this data indicates that the microbial population structure became more dissimilar over time.

PERMANOVA pair-wise comparisons of the variability in microbial population structure between distances based on an interaction of distance and time (Distance x Sampling Event) (Table 5.5) are shown in detail in appendix 1. These results indicate that only pair-wise comparisons which included either the infiltration galleries, extraction well or background bore were significant. Pair-wise comparisons were also undertaken on the independent affects of distances only (appendix 2) and on this occasion all samples displayed significantly different microbial populations based on DGGE/DNA at each distance tested from the infiltration gallery. Therefore these results suggest that an interaction of distance and time only occurred for the infiltration gallery, extraction well and background bore. The independent affect of distance only was very significant for all samples.

The results from Table 5.7 assess the differences in microbial populations obtained from 2.5m to 15m only (appendix 3) and therefore exclude the infiltration gallery, background and extraction well samples. The results from Table 5.7 are consistent with the findings shown in Table 5.5 which included the infiltration gallery, background and extraction well samples in that the independent factors of distance and sampling event were significant. However, in contrast with Table 5.5 there is now no evidence for an interaction of distance and time. The pair-wise comparisons associated with this PERMANOVA test, are shown in appendix 3. These results clearly show that microbial populations significantly varied between all distances and between each amalgamated sampling day tested. These results therefore provide further evidence that an interaction of distance and time only occurred for the infiltration gallery, background and extraction well samples only. The results also suggest there were significant differences between all distances and time when analysed independently thus suggesting a highly dynamic microbial population over distance and time.

**Table 5.4** Type 1 PERMANOVA – Perth MAR non-culture water samples

Source	df	SS	MS	F	P (permutation)
Sampling Day (Time)	13	31959	3458	2.78	0.001
Distance	9	56061	6229	7.05	0.001
Sampling Day x Distance	28	34230	1222	1.38	0.016
Residual	40	35327	883		
Total	90	1.57E5			

Experimental design included time and distance as fixed factors and an interaction of these as a variable factor

**Table 5.5** Type 3 PERMANOVA – Perth MAR non-culture water samples

Source	df	SS	MS	F	P (permutation)
Sampling Day (Time)	1	2662	2662.5	2.37	0.027
Distance	5	43136	8627.1	7.69	0.001
Sampling Day x Distance	11	25912	2355.7	2.10	0.001
Residual	56	62788	1121.2		
Total	75	1.4551E5			

Experimental design included time and distance as fixed factors and an interaction of these as a variable factor

- Samples FLB\_05, 07, 10 & 16 were removed from the analysis in order to obtain a Type 3 PERMANOVA (see method).
- Individual sampling events were amalgamated in order to obtain a Type 3 PERMANOVA as follows (see method):  
Sample 01 = 22<sup>nd</sup> November to 19<sup>th</sup> December (day 2 – 33)  
Sample 02 = 24<sup>th</sup> January to 20<sup>th</sup> February (day 68 – 95)  
Sample 03 = 7<sup>th</sup> March to 3<sup>rd</sup> April (day 110 – 137)

**Table 5.6** Distance x Sampling Event PERMANOVA Test based on differences between sampling events.

Amalgamated	Sampling	df	SS	MS	T	P (permutation)
Days for:						
Sampling Periods - 1, 2 & 3						
1, 2		5	10415	2083	1.3087	0.014
1, 3		5	17078	3415	1.8212	0.001
2, 3		6	10470	1745	1.2728	0.048

**Table 5.7** Type 3 PERMANOVA (excluding infiltration galleries, extraction well and background bore) - Perth MAR non-culture water samples

Source	df	SS	MS	F	P (permutation)
Sampling Day (Time)	1	2637	2637.5	2.43	0.033
Distance	2	6849	3424.5	3.16	0.002
Sampling Day x Distance	5	7667	1533.5	1.42	0.105
Residual	31	33571	1082.9		
Total	41	56368			

Experimental design included time and distance as fixed factors and an interaction of these as a variable factor

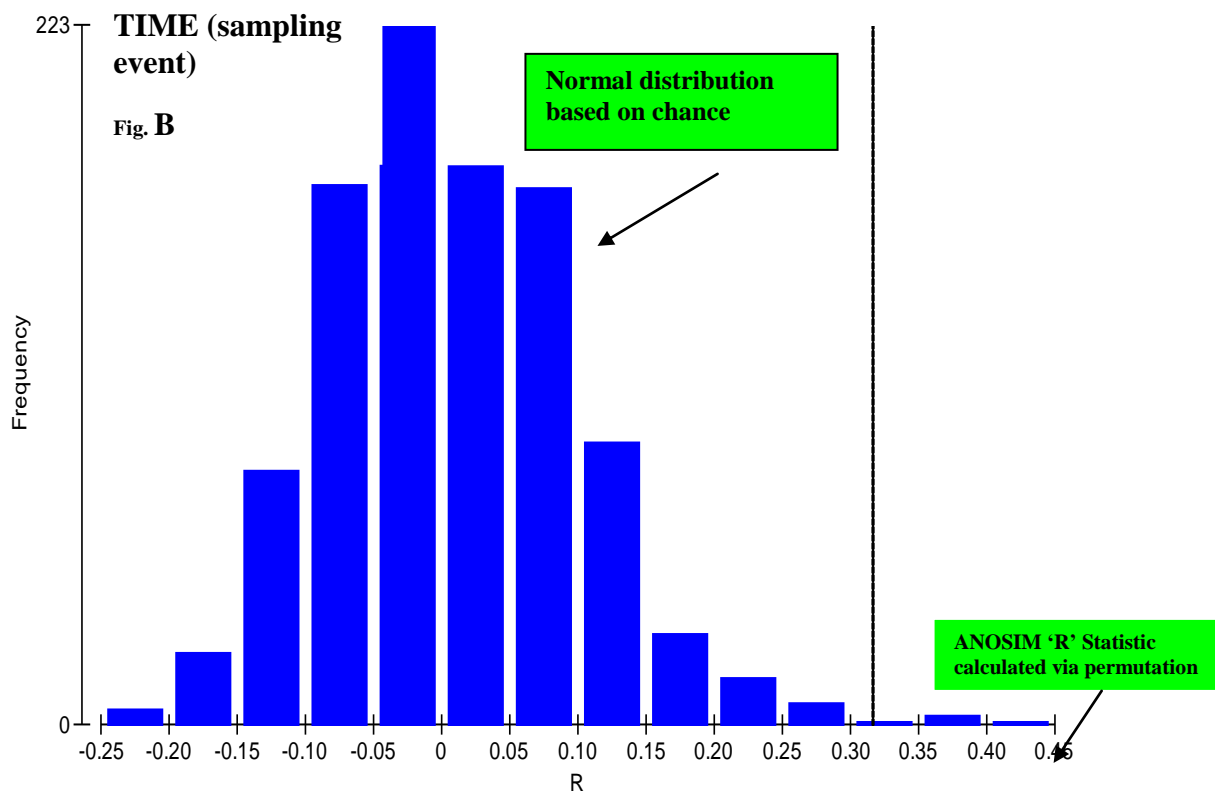
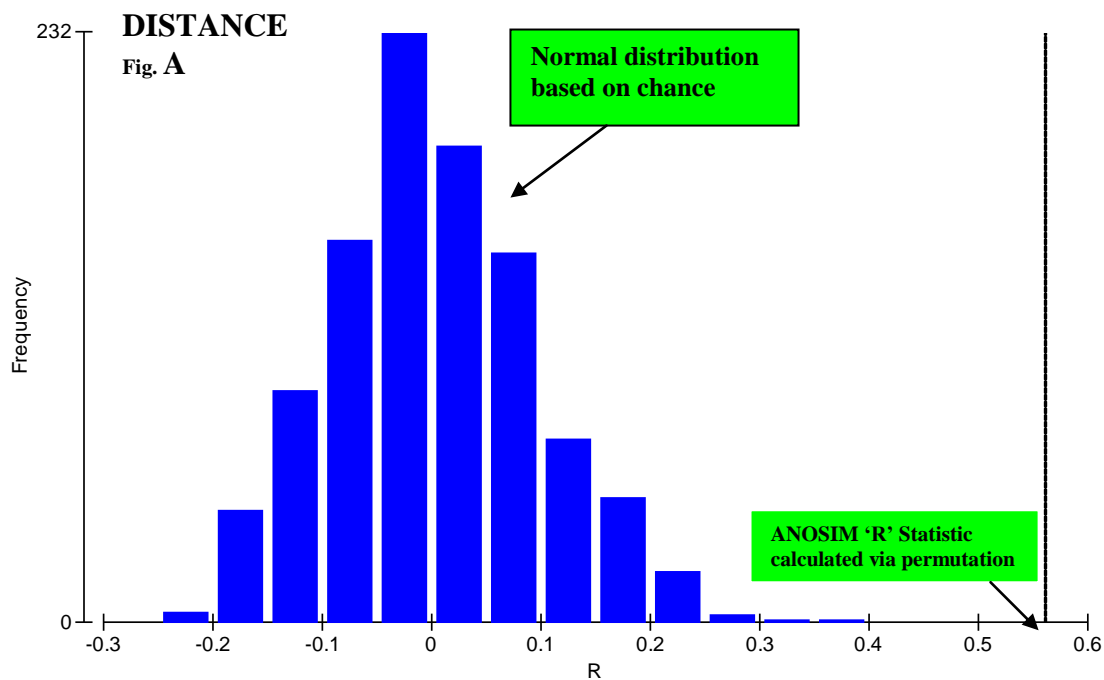
- Samples FLB\_05, 07, 10 & 16 were removed from the analysis in order to obtain a Type 3 PERMANOVA.
- Individual sampling events were amalgamated in order to obtain a Type 3 PERMANOVA as follows:

Sample 01 = 22<sup>nd</sup> November to 19<sup>th</sup> December (day 2 – 33)  
Sample 02 = 24<sup>th</sup> January to 20<sup>th</sup> February (day 68 – 95)  
Sample 03 = 7<sup>th</sup> March to 3<sup>rd</sup> April (day 110 – 137)

#### 5.2.5 ANOSIM - Statistical significance test for assessing microbial variation of Perth MAR non-culture water sample DGGE/DNA banding patterns for all distances over time

Figure 5-8(a) shows that the R statistic obtained for an ANOSIM test based on distance was 0.561. As can be seen from Figure 5-8(a) the normal distribution calculated for this test is placed well away from the R statistic obtained for the microbial data set for the factor distance. The P value for this statistic was 0.001 and therefore suggests very significant grouping of microbial samples based on distance. The pair-wise tests are shown in appendix 4. Figure 5-8(b) shows the R statistic obtained for an ANOSIM test based on sampling event was 0.317. As can be seen from Figure 5-8(b) the ‘end tail’ of the normal distribution calculated for this test, if it were to occur by chance, borders the calculated R statistic of 0.317. The P value calculated for this statistic is 0.05 and is therefore not strongly significant. The Pair-wise Tests are shown in appendix 5. These results suggest that there were significant grouping of microbial populations based on distance but differences between sampling events was not strongly significant. These results therefore help to support outcomes detailed from the PERMANOVA analyses which suggested that distance largely contributed to the significant differences in microbial population structures.

**Figure 5-8** ANOSIM Test calculates the normal distribution based on chance compared with the actual permutation test calculated for the distribution of (A) distance-based microbial data and (B) sampling event-based microbial data



5.2.6 PERMDISP - Statistical test for assessing microbial homogeneity of dispersion for all Perth MAR non-culture water sample DGGE/rDNA banding patterns for all distances over time.

Table 5.8 illustrates that the P value for both PERMDISP ‘F’ statistics were strongly significant therefore indicating that there are strong differences in homogeneity of dispersion in microbial populations between the time points and distances sampled. The pair-wise comparisons between sampling events, shown in appendix 6 indicates many significant results. In addition the pair-wise comparisons for the different distances are shown in appendix 7 which also illustrates significant variation in many pair-wise comparisons. Importantly when pair-wise comparisons are contrasted between the PERMANOVA (appendix’s 1-3) and PERMDISP (appendix 6 & 7) it can be shown that significance results between the two different statistical tests do not match. For example, distance 50m vs. 2.5m P value is not < 0.5 for both PERMANOVA and PERMDISP results. There are more instances of such mismatching between the two statistical techniques and therefore pair-wise significance tests between samples for both the PERMANOVA and PERMDISP tests result vary as to which pair-wise comparison are significant.

**Table 5.8** PERMDISP –Deviation of sample dispersion from the Centroid for Perth MAR non-culture water samples

	F Statistic	P (permutation)
Sampling Event	10.43	0.001
Distance	11.88	0.001

5.2.7 Plotting centroid PCO analysis of amplified Microbial rDNA/DGGE banding patterns for all Perth MAR non-culture Water Samples over time

In contrast to Table 5.3, Table 5.9 shows that 77% of the variation separating microbial community structures at each distance can now be explained over the first three PCO axes as opposed to five axes.

**Table 5.9** Percentage of variation explained by individual PCO axes obtained for CENTROID PCO analysis on microbial rDNA/DGGE banding patterns for all Perth non-culture Water Samples over time

Principal Coordinate Axes (PCO's – 1-3)	Percentage of Total Variation explained by each axis	Cumulative Percentage explained by PCO axes
1	33	33
2	25	58
3	19	77

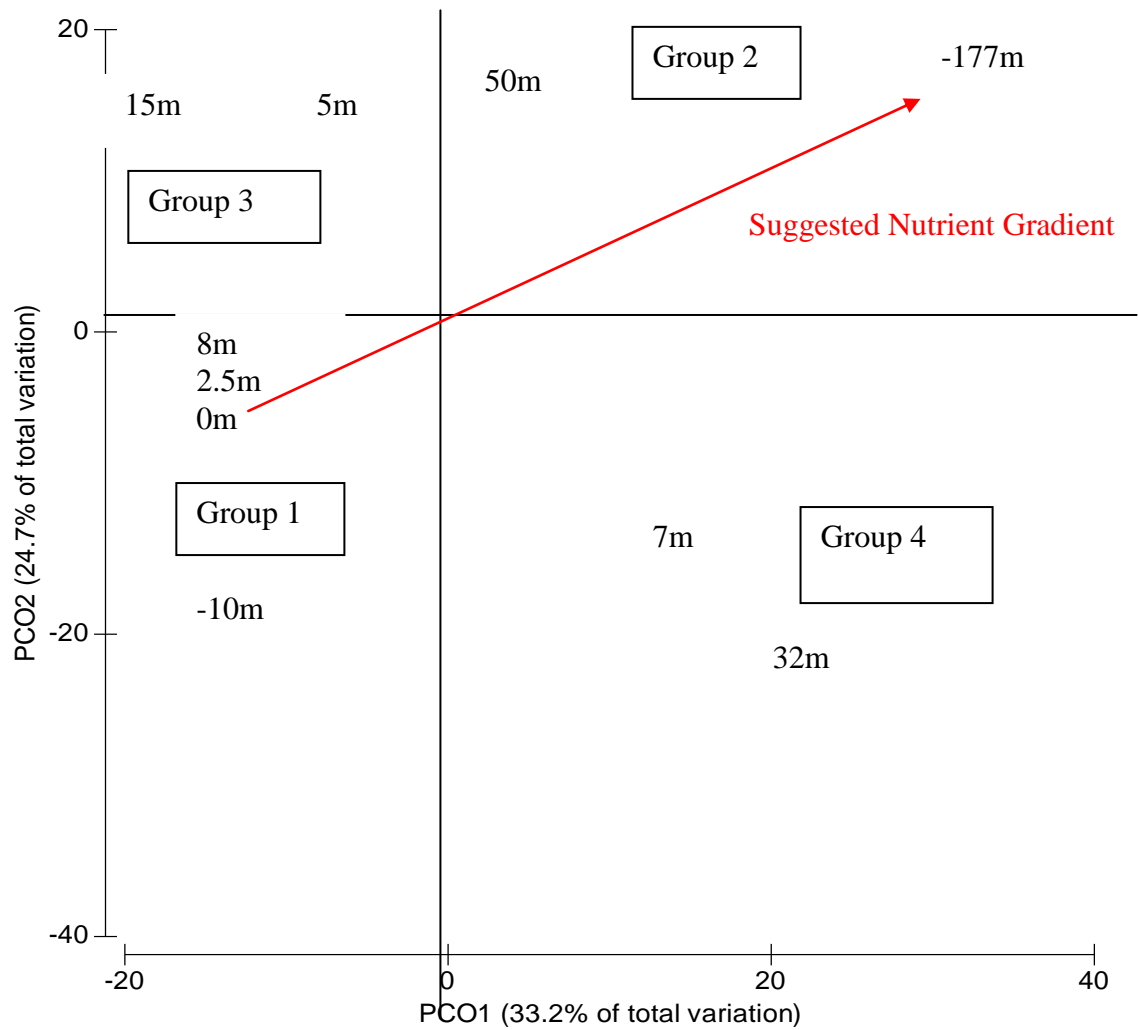
Figure 5-9 shows the PCO plot for PCO's 1 & 2 which describe 58% of the total microbial variation as shown in Table 5.9 for the reduced centroid similarity PCO matrix based on distance.

**PCO Group 1** – Microbial populations from the infiltration gallery, 2.5m, 8m and -10m were strongly grouped together as determined by the grouping structure of PCO (Figure 5-9). These microbial populations were always grouped together when different combinations of PCO 1, 2 & 3 were combined (data not shown). The centroid microbial population structure for 0m, 2.5m, 8m and -10m were therefore very similar for the total time analysed despite differing in distance and depth.

**PCO Group 2** – Microbial populations from the extraction bore (50m) and the background bore (-177m) were always placed well away from group 1 in another direction along one or two of the axes when different combinations of PCO 1, 2 & 3 were plotted. Therefore these microbial assemblages were strongly dissimilar to the microbial population structures from group 1.



**Figure 5-9 - 2D PCO plot for the reduced CENTROID similarity matrix based on distance from the infiltration gallery for microbial variation (rDNA/DGGE banding patterns) of Perth MAR non-culture water samples over time**



**Distances refer to: Distance from Infiltration gallery**

**Table 5.10 Interpreted PCO clusters based on matching positive and negative PCO 1 and 2 values from Figure 5-9.**

Distance (m)	Sample ID	Group	Distance (m)	Sample ID	Group
-10.0	FLB_05	1	-177.0	FLB_BG	2
0	FLB_GW	& 1	50.0	FLB_17	2
2.5	FLB_GE	1	5.0	FLB_06	3
8.0	FLB_01 & FLB_02	1	15.0	FLB_10 &	3
	FLB_08 & FLB_09			FLB_11	
			7.0	FLB_07	4
			32.0	FLB_16	4

**PCO Group 3** – Microbial populations at 5m and 15m from the infiltration gallery display similar variation in their centroid microbial analysis as they were placed together in group 3.

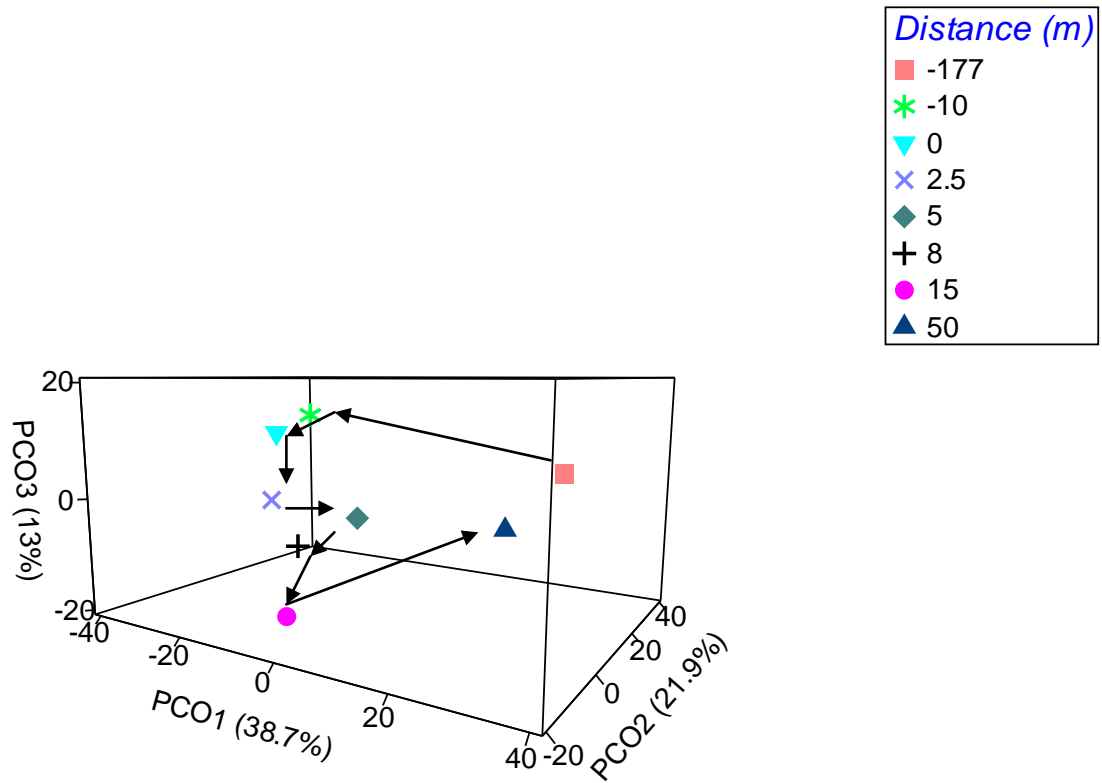
**PCO Group 4** – Microbial populations at 7m and 32m were placed together in group 4. It should be noted that these distances contained very few samples over time namely 7m at day 2 & 13 and 32m at sampling event 137 only.

The 3D PCO analysis shown in Figure 5-10 not only shows how microbial populations which are closest to the infiltration gallery indicated as group 1 in Figure 5-9 are very similar to each other, but also how the microbial populations vary in a successional manner according to distance. For example MDS points are placed according to their similarity to each other. The MDS points shown in Figure 5-10 are placed according to distance from the infiltration gallery. Thus microbial populations become less similar to the infiltration gallery community structure at greater distances from the source of the infiltrated plume.

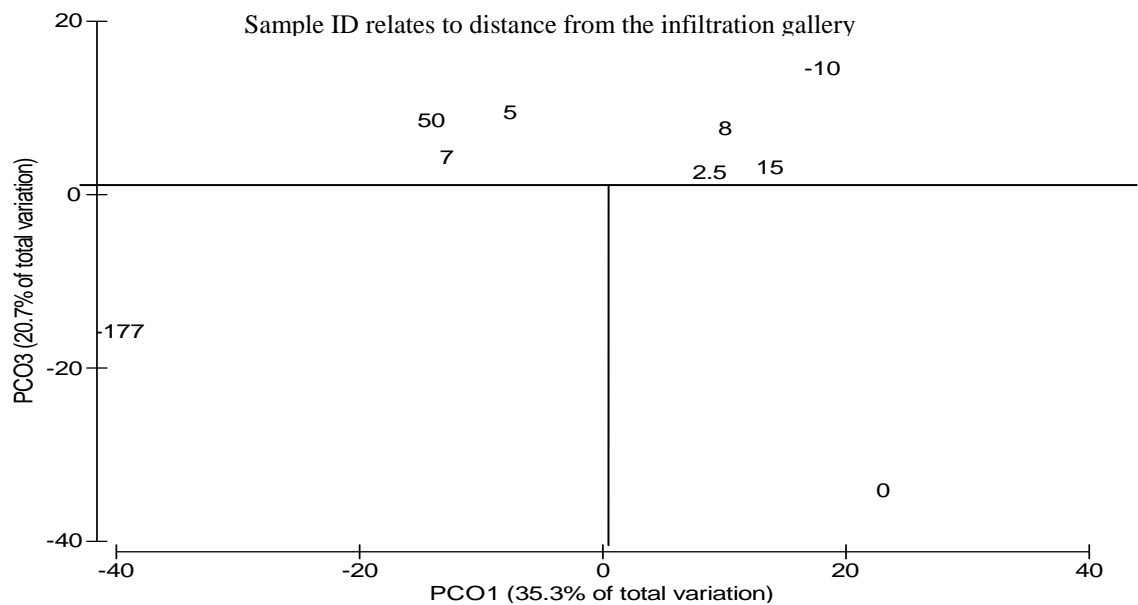
Geochemical analyses demonstrated that there was a notable change in aquifer chemistry at sampling event 68 (Figure 5-2 a&b). Therefore microbial populations were contrasted between distances before the notable changes in chemistry were observed. Microbial evaluation of results were therefore analysed prior to 24<sup>th</sup> January 2006 and are shown via PCO centroid analysis based on distance in Figure 5-11.

Figure 5-11 shows that between the sampling events of 2 to 33 the microbial populations in the infiltration galleries were very different to all other community structures when comparing PCO 1 & 3. This graphical representation could not be obtained when all data over the full duration of sampling period occurred (Figure 5-9). This therefore demonstrates how the microbial populations became more similar to the infiltration galleries over time after the initial period of chemical breakthrough was established somewhere between 19<sup>th</sup> December to 24<sup>th</sup> January.

**Figure 5-10** - 3D PCO plot for the reduced CENTROID similarity matrix based on distance from the infiltration gallery for microbial variation (rDNA/DGGE banding patterns) of Perth MAR non-culture water samples over time



**Figure 5-11** 2D PCO plot for the reduced CENTROID similarity matrix based on distance from the infiltration gallery for microbial variation (rDNA/DGGE banding patterns) of Perth MAR non-culture water samples - (22nd November to 19th December only – sampling events 2 - 33).



5.2.8 Concentrations of Individual Chemical Parameters Associated with MDS clustering of Microbial Populations

Individual chemical parameters were overlaid onto the microbial MDS plot in order to evaluate their potential association to any clustering of microbial populations. Table 5.11 details the alphabetical and numerical labelling of samples to sampling events.

**Table 5.11** Legend to be used in conjunction with all bubble plots linking chemical and microbial data.

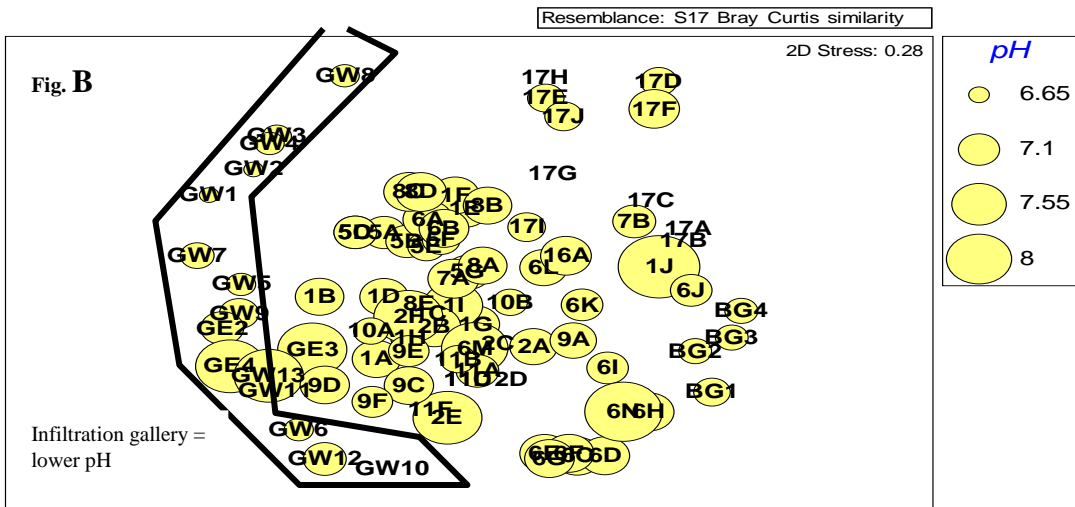
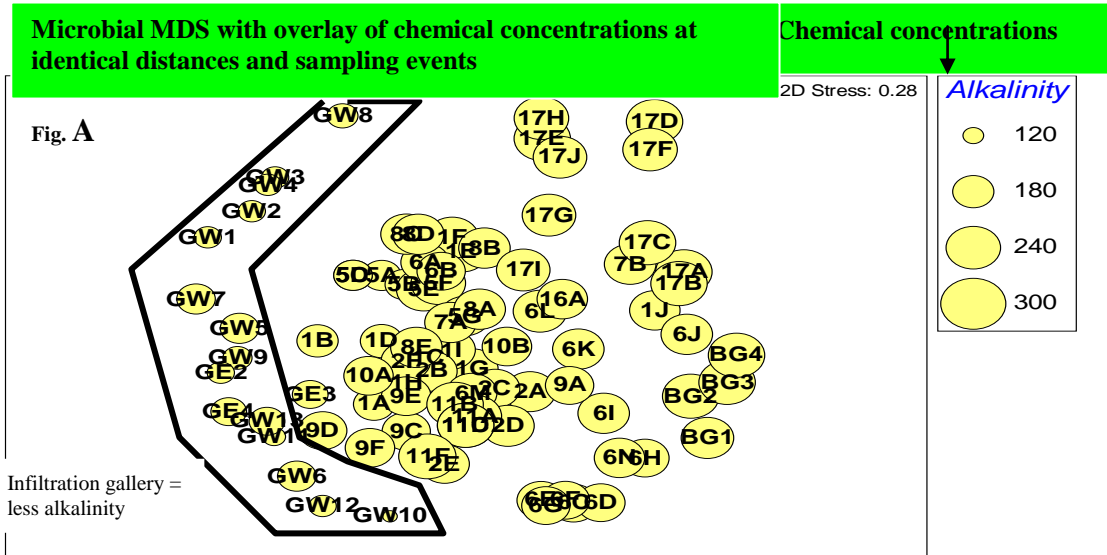
Sample *	Sample Event	Sample *	Sample Event	Sample *	Sample Event	Sample *	Sample Event	Sample *	Sample Event	Sample *	Sample Event
1A	2	5A	2	6J	32	9F	33	17H	110	GW7	68
1B	2	5B	2	6K	33	10A	33	17I	123	GW8	69
1C	13	5C	13	6L	68	10B	33	17J	137	GW9	81
1D	13	5D	13	6M	110	11A	81	BG1	2	GW10	95
1E	32	5E	32	6N	137	11B	95	BG2	33	GW11	110
1F	32	5F	32	7A	2	11C	110	BG3	69	GW12	123
1G	69	5G	68	7B	13	11D	110	BG4	123	GW13	137
1H	110	6A	13	8A	2	11F	123	GE2	123		
1I	123	6B	13	8B	2	16A	137	GE3	137		
1J	137	6C	13	8C	13	17A	26	GE4	137		
2A	68	6D	13	8D	13	17B	26	GW1	2		
2B	81	6E	13	8E	110	17C	26	GW2	2		
2C	110	6F	13	9A	2	17D	33	GW3	13		
2D	123	6G	13	9C	13	17E	33	GW4	13		
2E	137	6H	13	9D	13	17F	81	GW5	32		
2F	137	6I	32	9E	33	17G	95	GW6	32		

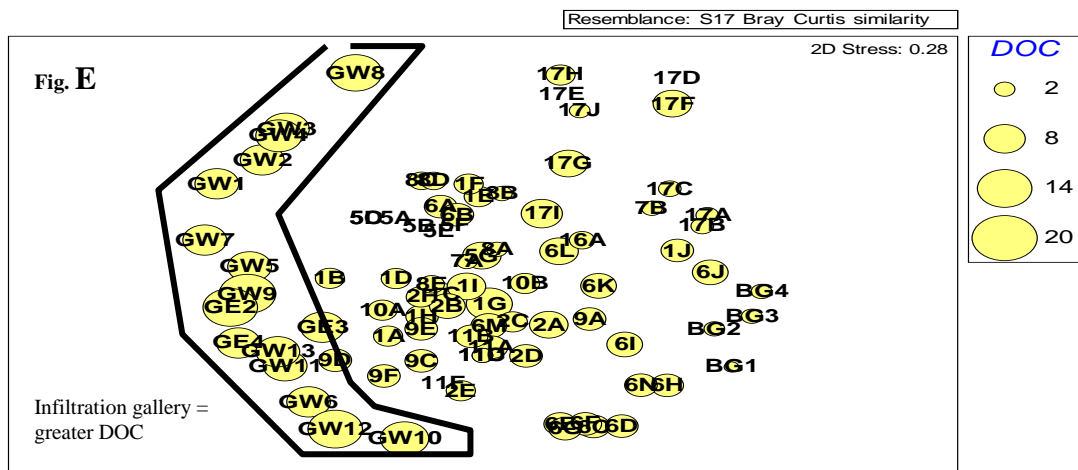
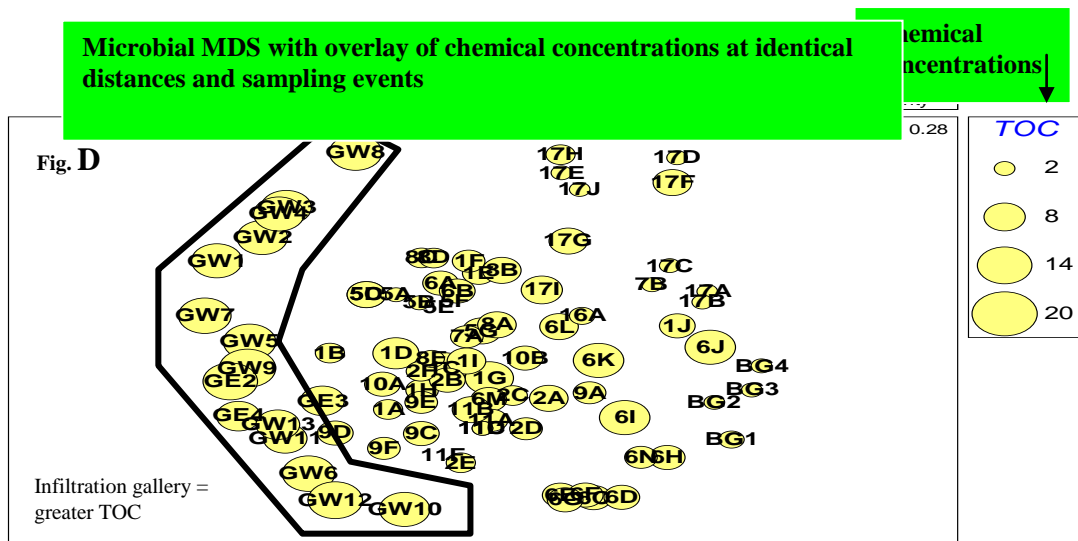
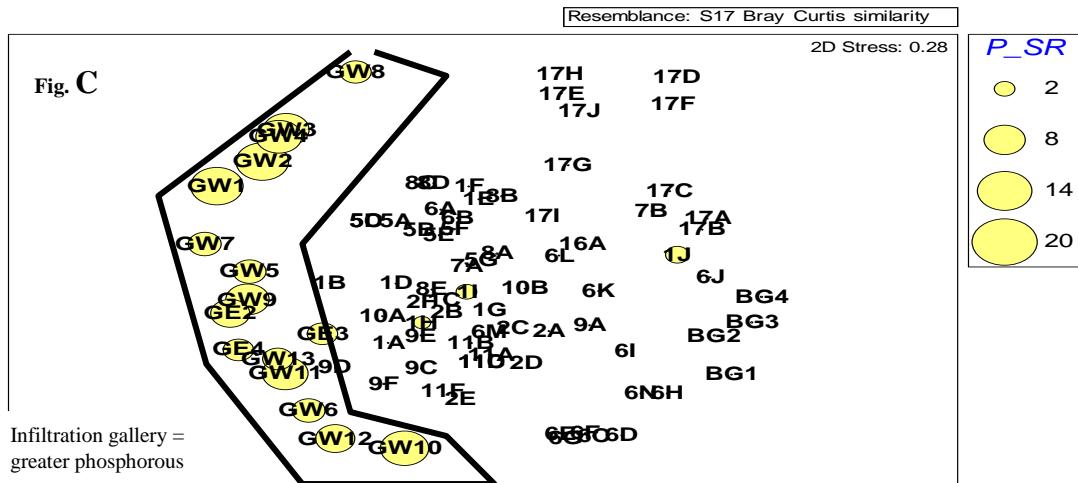
\* Number relates to Sample ID detailed in Table 5.1 e.g. 1A above = FLB\_01 at sampling event 02.

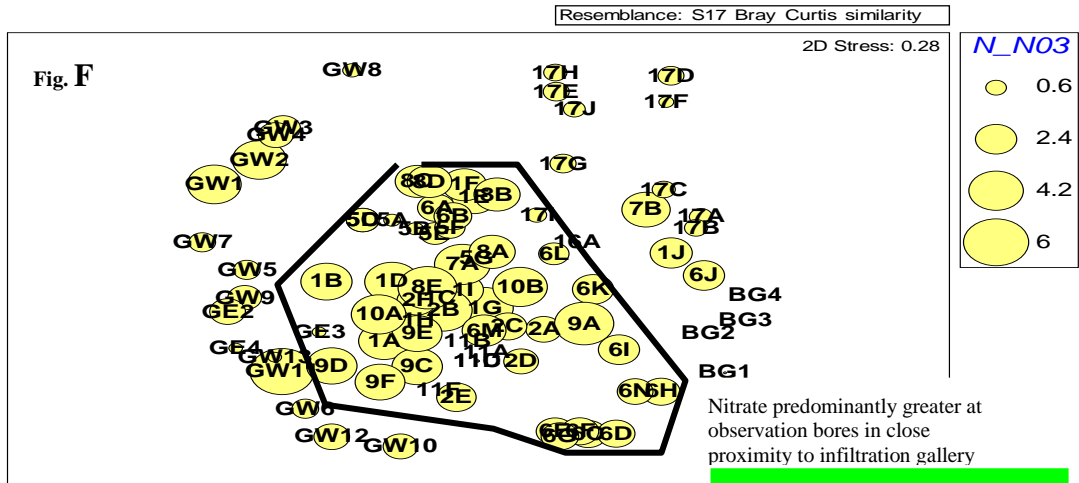
Table 5.11 is for the purpose of the bubble plots shown in Figure 5-12. Sample equates to sample ID as described in Table 5.1. Therefore sample 1 equates to sampling bore at 2.5m from the infiltration gallery. Alphabetical letters are attached to visually differentiate between each sampling event for the bubble plots. Therefore chemical data and concentrations of chemicals could be overlaid onto the microbial plot. Therefore the aquifer chemistry which was taken at the same time as each microbial sample can be compared to determine if microbial clusters were associated with geochemical trends e.g. infiltration gallery associated with greater concentrations of DOC (Figure 5-12 (e)).

Figure 5-12**Error! Not a valid bookmark self-reference.** (a-l) displays the variation in chemical parameter concentrations (Figure 5-4) as an overlay onto the MDS groupings of microbial DGGE/DNA initially displayed in the Figure 5-6. Figure 5-12(a,b&c) clearly show how different the infiltration gallery samples were microbiologically and chemically compared with most other observation wells. The concentrations of alkalinity and pH were less but phosphorous was at a greater concentration at the infiltration gallery compared with most other observation wells. In addition Figure 5-12(d&e) also shows how different the infiltration gallery microbial and chemical samples were to most other samples. Figure 5-12(d&e) contrasts with Figure 5-12(a,b&c) in that the observation wells in closest to the infiltration gallery e.g. 2.5m, 5m and 8m fluctuate in TOC and DOC concentrations over time. Although these observation wells fluctuate in TOC and DOC especially at later time periods (1D, 1G, 6I, 6J, 6K, 6L), overall the concentrations are less than shown for the infiltration gallery. Figure 5-12 (f) shows that the infiltration gallery samples varied in nitrate concentrations over time and predominantly the observation bores closest to the infiltration gallery e.g. 2.5m -15m contained higher concentrations of nitrate than the infiltration gallery samples. In contrast ammonia was present in the infiltration gallery samples and the concentrations appeared to increase for samples at later sampling periods (Figure 5-12L) but was not present in samples in close proximity to the infiltration gallery. Figure 5-12 (G) showed that redox increased over time at the infiltration gallery and these samples were predominantly clustered with non-infiltration gallery microbial populations as indicated on the MDS plot. Figure 5-12**Error! Not a valid bookmark self-reference.** (H) shows that there was a group of non-infiltration gallery microbial populations which were clustered next to the infiltration gallery populations which all showed elevated temperature readings. Figure 5-12 (H) shows that the infiltration gallery samples varied in chloride concentrations but there were a cluster of non-infiltration gallery samples with elevated levels of chloride as also indicated on the MDS plot. Figure 5-12 (J) shows that sulphate concentrations were very variable for most samples except the background samples. Figure 5-12**Error! Not a valid bookmark self-reference.** (K) clearly shows that samples below 18m depth e.g. BH11, BH16 and BH17 were very different to all other samples with respect to total iron concentrations. These results thus show how differences in chemical concentrations are associated with some of the differences in microbial populations described visually in the MDS plots.

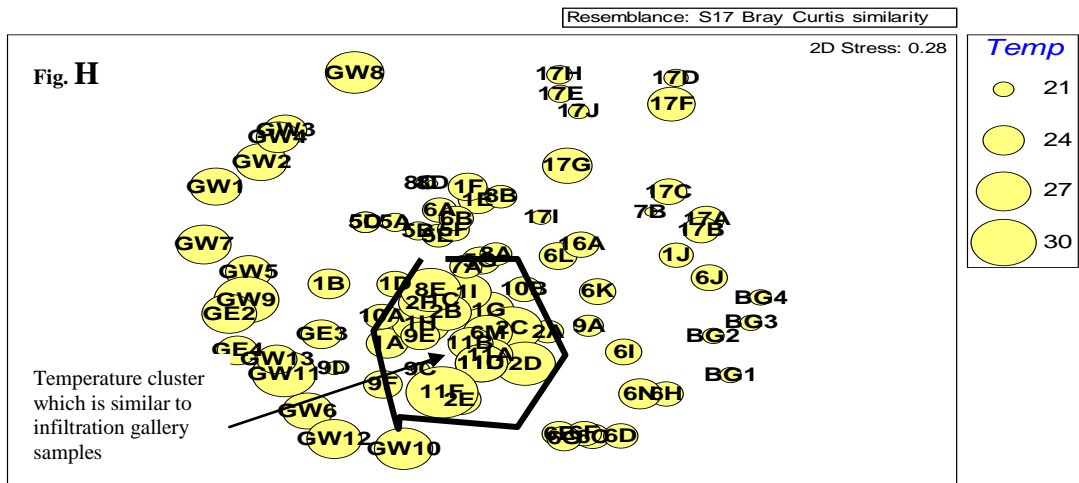
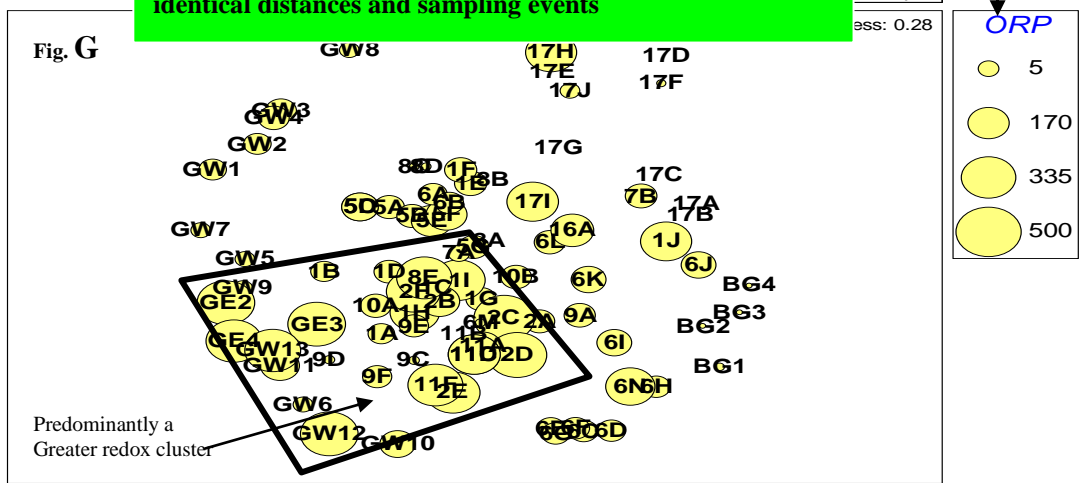
**Figure 5-12** 2D MDS Plot for amplified Microbial rDNA/DGGE banding patterns for all Water Samples with overlay of bubble plots for concentrations of (A) alkalinity, (B) pH, (C) P-SR, (D) TOC, (E) DOC, (F) NO<sub>3</sub>, (G) Eh, (H) Temp (I) Cl-, (J) SO<sub>4</sub>, (K) total Fe and (L) NH<sub>4</sub> chemical data.



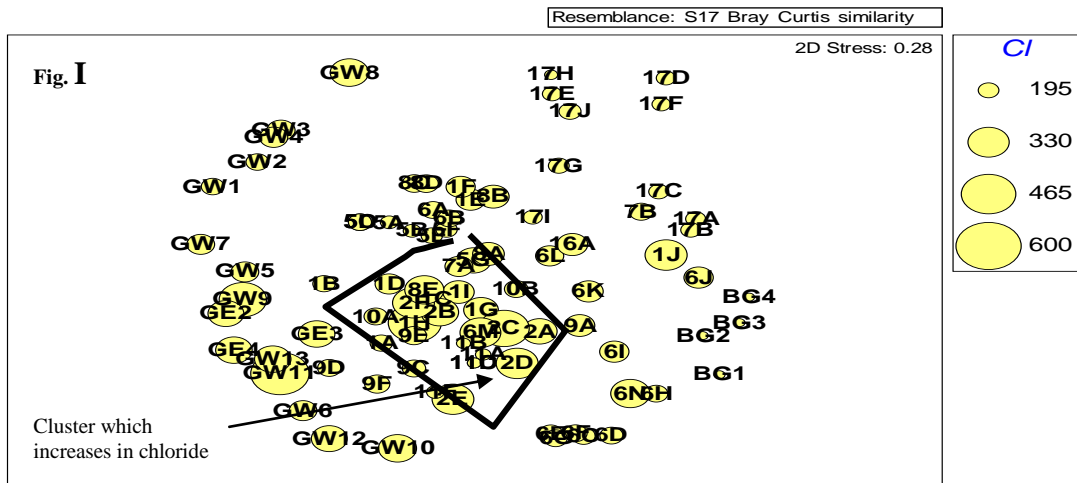




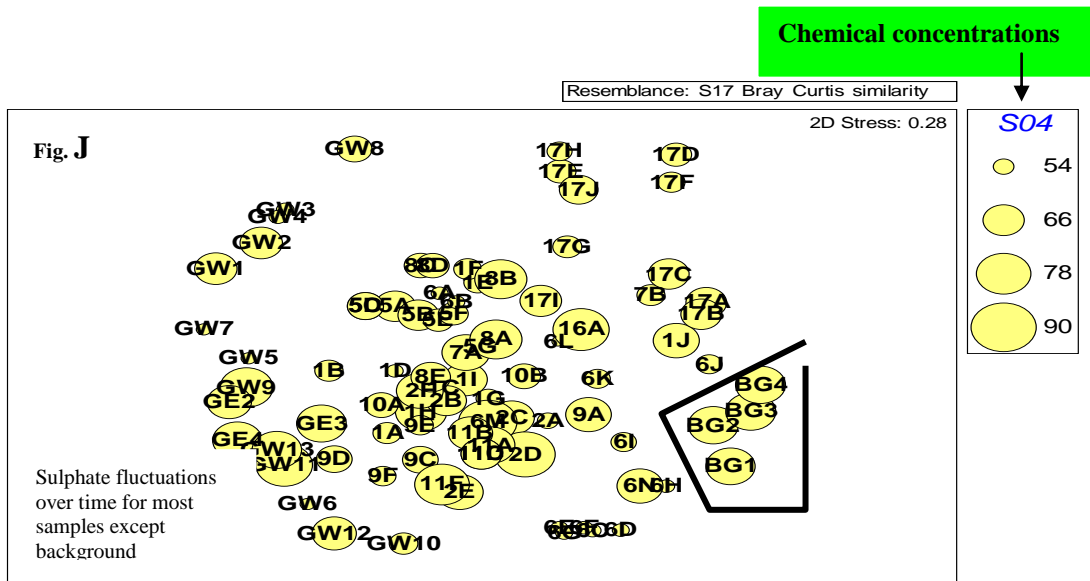
Chemical concentrations  
↓  
ORP

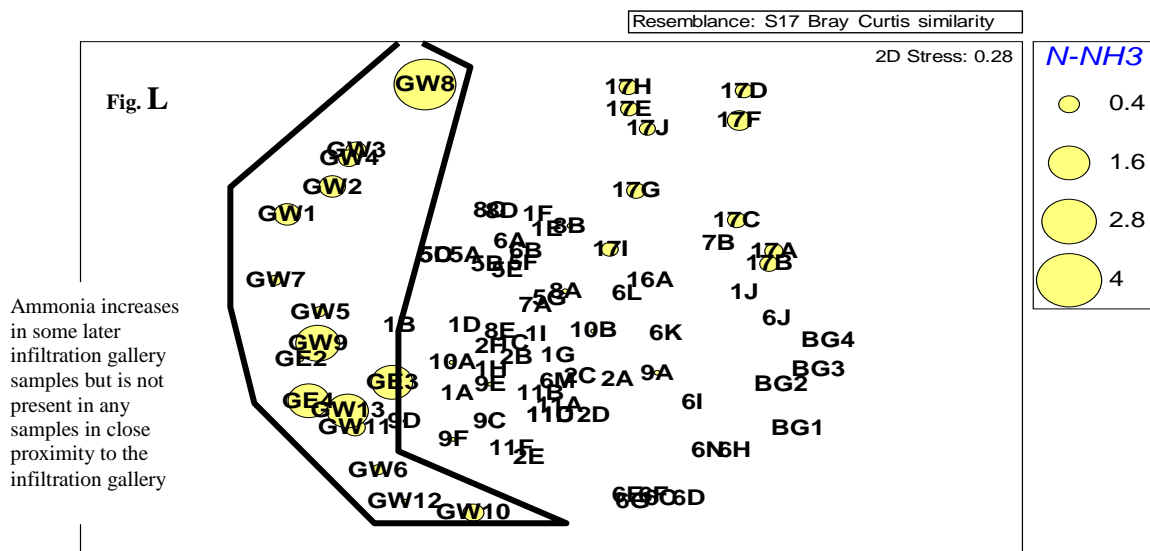
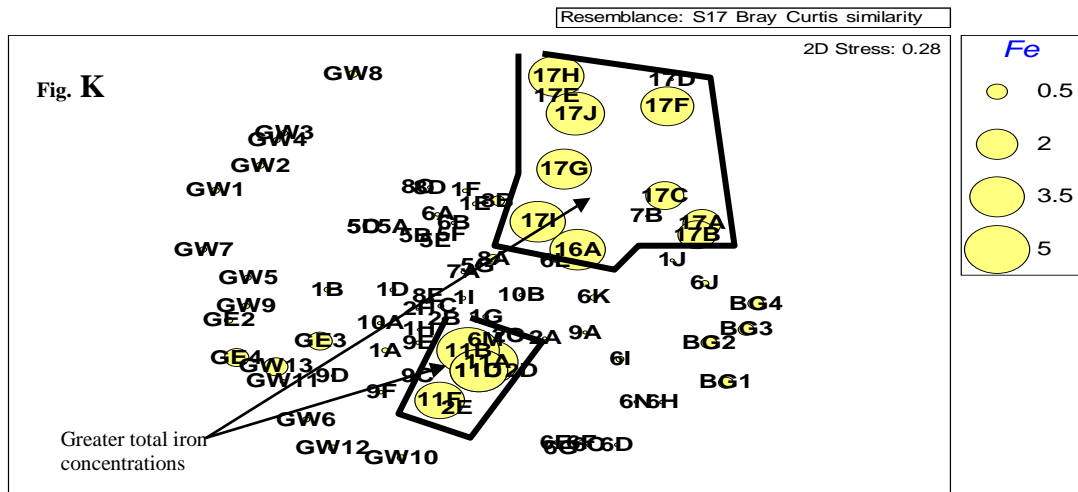






**Microbial MDS with overlay of chemical concentrations at identical distances and sampling events**





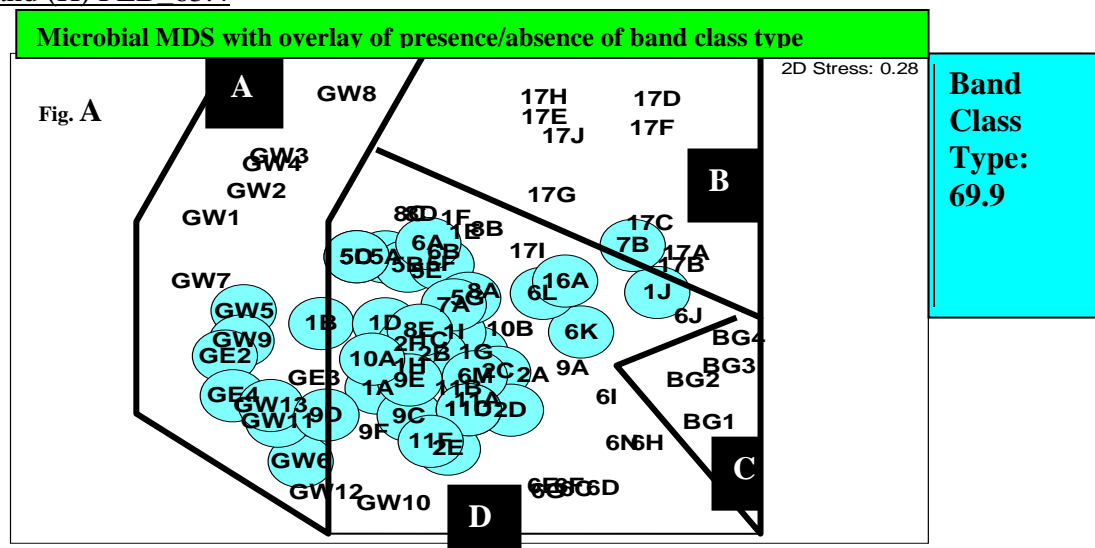
5.2.9 Separation of microbial species (band class types) at each distance and between sampling events

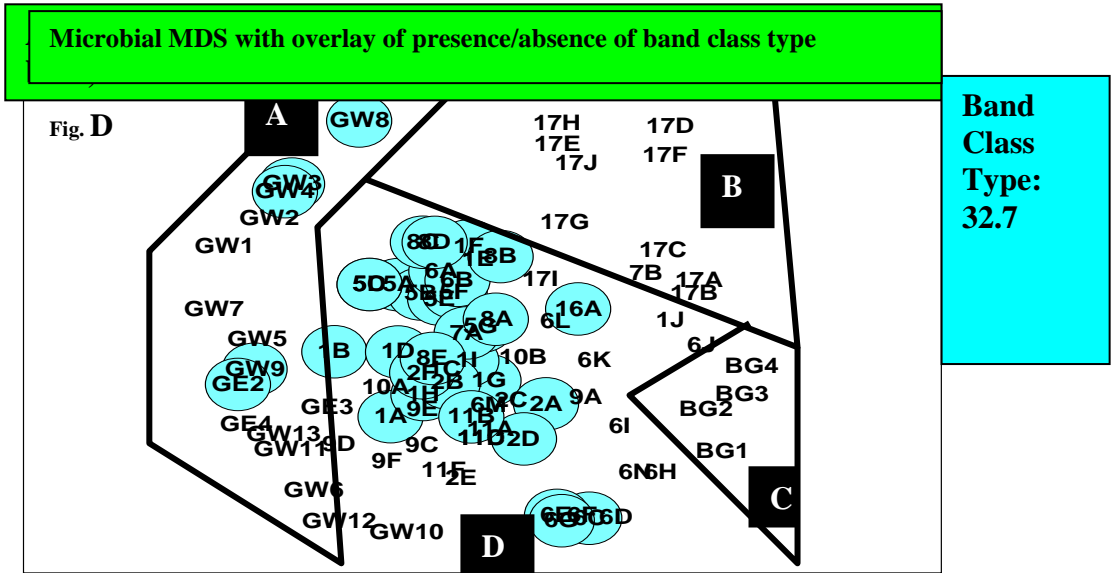
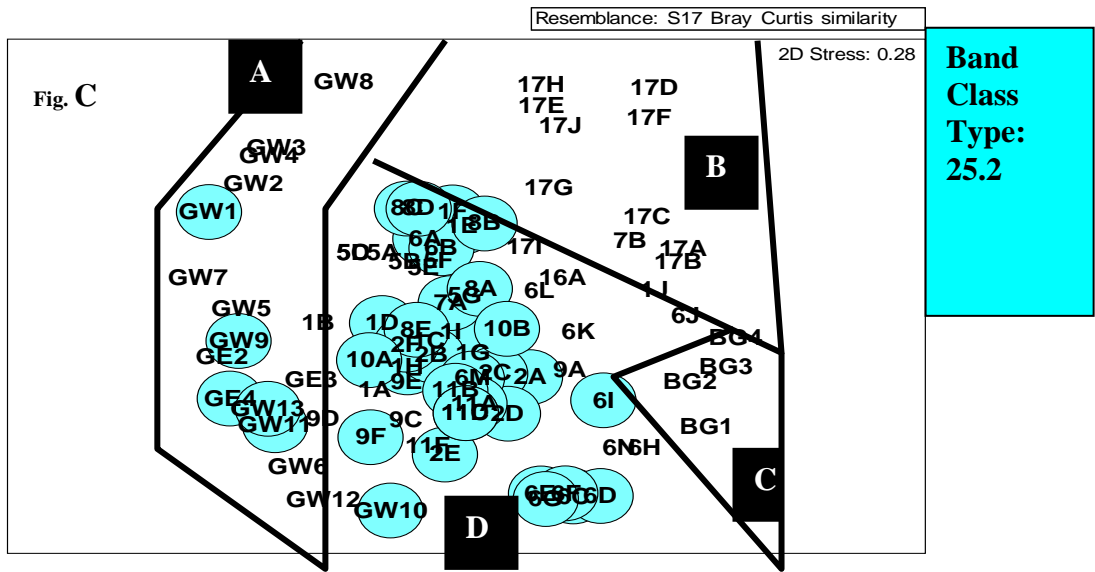
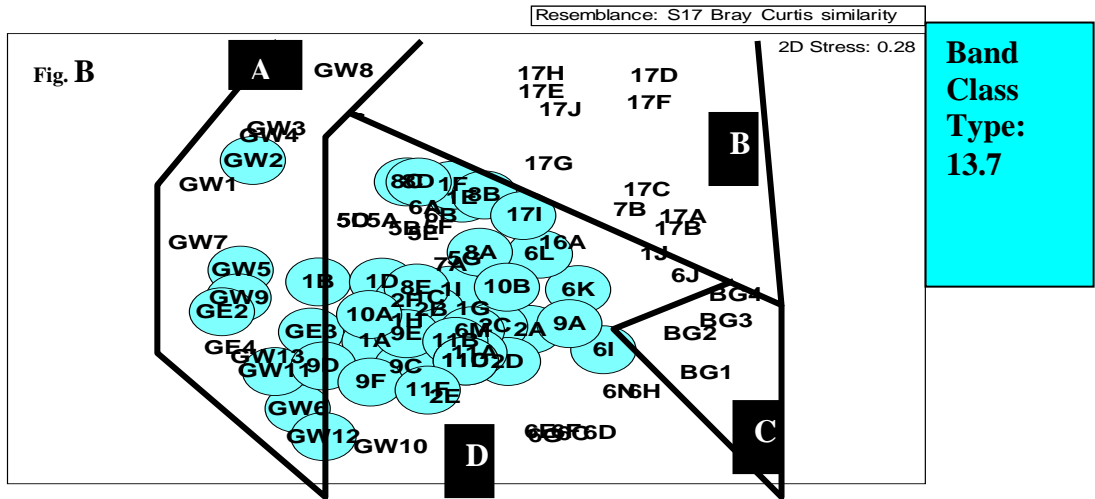
Figure 5-5 shows the binary matrix data for all non-culture water samples. Each band for each sample was assigned to a band class type. Total band classes for all water samples over time ranged from FLB\_0.4 to FLB\_98.2 as shown in Figure 5-5. The bubble plots below overlay band class types onto the microbial populations defined by the MDS plot previously described in Figure 5-6.

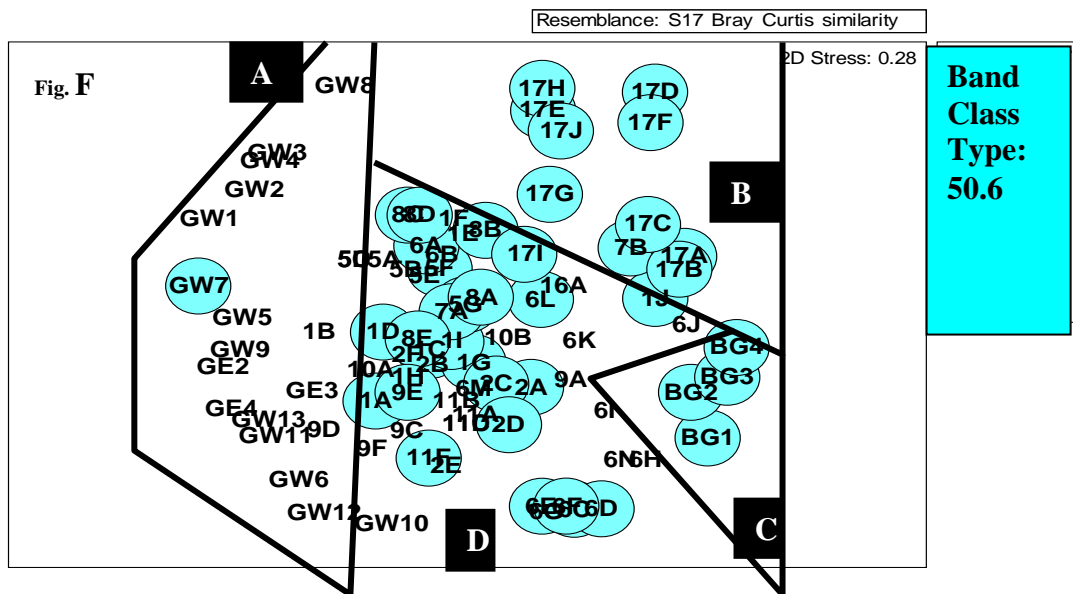
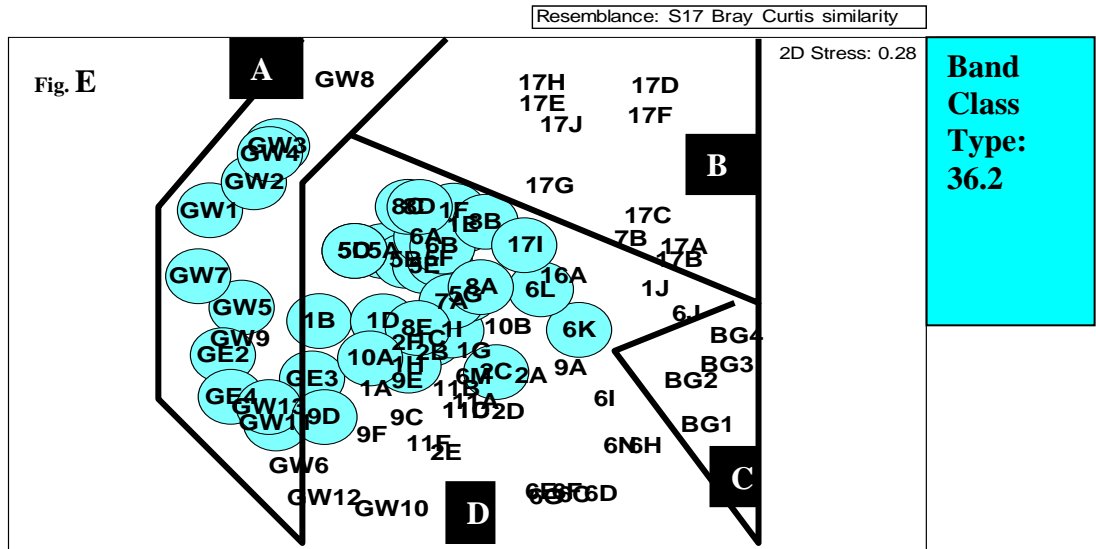
Figure 5-13 (A-M) displays the presence/absence of distinguishing DGGE/DNA bands (band class types) (Figure 5-5) as and overlay onto the MDS groupings of microbial DGGE/DNA initially displayed in the Figure 5-6. Figure 5-13 (A) shows band class type FLB: 69.9 which demonstrates that this microbial type was never present in

samples from the background and extraction well. Similar to band class type FLB: 69.9, band class type FLB: 13.7, 25.2, 32.7 36.2 shown in figures 13 (B,C,D,E) were also not present in the background and extraction well samples. The infiltration gallery samples varied in the numbers that contained these various band types. In contrast Figure 5-13 (F) illustrates a band class type that was not present in infiltration gallery samples except for GW7 (event 68). In addition this band class type was very prominent in the extraction well and background bore samples. Figure 5-13 (G) also illustrates a band class type (FLB: 58.2) that was not present in the infiltration gallery samples except for GE3 (sampling event 137). This band class type was also prevalent within the background bore samples although in contrast with band class type FLB: 50.6 it was not present within many extraction bore samples. Figure 5-13 (H) also illustrates a microbial type (band class type FLB: 63.4) that was also not predominately present within the infiltration gallery samples. In contrast with band class types (FLB: 50.6 and 58.2) the background samples did not contain this microbial type. Microbial types FLB:18, 44.4, 48, 56.2 and 85.2 demonstrate that the background and extraction well samples contained a limited number of band class types that were also present in the infiltration gallery samples. These five band class types represent 3.6% of the total 72 band class types identified in Figure 5-5. These results show how individual band class types are associated with the differences in microbial populations shown visually in the MDS plots.

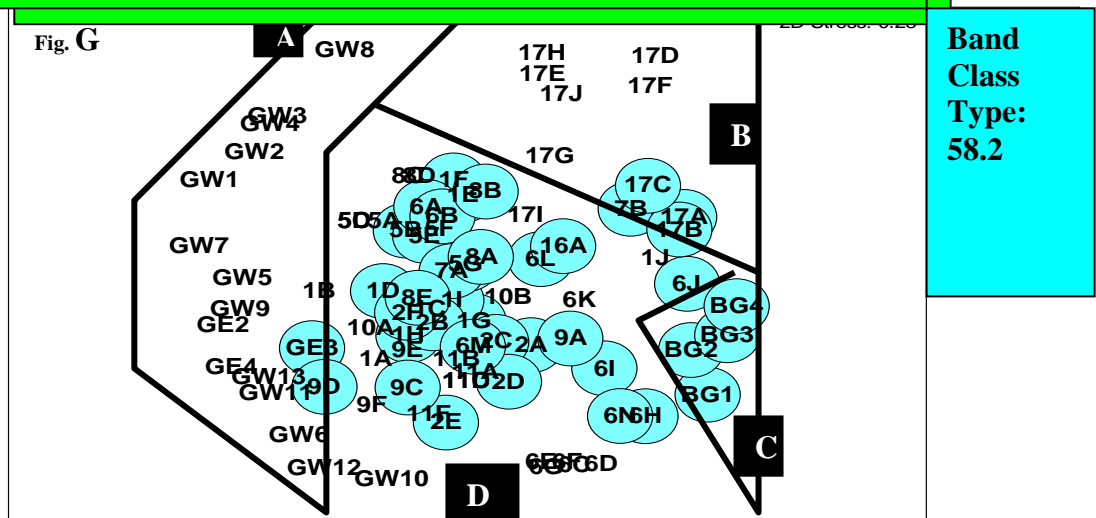
**Figure 5-13** 2D MDS Plot for amplified Microbial rDNA/DGGE banding patterns for all Water Samples over time with overlay of DNA band class type: (A) FLB:69.9, (B) FLB:13.7, (C) FLB:25.2, (D) FLB: 32.7, (E) FLB: 36.2, (F) FLB 50.6, (G) FLB 58.2 and (H) FLB 63.4

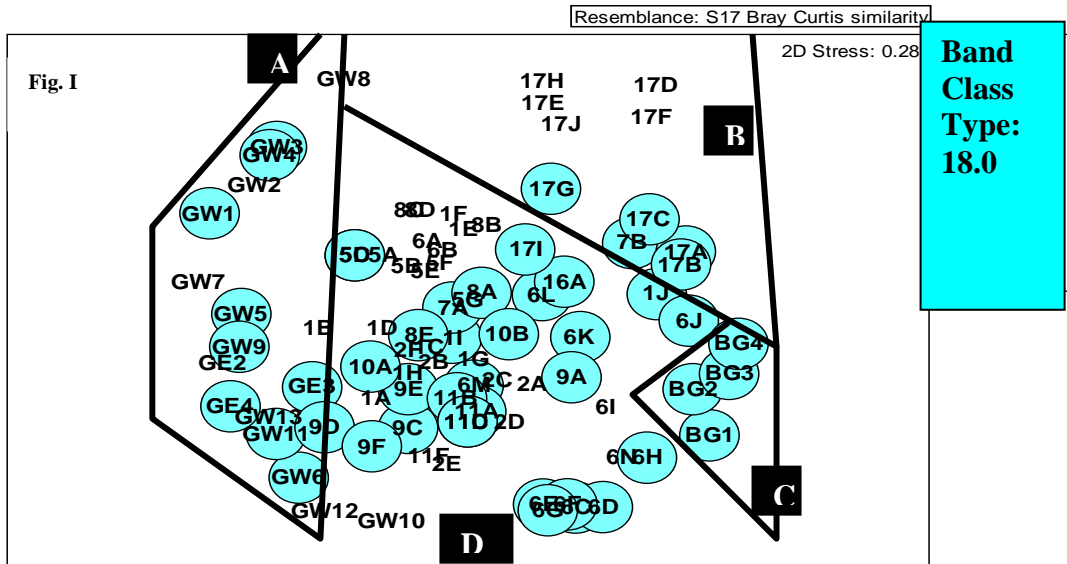
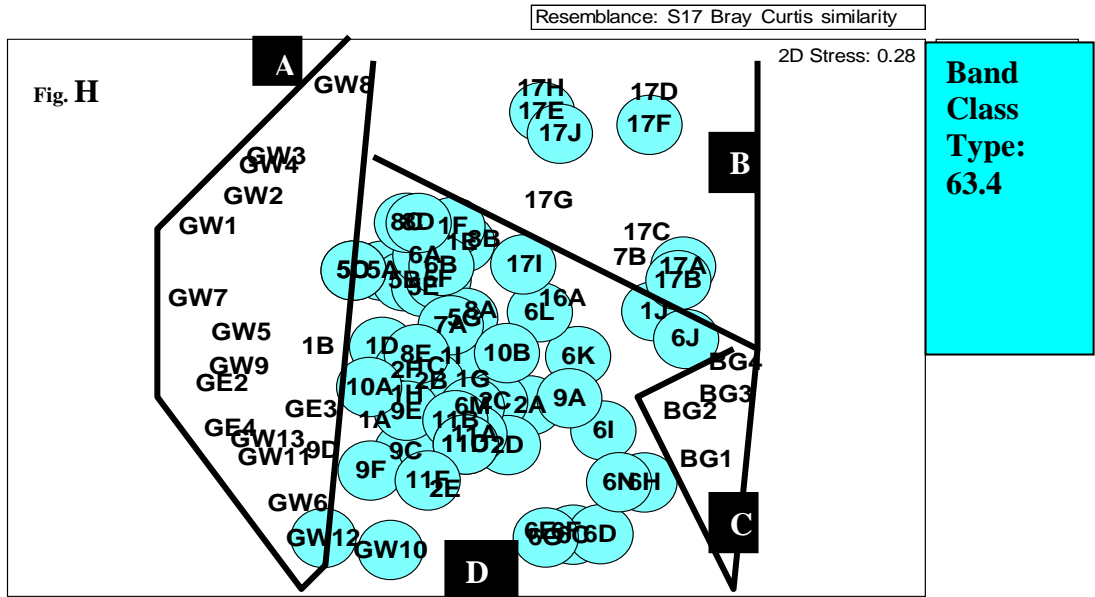




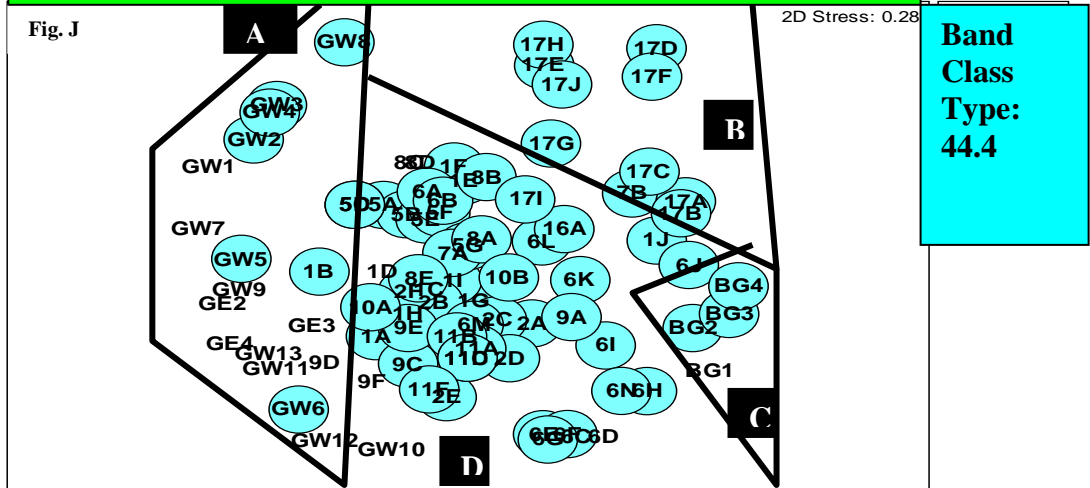


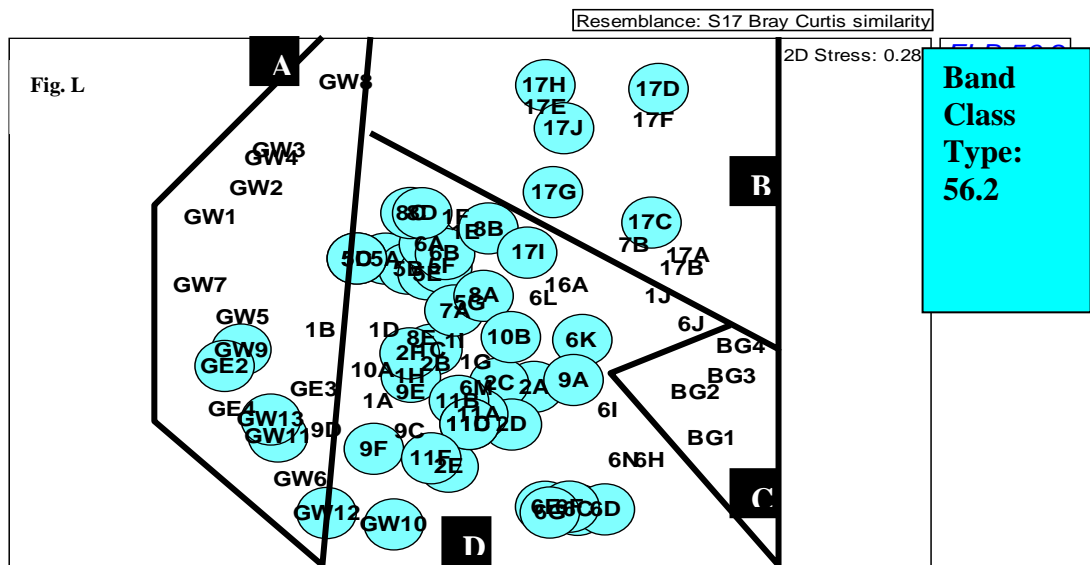
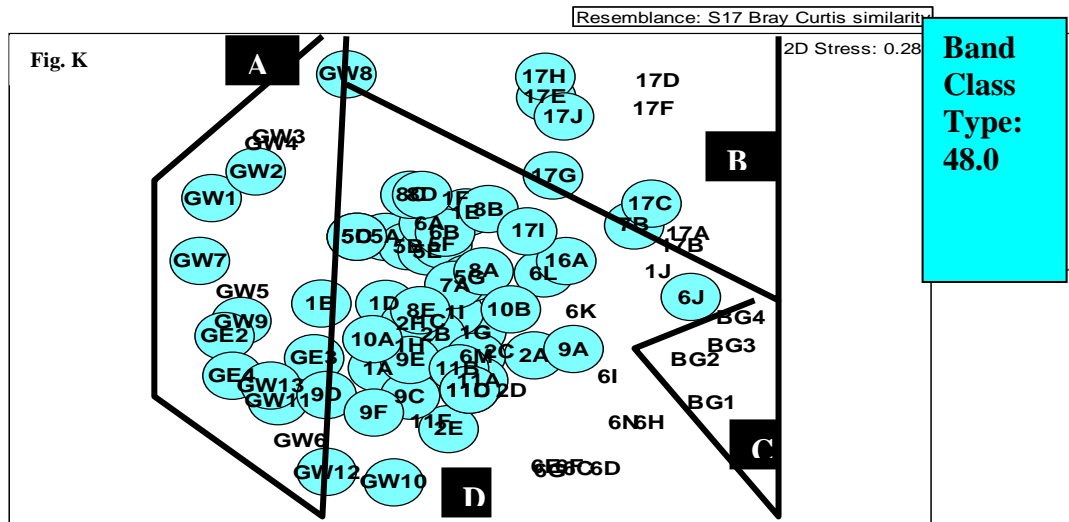
**A – Infiltration Collex B – Extraction Well C – Background**  
**Microbial MDS with overlay of presence/absence of band class type**



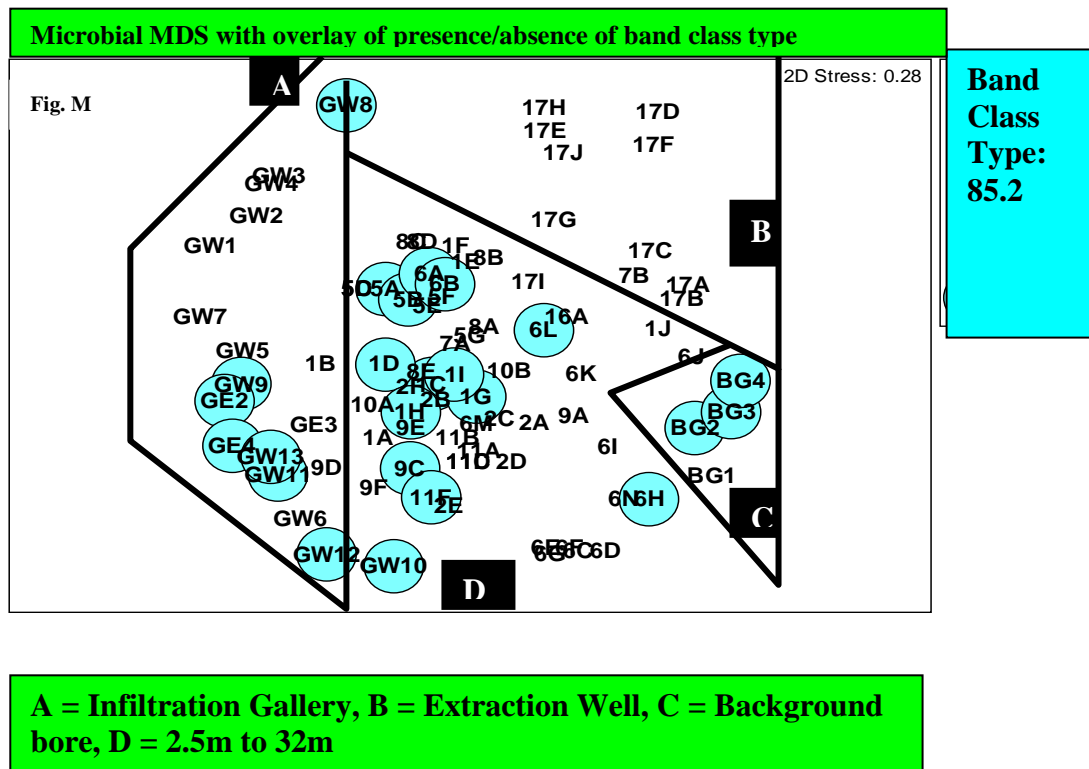


**A = Infiltration Gallery, B = Extraction Well, C = Background**  
**Microbial MDS with overlay of presence/absence of band class type**





**A = Infiltration Gallery, B = Extraction Well, C = Background bore, D = 2.5m to 32m**



*5.2.10 Does Groundwater Microbiology change Groundwater Chemistry? – Microbial and Chemical Variability Analysis of individual distances over time via MDS*

The specific time at which microbial and chemical data changed was investigated at individual distances. This was evaluated to investigate whether microbial populations changed in synch with overall chemical changes or conversely whether microbial populations changed before or after observed chemical changes at individual distances.

The MDS plot shown in Figure 5-14 (A) indicates that the microbial population from FLB\_01 at 2.5m initially changed at day thirteen and again at day 32. Microbial populations therefore changed before the overall chemical signature at 2.5m as shown in Figure 5-2 (B). The chemical signature at 2.5m changed from Group B to Group A sometime between sampling events 32 and 69. The microbial population after its initial changes at day 13 and 32 reverted back to its previous population as at day 2. The microbial community structure remained stable for a significant period from day 69 to 123. Finally the microbial population changed at day 137. In contrast the chemical signature at 2.5m gradually changed over time (Figure 5-2).

Figure 5-14 (B) clearly indicates that replicate samples for a given sampling event are highly reproducible. Duplicates samples which were taken on the same time e.g. day 2,

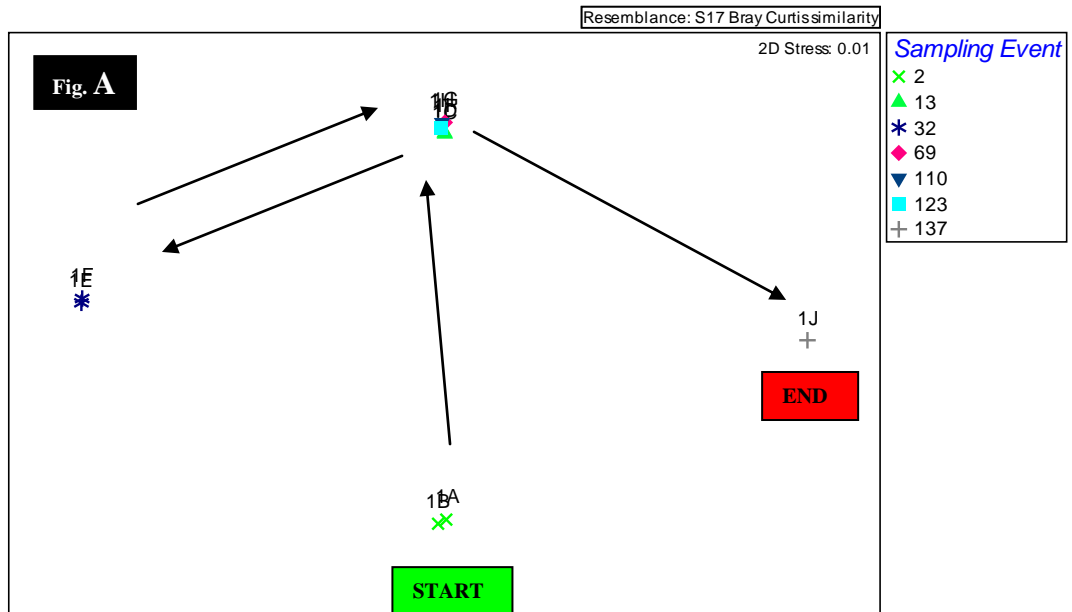


32, 137 clustered together in Figure 5-14 (B). In addition samples for FLB\_02 which are at the same distance as FLB\_01 (2.5m) correspond to sampling events for FLB\_01 as indicated via directionality of arrows when corresponding data between the two sampling stations are available. DNA evidence was not obtained at earlier sampling events for FLB\_02 (from day 68 only). The main difference between the two sampling stations is at sampling event 137. FLB\_01 reverted back to its starting population in contrast with FLB\_02 which was still clustered with previous microbial populations. FLB\_01 is thus more similar to the chemical data as shown in PCA Figure 5-2 (b). Figure 5-2 showed that 2.5m chemical samples began to retreat away from their positive association to nutrients. Despite this withdrawal aquifer chemistry did not revert to its exact starting values. Aquifer chemistry demonstrated gradual change whereas the microbial populations were very dynamic over time.

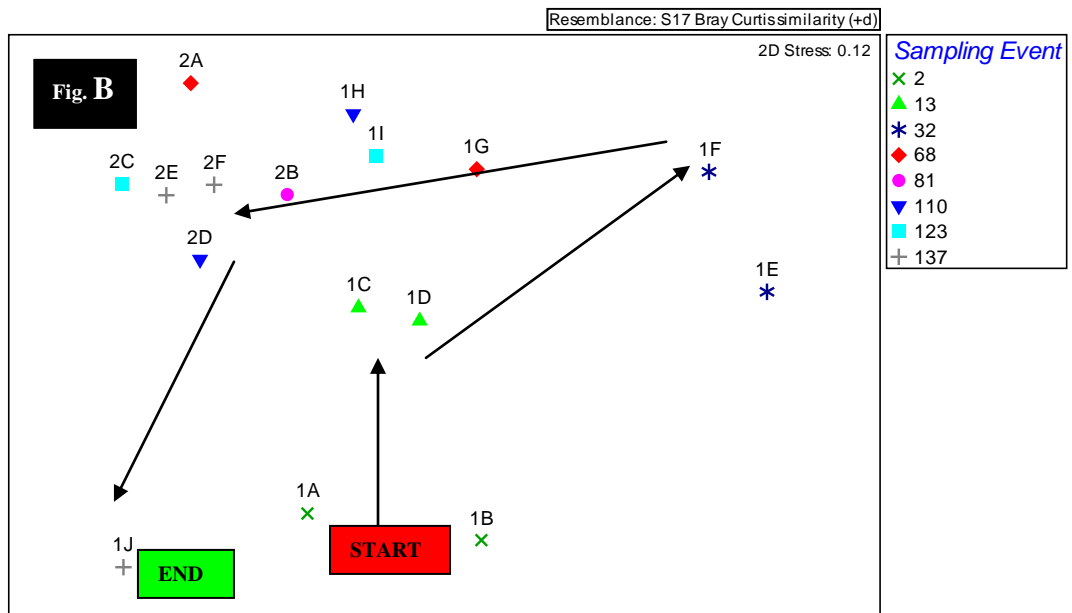
Figure 5-15 (A) shows how the microbial populations were initially relatively stable in the infiltration gallery from day 2 to 32. From day 32 the microbial populations were very dynamic and notably changed at each sampling event until some stability was obtained at day 95 to 137. The MDS plot clearly demonstrates that the microbial populations were very dynamic in the infiltration galleries over time. In addition the microbial population were more dynamic during mid-sampling (sampling events 32 - 95) and remained so for several weeks. The microbial populations were thus relatively stable at the beginning and towards the end of the sampling period.

Figure 5-15 (B) describes the MDS clustering of the chemical data previously demonstrated by principal component analysis in Figure 5-1 (A&B) so that microbial and chemical spatial MDS patterns could be directly compared. The chemical MDS plot clearly shows that the chemical data changed over time in the infiltration gallery. The chemical data was very stable from day 2 to day 60 in contrast with Figure 5-15 (A) where the microbial data changed after day 32. These results support data shown in Figure 5-14 (A) which clearly showed that the microbial population for FLB\_01 changed before the chemical data in Figure 5-2 (B) when MDS – microbial and PCA – chemical analyses were compared.

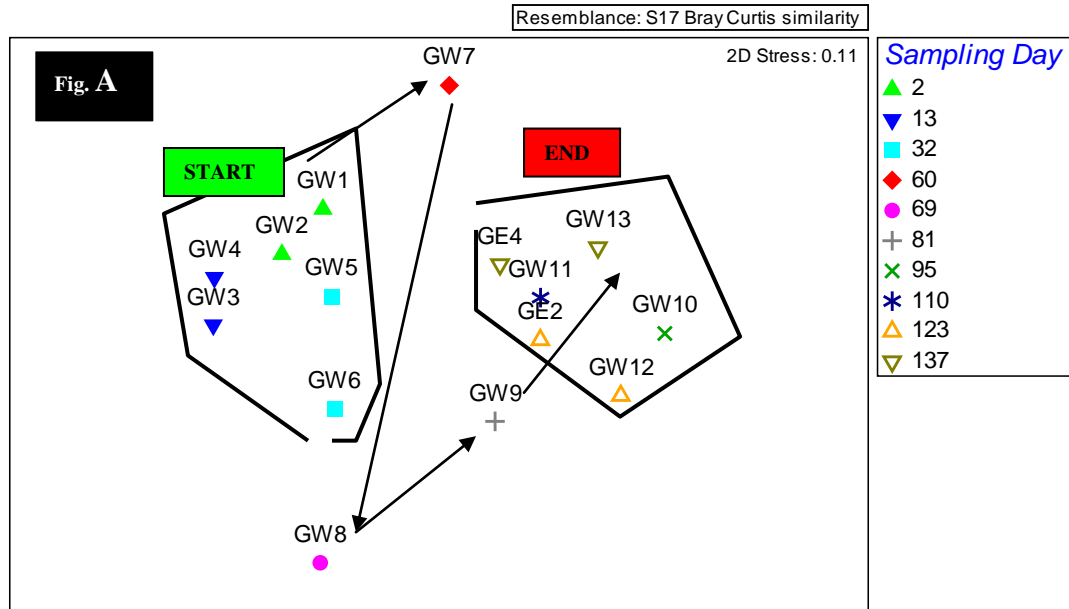
**Figure 5-14** Microbial population changes over time (days) assessed by variability in rDNA/DGGE banding patterns at (A) 2.5m (FLB\_01) and (B) 2.5m (FLB\_01 and FLB\_02) from Perth MAR non-culture water samples.



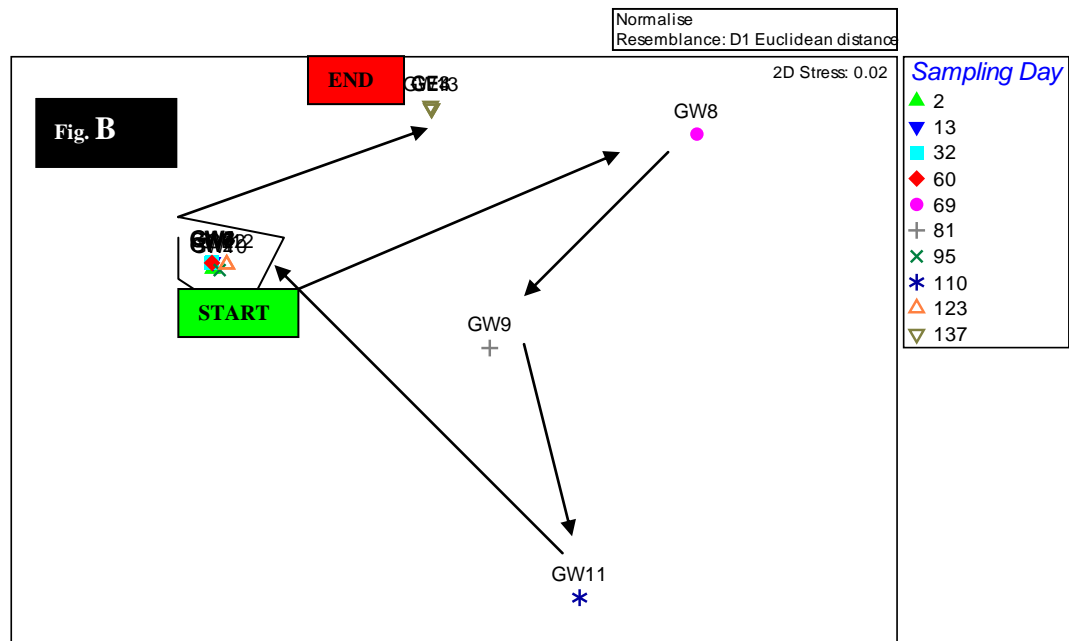
**A-J refers to sampling event indicated in table # as used for bubble plots  
1 = 2.5m distance, 11m depth. 2 = 2.5m distance, 9m depth**



**Figure 5-15** MDS for INFILTRATION GALLERY data only over time (days) for (A) microbial data – Microbial MDS via rDNA/DGGE banding patterns and (B) chemical data – Chemical MDS over time



**GW/GE (1-13) - refers to infiltration gallery sampling events indicated in Table 5.11 as used for bubble plots**



## 5.3 Perth MAR Microbial non-Culture DISCUSSION

### 5.3.1 *DGGE profiles*

Figure 5-5 shows the DGGE/DNA banding patterns and associated binary matrix table for all non-culture water samples from the Perth MAR infiltration site over time. Figure 5-5 illustrated that the extraction bore (FLB\_17) was clearly different from all other samples due to the presence of a dominant and consistent band that was evident over time. In contrast, all other sampling stations did not clearly demonstrate unique or different bands over time that were visually distinguishable. Kassen and Rainey, (2004) reviewed the ecology and genetics of natural microbial diversity in controlled laboratory experiments in order to understand the microbial diversity described in the environment. Consistent with these observations many of the groundwater population profiles at the MAR site were spatially and temporally diverse. Consequently, multivariate statistical techniques were used in order to determine spatial and temporal differences in microbial populations within and between samples.

### 5.3.2 *Microbial Populations at the Infiltration Gallery (positive control) versus the Background bore (negative control) and Extraction Well*

#### 5.3.2.1 Differences between extraction well, background bore and infiltration gallery

**The MDS analysis demonstrated that the infiltration gallery microbial populations were very different to the extraction and background bore populations over time (Figure 5-6). The extraction bore (group B) and background (group C) microbial populations were clustered furthest away from the infiltration gallery microbial population structures (group A). These spatial MDS arrangements suggest that the background bore and extraction well community structures were most dissimilar in microbial population structure to the infiltration gallery when compared with all other observation wells (group D). The PCO analyses shown in**

Figure 5-7 (A-C) also separated the infiltration gallery, extraction well and background bore microbial populations when different PCO axes were plotted. In addition the centroid PCO analysis based on distance (Figure 5-9) also placed the extraction well

and background bore microbial populations in the opposite direction on both axes to the infiltration gallery microbial community structure. These results thus support evidence that the background and extraction bores were most dissimilar in microbial population structure to the infiltration gallery compared with all other observation wells analysed. Figure 5-13 (A-M) also demonstrated some unique band class types which separated the microbial community structures from these different observation wells. PERMANOVA (Table 5.4 and Table 5.5) and ANOSIM (Figure 5-8) analyses also demonstrated that these microbial populations were significantly different between all distances and therefore the apparent visual differences in microbial populations shown via graphical representations could not have occurred by chance. Consequently the spatial and temporal microbial population structures of the infiltration gallery were significantly different to that of the extraction and background bores.

The infiltration gallery samples contained microbial populations associated with treated effluent only. Therefore the infiltration gallery samples were analogous to a positive control as the samples consisted solely of allochthonous nutrients and microbes associated with wastewater. In contrast the background bore located at 177m northeast and hydraulically up-gradient from the infiltration gallery was unaffected by treated effluent as described in the chemical analyses (Figure 5-1). The background bore was therefore analogous to a negative control as these samples only contained autochthonous nutrients and indigenous microbes from the groundwater at this location. The chemical analyses suggested that the background bore and extraction well samples were most dissimilar in chemical signature to that described for the infiltration gallery (Figure 5-1 a&b). The chemical analyses also suggested that samples from Group C in Figure 5-2 (a&b) which included the background bore and extraction well were unaffected by plume migration. The multivariate analyses for microbial populations (Figure 5-6 and Figure 5-9) supports the chemical evidence for dissimilarities between the infiltration gallery, extraction and background bores. These combined results thus indicated that the background and extraction wells were most dissimilar microbiologically and chemically to the infiltration gallery.

#### 5.3.2.2 Similarity between Extraction, Background and Infiltration Gallery

Microbial populations can be ubiquitous in contrasting environments providing they have fundamental environmental conditions for survival and growth (Roszak and

Colwell, 1987). For example, nitrate reducing bacteria can proliferate within wastewater treatment plants as the environmental conditions are ideally suited for nitrate-reducing bacteria. Additionally nitrate reducing bacteria may also be prevalent in groundwater providing nitrate and other resources are available. Nitrate reducing bacteria may be especially prevalent in groundwater which receives enhanced nitrate concentrations from pollution such as excessive fertilising and agricultural practices which can lead to nitrate entering the groundwater environment.

The infiltration gallery samples contained microbial populations associated with wastewater treatment only. Consequently the infiltration gallery samples did not contain any indigenous groundwater microbes from this infiltration MAR site. The occurrence of band class types between different distances are shown in Figure 5-13 (A-M). The results shown in Figure 5-13 (I-M) demonstrate that only five band class types (FLB: 18, 44.4, 48, 56.2 and 85.2) were present in both the positive (infiltration gallery) and negative control (background) samples and additionally in some extraction bore samples. Similar microbial types were therefore present between very different observation bores which were hydraulically unconnected with the infiltration gallery at time of sampling. The implication of similar microbial types between these very different observation bores will be discussed.

The treatment process for wastewater to secondary treated effluent uses nitrate reducing bacteria to reduce nitrate in order to lessen the environmental impacts of treated effluent disposal. The nitrate-reducing bacteria cultivated within the wastewater treatment process may be similar to indigenous groundwater species. Therefore similar microbial species could potentially occur between these two different environments. There is one group of nitrate-reducing bacteria that are gram negative and non-fermentative group, which include microbes such as *Pseudomonas* and *Alcaligenes*. These microbes are ubiquitous in water environments including groundwater and wastewater. Thus it is highly conceivable that these microbes were present in both water types.

Contrarily, the evidence that a few similar microbial types occurred between hydraulically unconnected sampling stations could also be the result of band matching error. GelComparII band matching criteria was set at 4% with 1% optimisation (section 3.13). Band matching was set at these values as increased band matching sensitivity

e.g. 0% band matching, separated DGGE ‘culture standards’ as being different between DGGE gels. A tolerance level for matching bands between gels was optimised so that bands could be accurately matched at a sensitivity that matched known similar DGGE bands e.g. the culture standards. Anything less than 4% sensitivity may thus have resulted in potential mismatching of bands to a band class type that was incorrect. There were 72 band class types in total (Figure 5-5) and only five band class types were identified that were consistently present between the infiltration, background and the extraction bores. Therefore only 3.6% of bands were consistently present between these hydraulically unconnected sampling stations. The 3.6% approximates the 4% tolerance levels set for band matching. Thus the 3.6% may represent the level of mismatching acknowledged as acceptable for designating bands to band class types, as determined by the culture standards. In addition DGGE studies combined with DNA sequencing have revealed that DNA from different microbial species that contain very similar sequences can migrate to the same position on the DGGE gel (Muyzer and Smalla, 1998). Therefore there was also a small level of experimental error that may have also contributed to the 3.6% of bands that occurred in very dissimilar sample types.

### 5.3.3 *Microbial Populations at the Infiltration Gallery (positive control) versus samples from 2.5m to 32m from the infiltration gallery.*

Monitoring bores were placed at various distances hydraulically down-gradient from the infiltration gallery. The directionality of groundwater naturally flowed towards the extraction well. The additional hydraulic gradient created from pumping also created enhanced conditions for plume migration from the infiltration gallery to the extraction bore (section 3.5). The migrating plume was shown to increase nutrients at monitoring bores from 2.5m to 8m over time (Figure 5-2b). In contrast, background groundwater was shown to be naturally low in nutrient status (Figure 5-4 A-R), thus bacterial proliferation may have been inhibited by low organic matter concentration, despite the availability of some electron acceptors. Therefore an elevation in nutrients within groundwater from infiltrated treated effluent had the potential to increase bacterial abundance. Additionally the indigenous microbial populations had the potential to dynamically change as the chemical plume migrated to greater distances from the infiltration gallery.

**Figure 5-6 demonstrated that microbial populations from 2.5m to 32m were clustered together forming a single population group denoted as D. The PCO analyses shown in**

Figure 5-7 (A-C) also demonstrated that these microbial populations could not be separated when different PCO axes were compared. Microbial populations from 2.5m to 32m therefore contrasted greatly with the infiltration gallery, extraction well and background bore microbial community structures because they did not form distinct distance based groups. These results suggested similarity in microbial population structure and/or dynamic temporal groundwater microbial variation from 2.5m to 32m. It is proposed that dynamic temporal microbial variation may have prevented the formation of discrete individual distance-based microbial populations between 2.5m to 32m. Pair-wise tests on PERMANOVA (appendix 2&3) suggested that there were significant differences in microbial population structure between all distances tested. Consequently these results indicate that the absence of specific MDS grouping for samples from 2.5m to 32m (Figure 5-6) was due to dynamic temporal variation.

In order to dissect the significant differences (appendix 2&3) in microbial community structures from observations bores at 2.5m to 32m from the infiltration gallery, a PCO centroid analysis was undertaken as shown in Figure 5-9. The centroid PCO method was able to explain a greater percentage of microbial variation over fewer PCO axes than non-centroid PCO analyses (Table 5.3 and Table 5.9). Consequently the centroid PCO analysis was able to separate microbial populations from 2.5m to 32m into three distinct groups, based on matching positive and negative values between PCO 1 & 2 axes. The infiltration gallery microbial populations were clustered with microbial communities from 2.5m, 8m and -10m in Group 1. These results suggested that despite significant differences (appendix 2&3) in microbial communities between distances, certain observation bores displayed greater similarity in microbial population structure. Microbial populations isolated from observation wells in close proximity to the infiltration gallery contained similar DGGE banding patterns, thus have similar microbial populations to that of the infiltration gallery.



Despite evidence for greater similarity in microbial populations in close proximity to the infiltration gallery, samples from 5m and 7m were not clustered with Group 1. This result may suggest preferential flow of treated effluent from the infiltration gallery to 2.5m and 8m due to the karstic nature of the aquifer. Thus these microbial populations were perhaps more impacted by treated effluent than those occurring at 5m and 7m. These results are in contrast to chemical data that showed that samples from 2.5m, 5m, 7m and 8m were placed together in Groups A and B (Figure 5-1A). Assimilable organic carbon (AOC) and biodegradable dissolved organic carbon (BDOC) were not measured and thus the type of carbon available may have differed between these observation wells that contributed to the observed differences in microbial populations. The PCA analysis showed that the overall 'chemical signature' at 5m prior to MAR commissioning was slightly different to that of all other background readings for samples from 2.5m to 32m (Figure 5-3 A&B). Consequently the microbial populations at 5m prior to MAR commissioning may have been significantly different to samples from 2.5m and 8m due to differences in ambient groundwater chemistry. These differences may have been a function of variation in depth profile (Table 5.1). As a result the indigenous microbial populations at 5m may have also behaved differently in response to the migrating nutrient plume. These results highlight the potential need to analyse BDOC and AOC in future studies of this kind.

Contrarily, microbial populations at 2.5m and 8m may have consisted of microbes associated with wastewater which migrated with the infiltrated chemical plume. Consequently it could be suggested that the microbial populations associated with treated effluent, which may have migrated with the plume, out-competed indigenous microbes at 2.5m and 8m. Alternatively, samples from 2.5m and 8m perhaps contained very similar indigenous microbial populations to those present in the treated effluent before migration of the plume that responded positively to the increase in nutrients. It is therefore suggested that future studies at MAR sites should comprehensively analyse the spatial and temporal chemical and microbial population profiles at all observation wells over a specific time period before MAR commissioning. Additionally microbial profiles of treated effluent direct from the wastewater plant could also be evaluated prior to MAR commissioning to fully evaluate natural spatial and temporal variations.

The microbial PCO centroid analysis also contrasted with the chemical analysis with regards to 15m samples (FLB\_11). The PCO centroid microbial analysis placed the 15m samples with the 5m samples (Group 3) and 7m with 32m (Group 4). The chemical analysis predominantly placed 15m and 32m samples with the background and extraction bore samples. Therefore these samples were implicated as being unaffected by plume migration. The overall chemical signature at 5m migrated from Groups A to B to C (Figure 5-3 A & B) and thus progressively became more positively associated with nutrients over time. Therefore these results suggest that although groundwater at 5m received enhanced nutrients from the migrating plume, the microbial populations were similar to those from a sampling station shown to be unaffected by plume migration. The results therefore suggest that the indigenous microbial populations at 5m perhaps out-competed microbial populations associated with treated effluent that migrated with the plume. Therefore the microbial populations at 5m responded differently to the migrating plume than shown for microbial populations at 2.5m and 8m. This reinforces the suggestion of preferential flow in the karstic aquifer to some of the observation wells. The results also show that microbial populations from a sampling station shown to be unaffected by plume migration e.g. 15m (FLB\_11) and 32m (FLB\_16) contained different microbial populations. Therefore the results are showing that microbial populations are naturally more diverse than ambient groundwater chemistry at these distances.

In summary, although the centroid PCO analysis was able to separate microbial population structures from 2.5m to 32m, the clustering of samples did not exactly correspond to aquifer chemical analysis. There was dissimilarity between expected and observed results for changes in microbial populations, based on variation in chemical signature analyses. Discrepancies between groundwater microbiology and geochemistry may have been due to greater dynamic changes in microbial ecology than groundwater chemistry. The PCO centroid analysis is analogous to an overall average population over time .e.g. an averaging of the results. Thus the PCO centroid analysis was able to dissect the dynamic variation determined in the MDS plot (Figure 5-6). The use of 'averaging' may introduce errors by over-simplifying microbial differences over time. Therefore there could be discrepancies when considering averages unlike the chemical data that did not require averaging. Despite the differences between statistical methods used, the chemical and microbial analyses corresponded in many ways in

separating similarities and dissimilarities between different distance-based observation wells.

#### 5.3.4 Dynamics of Spatial and temporal variation in Microbial Populations compared to changes in nutrient concentrations

**Chemical changes were easily evaluated by a simple PCA which separated samples into distinct groups without requiring centroid analysis. These results suggested a progressive spatial and temporal change in overall aquifer chemistry, thus indicating slow-moving changes. In contrast the initial MDS plot (Figure 5-6) and PCO plots (**

Figure 5-7 A-C) were unable to confidently separate microbial populations between all distances into population groups. Despite the absence of distinct distance based groups for all observation bores, the infiltration gallery, extraction and background bores appeared to be very different. However, despite these indications the MDS stress value was high (0.28) and thus MDS interpretation should be viewed with caution. Additionally, samples from group D which ranged from 2.5m to 32m were not separated into discrete groups. Dynamic spatial and temporal variation in microbial communities was demonstrated in Table 5.3 as 72% of total microbial variation could only be explained over five PCO axes. These results indicated dynamic changes in microbial populations over distance and time. The PERMANOVA tests (Table 5.4, Table 5.5 and Table 5.7) demonstrated that time (e.g. sampling events) in addition to distance was a significant factor that caused variation in microbial population structures. The PERMANOVA results therefore suggested there were significant spatial and temporal variation in microbial populations. The PERMDISP test also demonstrated that there were significant differences in homogeneity of microbial dispersion between the time points and distances sampled. Therefore PERMDISP also provided evidence for a highly dynamic microbial community. Overall the microbial analyses indicated that variation in microbial populations was more dynamic than changes in chemical signature over time. It suggested that the complexity differences between the two different data sets contributed to the few discrepancies observed

between microbial and chemical clustering of distance-based samples. Variations between microbial population dynamics in association with individual chemical parameters will be discussed in section 5.3.8.

**Summary** – The key similarity between aquifer microbial population dynamics and aquifer chemistry is that the extraction and background bore were most dissimilar to the infiltration gallery samples. Fundamental differences between microbial and chemical analyses were that microbial samples from 2.5m to 32m showed greater dynamic variation over time. In addition the microbial PCO centroid analysis demonstrated that samples from 2.5m and 8m were very strongly associated with the infiltration gallery samples over time. In contrast the infiltration gallery PCA chemical analysis suggested the infiltration gallery samples were very different to all other samples. The results also provided evidence to suggest that groundwater affected by plume migration (5m) contained a microbial population structure which was more similar to groundwater unaffected by plume migration (15m-FLB\_11). These results suggest microbial populations can respond differently to the migrating plume thus contributing to more dynamic changes than shown for variations in overall chemical signature.

#### 5.3.5 Sampling Events 2 - 33 – Evidence for Lag Period before Microbial Populations Changes in Response to the Migrating Nutrient Plume –

The microbial analysis shown in Figure 5-9 illustrated microbial differences from sampling events 2 – 137. Figure 5-11 in contrast detailed the microbial differences from sampling events 2 – 33 only. The two Figures contrasted specifically with regards to the infiltration gallery samples. Figure 5-11 demonstrated that during earlier sampling events the infiltration gallery microbial populations were very different to all other samples. In contrast Figure Figure 5-9 demonstrated that the infiltration gallery samples were very similar to 2.5m and 8m samples over the total sampling events (2-137). The chemical signature of the infiltration gallery samples was shown to be very different to all other samples over total time. The microbial analysis thus more closely replicated the ‘chemical signature’ between sampling events 2 – 33. These results suggest that microbial analysis of changes in biodiversity were more sensitive to allochthonous nutrients than analysing the changes in chemical signature. Therefore

small changes in chemistry can significantly change microbial biodiversity to that described at the enhanced nutrient rich source e.g. infiltration gallery.

Amalgamation of sampling events was undertaken so that at Type III, PERMANOVA could be obtained as it is considered more robust than a Type I. Pair-wise tests are shown in Table 5.6 which indicated that the microbial population structures were most dissimilar when comparing the initial sampling events (Sampling Period 1) with the final sampling events (Sampling Period 3). These results therefore suggest that the microbial populations became more dissimilar to this original microbial population structure as time progressed. The chemical plume was shown to impact monitoring bores to a greater extent as time progressed. Therefore as the chemical plume migrated with time, the microbial populations in each bore became more dissimilar to the original starting population. These results thus suggest that microbial populations quickly became adapted to the enhanced nutrient supply from infiltrated plume migration similar to microbial acclimation previously described for an aquifer contaminated by coal tar derivatives (Madsen *et al.*, 1991).

**Summary** – Observation wells in close proximity to the infiltration gallery became more similar in microbial population structure to the infiltration gallery populations after sampling event 33. This shift in microbial population structure coincided with the shift in chemical signature discussed for observation bores 2.5 to 8m (Figure 5-2 A&B) e.g. aquifer chemistry shifted from Group B to Group A. Comparisons between the PCA chemical analyses and microbial centroid PCO analyses demonstrated that changes in aquifer chemistry and shifts in microbial population dynamics both occurred sometime after sampling event 33. These results suggest that microbial populations changed due to a greater impact from the migrating chemical plume over time.

### 5.3.6 Discrepancies between overall chemical signature and microbial population dynamics

Microbial samples from 5m were not clustered with samples from 2.5m, despite similarity in their overall chemical signature over time (Figure 5-1A&B and Figure 5-2 A&B). Dynamic temporal variation in microbial population dynamics was implicated in 5.3.4. In order to fully investigate the small differences in overall trends in aquifer chemistry and microbial population dynamics between the 2.5m and 5m monitoring bores, individual chemical parameters were investigated in Figure 5-4 A-R. These

parameters were individually analysed to ascertain differences in groundwater chemistry at these two sites which were undetected in the overall ‘chemical signature’.

Temporal variation in individual chemical parameters was investigated between distances with consideration for their potential affect on groundwater microbiology. A notable difference between individual chemical parameters between 2.5m and 5m occurred for nitrate (Figure 5-4E). The level of nitrate concentration from day 81 steadily decreased at 5m whereas there were steep increases and decreases observed at 2.5m. These results indicated that nitrate was being utilised at different rates. Therefore, perhaps groundwater at 2.5m and 5m contained a different nitrate-reducing bacterial community. Alternatively the nitrate-reducing microbial populations at 2.5m and 5m contained similar microbial communities, but metabolised nitrate at different rates. This may have been a consequence of differences in appropriate organic carbon concentrations between these distances to drive nitrate reduction. TOC and DOC only were analysed and not biodegradable (BDOC) and assimilable organic carbon (AOC). Therefore these observation wells may have contained different organic carbon sources which directly affected the microbial communities that developed.

The variation in nitrate concentration between locations which were shown to be similar in their overall temporal chemical signature (Figure 5-3), indicated that microbial species may have changed groundwater chemistry at specific locations. These differences may therefore explain why microbial and chemical trends do not exactly replicate each other. Therefore microbial analysis demonstrated biodiversity differences that were not supported by chemical trends which combined all chemical parameters. These results demonstrate that the overall chemical signature, individual chemical parameters and changes in microbial population dynamics need to be individually and comparatively investigated for a more comprehensive understanding of aquifer biogeochemical processes.

#### *5.3.7 Distance-based Successional Changes in Microbial Populations Dynamics*

The 3D PCO centroid analysis evaluated the microbial communities to a distance of 15m from the infiltration gallery (Figure 5-10). The trajectory line based on distance indicated that samples which are geographically in closer proximity were more similar to each to other. This graphical representation visually indicated that microbial

populations changed in a successional distance-based manner from the infiltration gallery. Chemical plume migration was also shown to be related to distance from the infiltration gallery (Figure 5-2). These combined results therefore suggest that microbial populations changed in a successional distance based manner concomitant with chemical plume migration. In addition the PERMANOVA analyses (Table 5.4, Table 5.5 and Table 5.7) indicated that distance was the dominant factor causing significant differences in microbial population structure. Simply, samples closest to the infiltration gallery were more similar microbiologically and chemically than samples at further distance from the infiltration gallery.

### 5.3.8 *Individual Chemical Parameters Associated with MDS clustering of Microbial Populations*

#### 5.3.8.1 DOC, TOC and phosphorous

**DOC, TOC and phosphorous** were associated with treated effluent as demonstrated in PCA Figure 5-1 (A). These chemical variables were negatively associated to Group C samples which were implicated as being unaffected by plume migration. In contrast, the infiltration gallery samples were predominantly positively associated. Figure 5-12 (C,D & E) visually demonstrated the greater concentrations of phosphorous, TOC and DOC which were associated with the gallery samples. This graphical representation of combined chemical and microbial data illustrates how individual chemical parameters were related to distinct microbial populations present at different distance based monitoring bores. It can therefore be hypothesised that the elevated concentrations of these chemical parameters at the infiltration gallery contributed to the different microbial community structure determined for this monitoring bore.

Phosphate was predominantly utilised at the infiltration gallery with the exception of breakthrough to 2.5m at sampling event 110. These results suggest that phosphate was quickly utilised via microbial metabolism within the infiltration gallery or that it was removed geochemically. The results indicate that groundwater did not contain phosphate at distance from the infiltration galleries. Phosphate is required for microbial metabolism, therefore it is suggested that any concentrations available in groundwater were quickly utilised via groundwater microbes thus resulting in undetectable concentrations. Thus it is hypothesised that phosphate was very transient due to naturally low concentrations in groundwater with any increased concentrations

of phosphate being quickly consumed via microbial metabolism. Therefore phosphate was not detected due to the transient nature of the oligotrophic aquatic environments (Jordan and Joint, 1998). Alternatively phosphate can be removed by adsorption to iron coated sand and limestone, which may also have occurred during the infiltration process. Thus a significant geochemical reduction may have been the cause of the phosphate reduction. As microbes utilise phosphate in small amounts it is suggested that geochemical loss was greater than microbial action.

TOC and DOC shown in Figure 5-12 (D) and (E) indicated that samples from 2.5m to 8m varied in their concentrations over time and between distances. These differences occurred despite similar overall chemical signature trends. Microbial populations shown in Figure 5-12 (D) and (E) did not cluster according to their respective TOC and DOC concentrations which again may be a reflection of their respective AOC and BDOC concentrations (which was not measured). Background water contained low concentrations of TOC and DOC; consequently it is suggested that migration of TOC and DOC from the infiltration gallery would fuel an increase in microbial numbers. The easily assimilable proportion of the migrating organic carbon would thus be rapidly utilised leaving refractory material. The removal of easily assimilable carbon would thus lead to a decline in many microbial populations relying on this nutrient. A decline in microbial populations could thus result in an increase in TOC concentration. It is suggested that concentrations of TOC would increase due to a reduced removal rate from a decline in microbial populations and the constant supply from the infiltration gallery. The increase in TOC would thus lead to a concomitant increase in microbial populations that were able to competitively compete for the easily assimilable proportion of incoming TOC. Thus it is proposed that episodic cycles occurred that consisted of microbial population increases and declines, based on the increase and decrease of TOC concentrations. These episodic cycles would thus lead to concomitant changes in microbial populations thus resulting in a very dynamic system with constant fluxes in TOC concentration and the proportion and availability of easily assimilable and refractory organic carbon. These changes in environmental parameters essential for microbial metabolism would thus lead to continual competitive changes in microbial populations. Due to the highly dynamic nature of the microbial system they were not clustered according to TOC and DOC suggesting that the biogeochemistry of the aquifer was complex.



### 5.3.8.2 Total Iron and Temperature

**Total Iron and Temperature-** Figure 5-12(K) shows that the extraction bore and samples at 15m distance, 19m depth (FLB\_11) and 32m distance, 18m depth (FLB\_16) contained much higher concentrations of total iron. Chemical results suggested that these sampling stations were unaffected by plume migration. Figure 5-4 (H) illustrated how these sampling stations increased in total iron over time. The total iron concentrations were initially low but increased as infiltration and extraction progressed over time. This observation suggests a possible side effect from an unknown factor associated with the MAR that may be site specific. It is suggested that extracting water from the aquifer affected observation bores FLB\_11 and 16 despite evidence that they were unaffected by plume migration. The increase in total iron concentration and temperature as time progressed indicates that groundwater chemistry changed despite being unaffected by plume migration. These results suggest that the hydraulic gradient formed from pumping created a localised gradient for preferential flow of groundwater (non-plume related) containing higher concentrations of reduced iron into this observation well. Due to the increase in temperature at FLB\_11 (increase in groundwater temperature with depth) it may be hypothesised that water was drawn from groundwater table via a preferential flow path created by a fissure in the limestone. It is hypothesised that this did not occur immediately when pumping began as the fissure is perhaps not large and was clogged with aquifer material and calcification. The continual pumping and hydraulic pull formed from the hydraulic gradient dislodged these materials thus creating less resistance therefore increasing aquifer transmissivity in this localised fissure. Conversely the hydraulic gradient may have pulled water from a deeper aquifer via a limestone fracture. Bore water in the Perth area characteristically contains high iron concentrations (Appleyard *et al.*, 2004). Bore water also has elevated temperatures due to depth, consequently Perth bore water contains elevated temperature and total iron concentrations and thus may have penetrated these observation wells.

The increase in total iron at FLB\_11 was replicated in the extraction bore which was affected by clogging issues related to iron (unpublished data). As already discussed, the hydraulic gradient pulled groundwater from different depths and distances to that described for the extraction bore (section 3.5). It is therefore suggested that groundwater from FLB\_11 was pulled into the extraction well water along with other

groundwater at this site. Additionally, elevated oxygen levels were established at the extraction bore due to pumping. It is therefore hypothesised that an increase in oxygen and iron concentrations thus resulted in favourable conditions for iron oxidising bacteria. It is speculated that the dominant and consistent band that occurred at the extraction well (Figure 5-5) belonged to a group of iron-oxidising bacteria and further work will sequence this DNA. Despite samples from the extraction bore, FLB\_11 and 16 containing elevated total iron concentrations they were not grouped together on the MDS plot (Figure 5-12 K). These results thus again illustrate that aquifer biogeochemistry is complex and can often not be explained by changes in individual chemical parameters.

#### 5.3.8.3 Nitrate, Ammonia and Nitrification

**Nitrate, Ammonia and Nitrification** – Figure 5-12 (F) demonstrated that nitrate was predominantly low (<2mg/L) at the infiltration gallery except for the very initial samples at day 2 and for a later sampling event at day 110. In addition the background and extraction bores, FLB\_11 and FLB\_16 also contained very low nitrate concentrations. These samples formed Group C in the chemical PCA analysis shown in Figure 5-2 (A) and (B) which were implicated as unaffected by plume migration. Therefore the infiltration gallery and samples unaffected by the nutrient plume contained low concentrations of nitrate (<2mg/L). In contrast samples in close proximity to the infiltration gallery at 2.5m to 8m and additionally FLB\_10 contained higher concentrations of nitrate. Figure 5-4 (E) more clearly shows the higher levels of nitrate concentration for these samples and additionally temporal fluctuations which displayed deep peaks and troughs.

Nitrate concentrations were greater at observation wells from 2.5m to 8m from day 2 than their respective background readings as indicated in the overall chemical signature PCA plot Figure 5-3 (A) and (B). These results suggest that nitrate increased at sampling stations in close proximity to the infiltration gallery from day 02 despite low concentrations in the infiltration gallery. Therefore these results suggest that an unknown factor associated with the plume, created conditions that elevated nitrate levels in ambient groundwater. Nitrate concentrations only increased in groundwater at sampling stations that received the migrating chemical plume.

Nitrate may be formed from the oxidation of ammonia to an intermediate nitrite product which is then transformed into nitrate. *Nitrosomonas* and *Nitrobacter* bacterial species are able to form nitrate using this bacterial nitrification process (Bothe *et al.*, 2000). Figure 5-4 (G) shows that ammonia fluctuated greatly in the infiltration gallery but was never present in samples from 2.5m to 8m over time at detectable concentrations. Therefore ammonia disappeared from the system. Figure 5-4 (G) demonstrated a peak in ammonia between sampling event 32 and 69 at the infiltration gallery. There was a subsequent increase in nitrate at the infiltration gallery from day 69 and a substantial increase between sampling events 95 and 110. The increase in nitrate at the infiltration gallery resulted in a decline in sampling stations in closest proximity to the infiltration gallery. These results suggest that nitrification of ammonia contained with the treated effluent was taking place periodically between the infiltration gallery and observation bores from 2.5m to 8m. A substantial increase in nitrate did occur at sampling event 110 at the infiltration gallery. The increase in nitrate at the infiltration gallery resulted in a decline in ammonia due to nitrification also at the infiltration gallery. These results coincided with a decline in nitrate at observation bores receiving the plume. It is proposed that a decline in nitrate at observation bores receiving the plume was due to nitrification at the infiltration gallery. Nitrification at the infiltration gallery thus utilised ammonia that was previously migrating with the plume and subsequently being converted to nitrate in groundwater that received the plume. In summary it is hypothesised that ammonia predominantly migrated with the chemical plume to the soil beneath the infiltration galleries. Here, and in the near aquifer zone ammonia was quickly converted to nitrate by nitrifying bacteria. Nitrification also periodically occurred in the infiltration gallery. The greater concentrations of groundwater nitrate from 2.5m to 8m compared with the infiltration gallery suggested that nitrification was predominantly taking place at these observation wells rather than directly at the infiltration gallery. Thus it is very plausible that nitrification occurred during infiltration through the unsaturated zone. Nitrate remained low in sampling stations FLB\_11, 16 and the extraction bore as these samples stations were unaffected by plume migration and therefore did not have the potential to convert ammonia to nitrate as the ammonia was supplied by the infiltration gallery.

#### 5.3.8.4 Alkalinity, Chloride, Electrical Conductivity and TDS

**Alkalinity, Chloride, Electrical Conductivity and TDS** - Alkalinity demonstrated a very strong PCA association with Group C in Figure 5-1 (A). Thus samples that were implicated as being unaffected by plume migration had a strong association to alkalinity, whereas the infiltration gallery had had a strong negative association. Figure 5-4 (N) demonstrates the much lower concentrations of this analyte for the infiltration gallery compared with all other samples. Correlation analyses shown in Table 5.7 demonstrated that alkalinity was negatively correlated to DOC, TOC and phosphorous. Unlike DOC, TOC and phosphorous, alkalinity concentrations did not change in 2.5m to 8m at later sampling events and could therefore not be related to plume migration. Samples from 2.5m to 8m did not decrease in alkalinity concentration concomitant with plume migration. Therefore alkalinity could not be used as a tracer to track plume migration from the infiltration gallery. It is therefore suggested alkalinity is not stable once mixed with ambient groundwater/aquifer limestone where chemical components that constitutes alkalinity mix and react with other chemical analytes within groundwater.

Chloride, electrical conductivity and TDS were all highly correlated as shown in Table 5.7. These analytes are chemically related and therefore the high correlation values suggest that the chemical data was robust and reliable. Chloride concentrations were low in the background and extraction well (Figure 5-4L) but much higher in the infiltration gallery. Therefore it is suggested that chloride was associated with treated effluent which was not replicated in ambient groundwater. Figure 5-12(I) indicated that that a cluster of microbial samples were associated to increased levels of chloride concentrations. Peaks and troughs in chloride concentrations at the infiltration gallery were replicated in samples implicated in receiving plume migration, but varied in the concentration of chloride present. Therefore unlike alkalinity, chloride, which is also associated with treated effluent compared with groundwater, was conserved during plume migration. It is suggested that the reduced concentrations at distance from the infiltration gallery is indicative of dilution caused by treated effluent mixing/displacing ambient groundwater, thus reducing its overall concentration. These results also suggest that chloride was implicated in clustering microbial samples which were associated with plume migration. Therefore chloride can be used as a conservative tracer for microbial clustering of samples responding to and/or migrating with the effluent plume.

### 5.3.9 Sulphate

**Sulphate** – Sulphate concentrations fluctuated over time for most samples (Figure 5-4 D). The dissolved oxygen and redox levels (Figure 5-4 F&S) suggested that sulphate reduction should not have been a favourable groundwater metabolic process. In contrast to many electron acceptors (e.g. nitrate), sulphate concentrations were abundant in background groundwater and observation wells unaffected by the nutrient plume. Sulphate reduction may thus have been inhibited due to the limited availability of TOC and DOC in addition to redox and dissolved oxygen levels.

The possibility of active sulphate reduction within the groundwater environment at the Perth MAR site will be discussed in greater detail in the culture analysis section as sulphate reducers were cultured from all observation bores at various sampling events. Never-the-less because sulphate reducing bacteria were present in groundwater samples this indicated that the redox potential of the groundwater environment was not toxic to these anaerobic bacteria. These results therefore suggest that sulphate reduction may have been actively occurring in favourable micro niches periodically over time.

### 5.3.10 Comparisons of Microbial and Chemical Variability at select sampling stations.

There was an initial change in overall chemical signature from the background readings at sampling event 02 (Figure 5-3 A&B) for samples from 2.5m. Samples from 2.5m also migrated from Group B to Group A (Group A being more positively associated with nutrients) sometime between sampling events 33 and 68. Based on these changes in overall chemical signature it was suggested that breakthrough of the nutrient plume from the infiltration gallery had already occurred at sampling event 02 but the groundwater became more positively associated to nutrients sometime between sampling events 33 and 68. Microbial analysis was undertaken from sampling event 02. The MDS plot shown in Figure 5-14 (A) indicated that the microbial community structure initially changed at 2.5m at day 13 and again at day 32. These results therefore indicated that microbial populations changed before the chemical signature migrated from Group B to Group A.

After the migration from Group B to Group A at day 69, the chemical signature of samples at 2.5m gradually changed over time e.g. predominantly these samples

gradually became more positively associated with nutrients over time. In contrast after initial changes at day 13 and 32, the microbial population structure reverted back to its previous overall structure as at day 2. Microbial populations at 2.5m then remained stable for a significant period from day 69 to 123. The microbial population finally changed at day 137. The comparative microbial and chemical analysis suggests that their respective temporal changes were different. The overall chemical signature progressively changed over time whereas changes in microbial populations were not progressive or systematic in anyway. They were observed to randomly change in a dynamic fashion. The comparative microbial and chemical analysis also suggests that microbes may change groundwater chemistry as the microbial population structures changed before the overall chemical signature migrated from Group B to Group A.

Figure 5-14 (B) demonstrated that duplicate samples which were taken at the same time e.g. day 2, 32, 137 were clustered together. These results indicated that replicate samples for a given sampling event were highly reproducible. Microbial MDS, PCO, PERMANOVA, ANOSIM and PERMDISP analyses incorporated a distance at 2.5m. Samples from FLB\_01 and 02 were both located at the same distance from the infiltration gallery (2.5m) but differed in depth namely 11m and 9m respectively (Figure 5-23). Both these sampling stations were amalgamated for the overall defined distance at 2.5m used for all analyses. Figure 5-14 (B) demonstrated that these two observation bores which were situated at the same distance but at slightly different depths contained similar microbial populations at duplicated sampling events. Therefore not only did replicate samples cluster together but additionally samples at identical distances but with slight differences in depth also contained very similar microbial populations over time.

Figure 5-15 (A) demonstrated microbial population changed at the infiltration gallery over time whereas Figure 5-15 (B) showed the variation in overall chemical signature for complementary sampling events. These results also showed that microbial populations changed before changes in the overall aquifer chemical signature (e.g. day 60 as opposed to day 69). These results may therefore also indicate that microbial populations were changing groundwater chemistry. These results may thus indicate that changes in microbial biodiversity (changes in biodiversity at the lowest trophic level) can perhaps be used as indicators to track plume migration.

### 5.3.11 Conclusions

- 1) Spatial and temporal change in microbial community structures was more dynamic than changes in overall chemical signature.
- 2) Spatial diversity was determined as the greatest contributing factor causing significant differences in microbial community structures.
- 3) The microbial populations changed in a successional manner which could be related to changes in aquifer geochemistry.
- 4) Shifts in microbial biodiversity were sensitive to detecting small changes in environmental conditions that could be related to individual environmental parameters.

- 5) DGGE analysis was unable to determine the magnitude of microbial population differences between different distances, whereas chemical analysis clearly separated the infiltration gallery samples as being very different.
- 6) Microbial populations changed before the overall chemical signature at specific observation wells analysed (2.5m and infiltration gallery) suggesting microbes changed groundwater chemistry.

## 5.4 Perth MAR Microbial RESULTS for Fermentative Cultures

### 5.4.1 Microbial Diversity of Perth MAR Infiltration Site for Fermentative Cultures via DGGE rDNA banding patterns

The DGGE DNA/PCR banding patterns for all fermentative bacterial cultures shown in Figure 5-16 illustrates that there were twenty seven band class types ranging from FLB\_2.9 to FLB\_97.2. The band class types visually varied in band intensity and types present within and between observation wells over time. A MDS was undertaken on the binary matrix data to ascertain similarities and differences in microbial population structure between samples.

### 5.4.2 Multidimensional Scaling analysis of microbial DGGE rDNA banding patterns for Perth MAR Fermentative cultures over time

The 2D MDS plot shown in Figure 5-17 did not clearly indicate specific fermentative bacterial grouping structures at a confident MDS stress level. Despite a high MDS

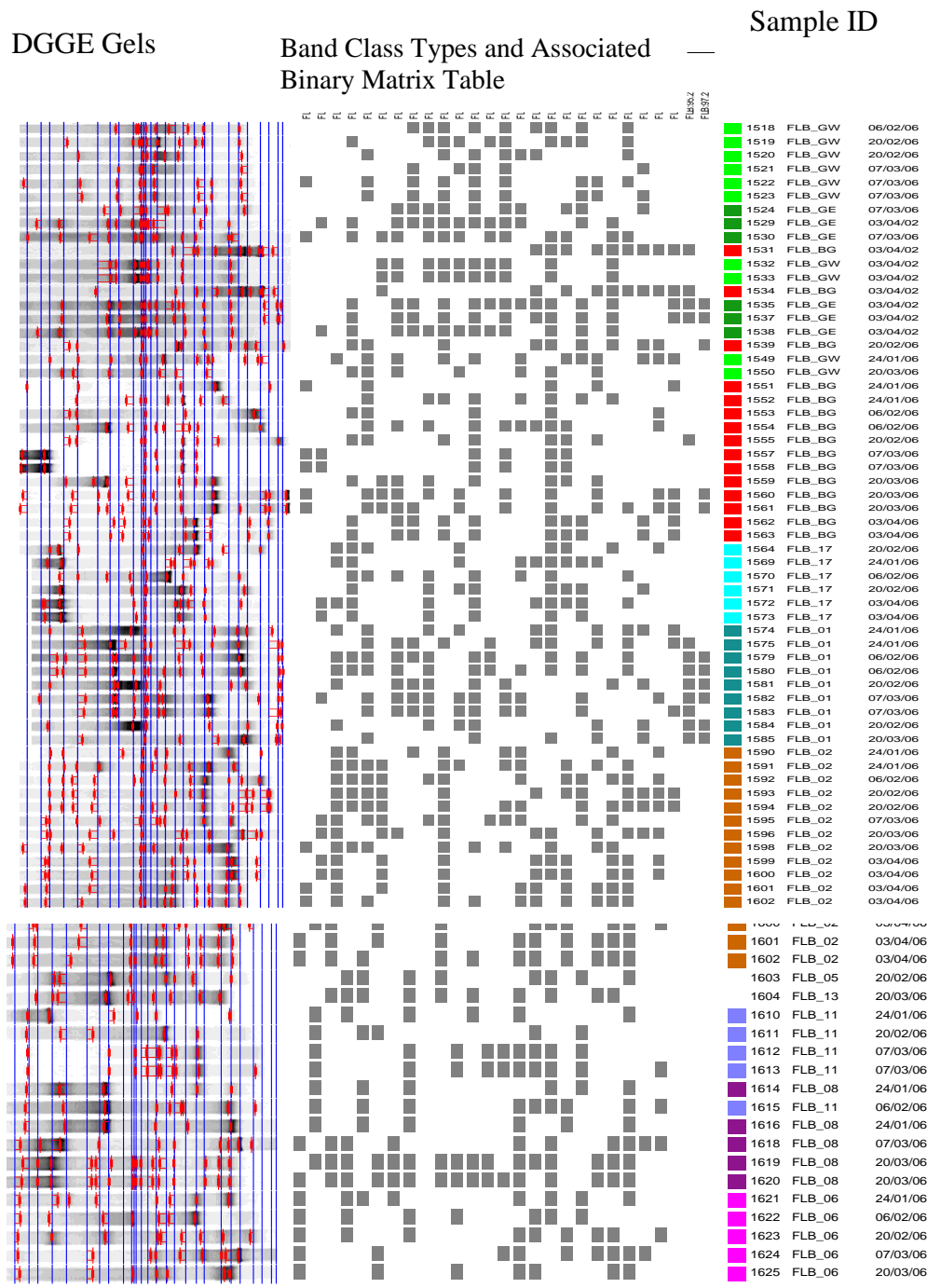


stress level initial visual interpretation suggests clustering of some fermentative bacterial community structures namely at the infiltration gallery (Group A), background bore (Group B) and a separation of the fermentative bacterial populations at 2.5m namely FLB\_01 (Group C) & FLB\_02 (Group D). Fermentative bacterial community structures from all other distances did not form distinct population groups and were randomly scattered across the MDS plot.

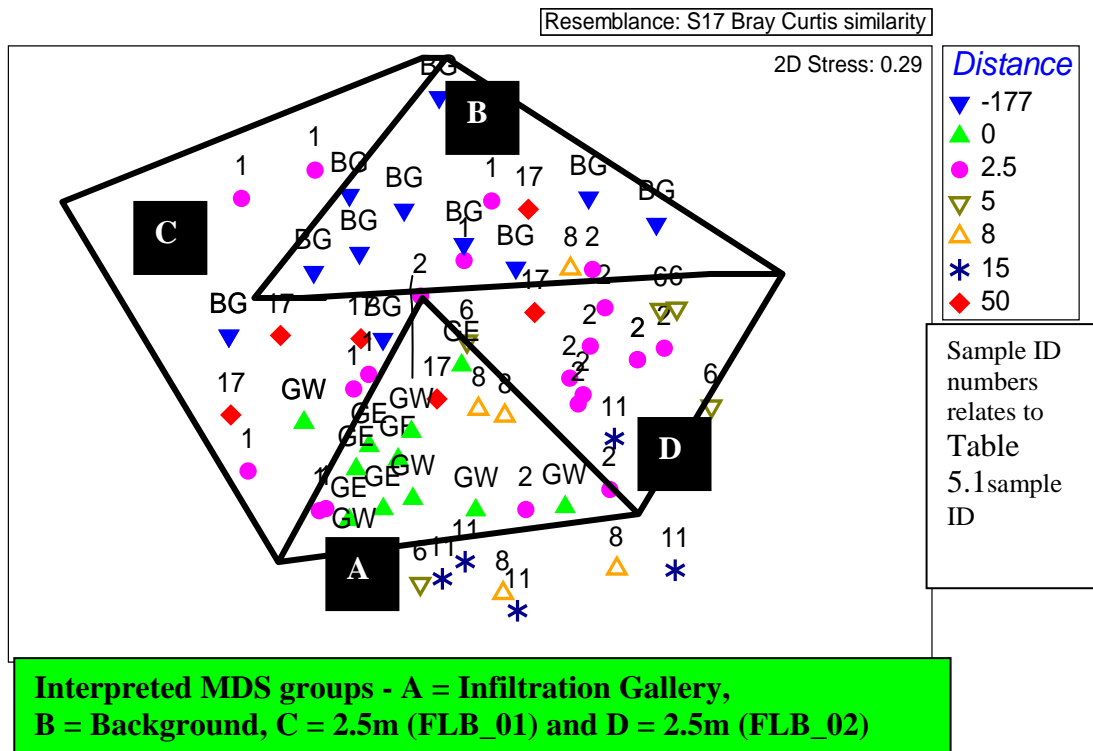
#### 5.4.3 Plotting centroid PCO analysis of amplified Microbial rDNA/DGGE banding patterns for all Perth MAR Fermentative Cultures over time

Figure 5-18 shows the overall similarity between fermentative bacterial community structures at different distances over time. PCO 1 and PCO 2 described 57% of the total variation in fermentative bacterial community structure between the distance-based observation wells over time. The results suggest that the fermentative bacterial populations from 2.5m were most similar to the fermentative bacterial community structure at the infiltration gallery. In contrast the background and extraction bore fermentative bacterial community structure were positioned away from the infiltration gallery and 2.5m bacterial populations. These results thus suggest that these bacterial populations were most dissimilar to the infiltration gallery and 2.5m fermentative community structures. Fermentative cultures from 5m and 8m were not placed in close proximity to the 2.5m fermentative bacterial population structure and therefore did not fall within the suggested nutrient gradient from the positive (infiltration gallery) to the negative control (background).

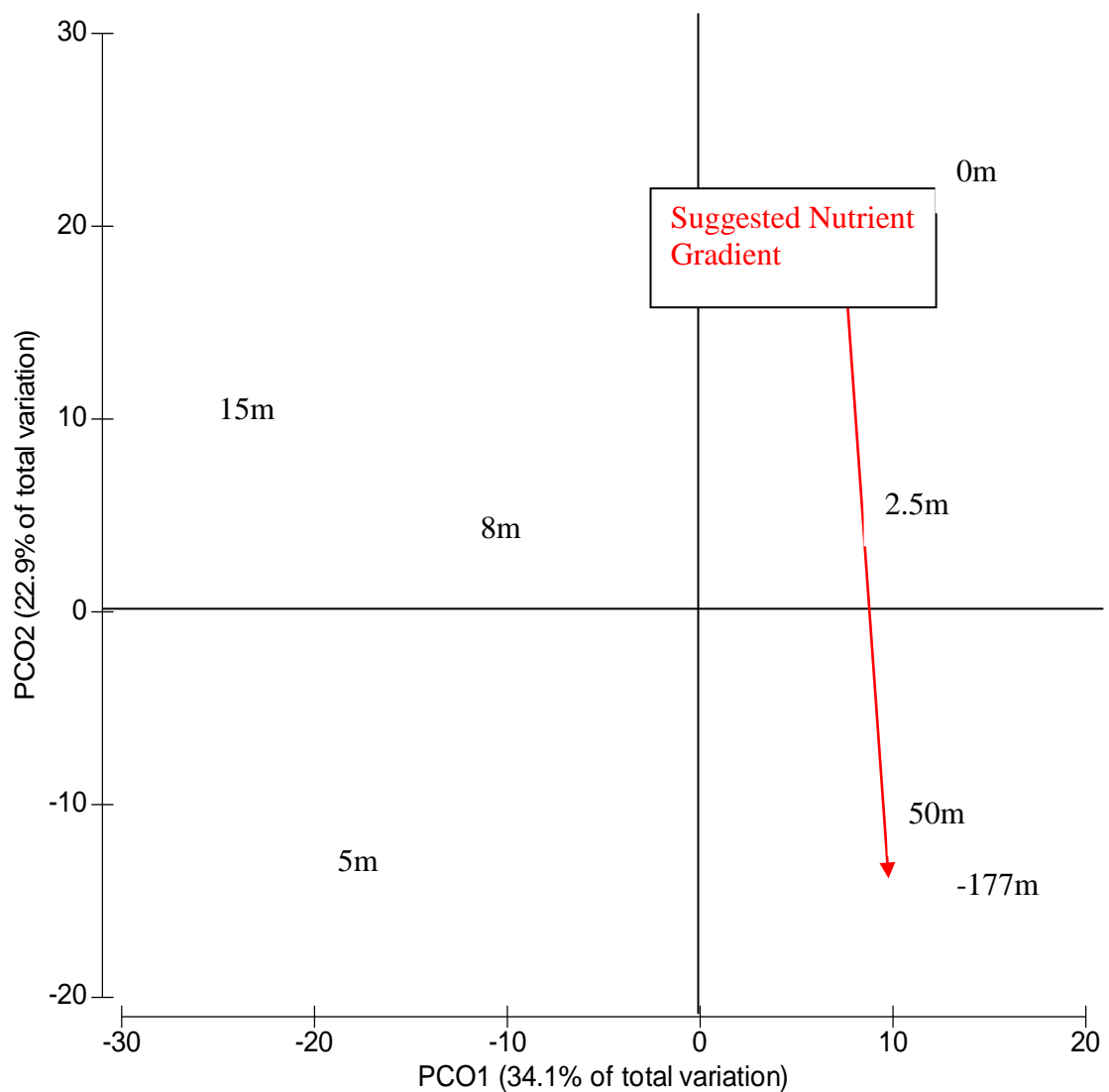
**Figure 5-16** DGGE of bacterial rDNA/PCR banding patterns and associated binary matrix table for band matching data for all fermentative cultures from the Perth MAR infiltration site over time.



**Figure 5-17** 2D MDS Plot for amplified Microbial rDNA/DGGE banding patterns for all Perth MAR Fermentative Cultures over time



**Figure 5-18 - 2D PCO plot for the reduced CENTROID similarity matrix based on distance from the infiltration gallery for microbial variation (rDNA/DGGE banding patterns) of Perth MAR fermentative cultures over time.**



**Distances refer to: Distance from Infiltration gallery**

5.4.4 PERMANOVA – Statistical significance test for assessing microbial variation of Perth MAR fermentative culture DGGE/rDNA banding patterns for all distances over time.

The statistical design for analysing differences between the fermentative bacterial populations consisted of factors (i) time (ii) distance and (iii) an interaction of time and distance. This experimental design was used for all PERMANOVA analyses for non-culture and culture samples.

The results from Table 5.12 indicate that the fermentative bacterial community structures varied significantly due to an interaction of distance and time. The results also indicate that there was a significant affect from distance and time independently and that distance had the largest affect on the variation in fermentative community structure (largest F value and lowest P value). These results replicate the outcome of the PERMANOVA tests for non-culture water samples as shown in Table 5.4 and Table 5.5.

The pair-wise tests for the independent affect of distance alone contributing to the differences in fermentative community structures are shown in detail in Appendix 9. These results indicate that predominantly there were significant differences in fermentative bacterial populations between most distances. Insignificant differences were detected in pair-wise comparison between 2.5m & 8m and 5m & 8m. Pair-wise tests for the interaction of distance and time based on **distance** are shown in detail in Appendix 10. These results indicate that there was minimal interaction occurring between distance and time that contributed to the significance difference in fermentative bacterial community structure. Pair-wise tests for the interaction of distance and time based on **time** are also shown in detail in Appendix 10. These results show that a significant difference in fermentative community structure only occurred between amalgamated sampling time 2 & 3.

#### 5.4.5 *PERMDISP – Statistical test for assessing microbial homogeneity of dispersion for all Perth MAR fermentative culture DGGE/rDNA banding patterns for all distances over time.*

PERMDISP was used to statistically test for the homogeneity of multivariate dispersion of fermentative bacterial DGGE rDNA banding patterns over time for each distance. Table 5.13 illustrates that the P value for the distance-based F statistic was significant for homogeneity of dispersion. In contrast a highly significant difference was not obtained for sampling event. These results thus suggest that the fermentative bacterial populations were relatively homogenous over time at each distance but there was a significant difference overall in the homogeneity of dispersion between the various distances. Pair-wise comparisons for both distance and sampling event are shown in detail in Appendix 11 and 12. Comparison of pair-wise tests between the PERMANOVA (Appendix 9 & 10) and PERMDISP (Appendix 11 & 12) statistical tests show that they were not identical. Therefore these results indicate that the

microbial populations isolated from fermentative media were not similar over distance as a consequence of location (PERMANOVA) and dispersion (PERMDISP).

**Table 5.12** Type 3 PERMANOVA – Perth MAR fermentative cultures

Source	df	SS	MS	F	P (permutation)
Distance	4	27328	6832	4.75	0.001
Sampling Event	1	3771	3771	2.62	0.018
Distance x Sampling Event	9	18744	2083	1.45	0.034
Res	50	71829	1437		
Total	66	1.3729E5			

Experimental design included time and distance as fixed factors and an interaction of these as a variable factor.

- Individual sampling events were amalgamated as follows so that pair-wise comparisons could be obtained on a Type 3 PERMANOVA:

Sample 01 = 24<sup>th</sup> January to 6<sup>th</sup> February (day 68 – 81)  
 Sample 02 = 20<sup>th</sup> February to 7<sup>th</sup> March (day 95 – 110)  
 Sample 03 = 20<sup>th</sup> March to 3<sup>rd</sup> April (day 123 – 137)

**Table 5.13** PERMDISP – Deviation of sample dispersion from the Centroid for Perth MAR fermentative cultures

	F Statistic	P (permutation)
Sampling Event	2.58	0.081
Distance	4.83	0.007

5.4.6 Separate DGGE/rDNA microbial MDS analyses between (i) background and infiltration gallery (ii) background, infiltration gallery and extraction bore and (iii) all other samples excluding background, infiltration gallery and extraction bore.

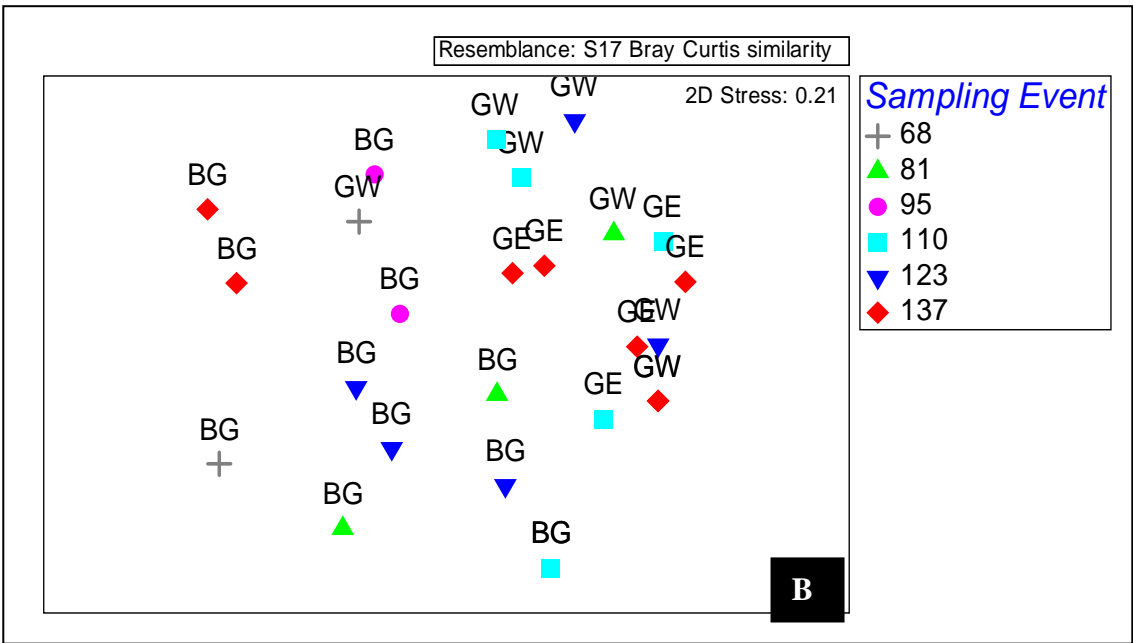
(i) **Background vs. Infiltration Gallery** - Figure 5-19 shows that the background fermentative bacterial populations were more widely dispersed across the MDS plot compared with fermentative community structures from the infiltration gallery. Despite variation in dispersion these samples could be separated into two groups namely the background (Group A) and infiltration gallery (Group B) samples.

(ii) **Background vs. Infiltration Gallery vs. Extraction Bore** - Figure 5-20 shows that the extraction bore samples were more similar to the background samples (Group C) than the infiltration gallery (Group A). An exception is an infiltration gallery

fermentative population structure at sampling event 69 which clustered with the background fermentative bacterial populations. Therefore this population was very different to all other infiltration gallery fermentative bacterial community structures. In addition the background fermentative populations were more widely dispersed than the extraction and infiltration gallery fermentative bacterial populations as these cultures were spread over two groups namely Group B and Group C.

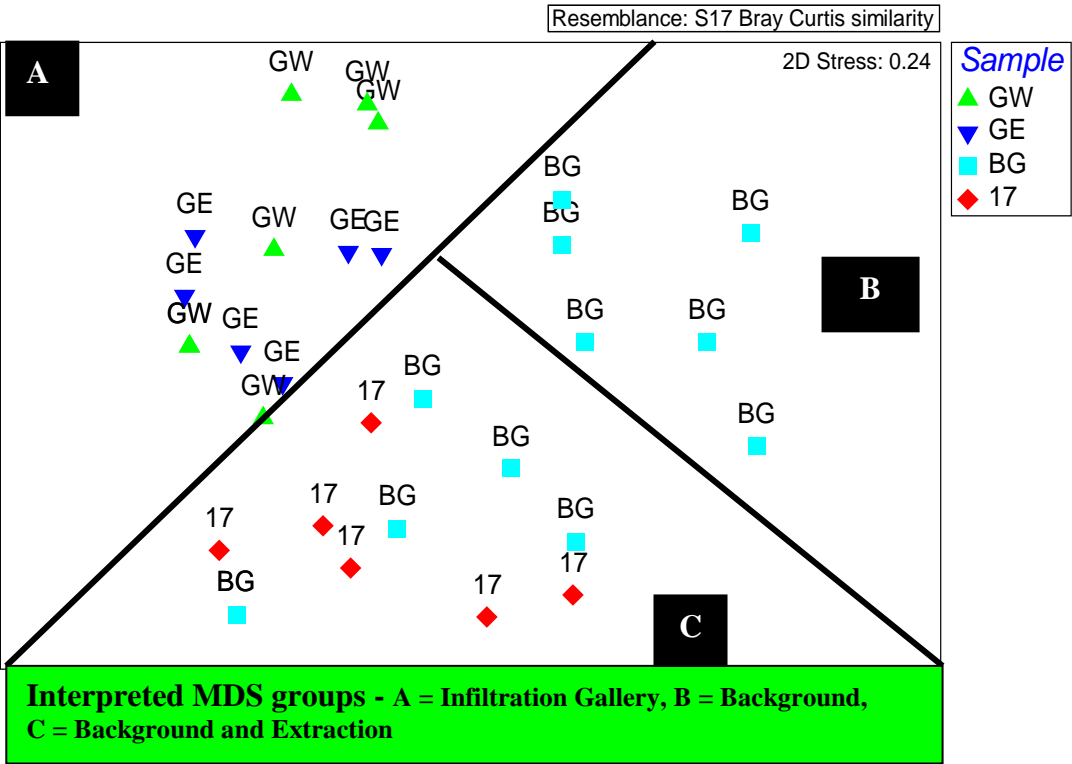
(iii) Comparison of all sites excluding Background, Infiltration Gallery and Extraction well - Figure 5-21 indicates potential groupings between fermentative bacterial populations other than the most dissimilar sampling stations (infiltration galleries, extraction and background well - Figure 5-20). For examples the fermentative bacterial population structures at 2.5m were separated into their respective sampling stations namely FLB\_01 (Group A) & FLB\_02 (Group B). In addition fermentative bacterial populations at 15m (Group C) were clustered together. In contrast fermentative bacterial population structures from 5m & 8m were more variable over time as these population structures were spread across Groups B and C.

**Figure 5-19** 2D MDS Plot for amplified Microbial rDNA/DGGE banding patterns for Perth MAR Background (BG) and Infiltration Gallery (GW/GE) Fermentative Cultures over time



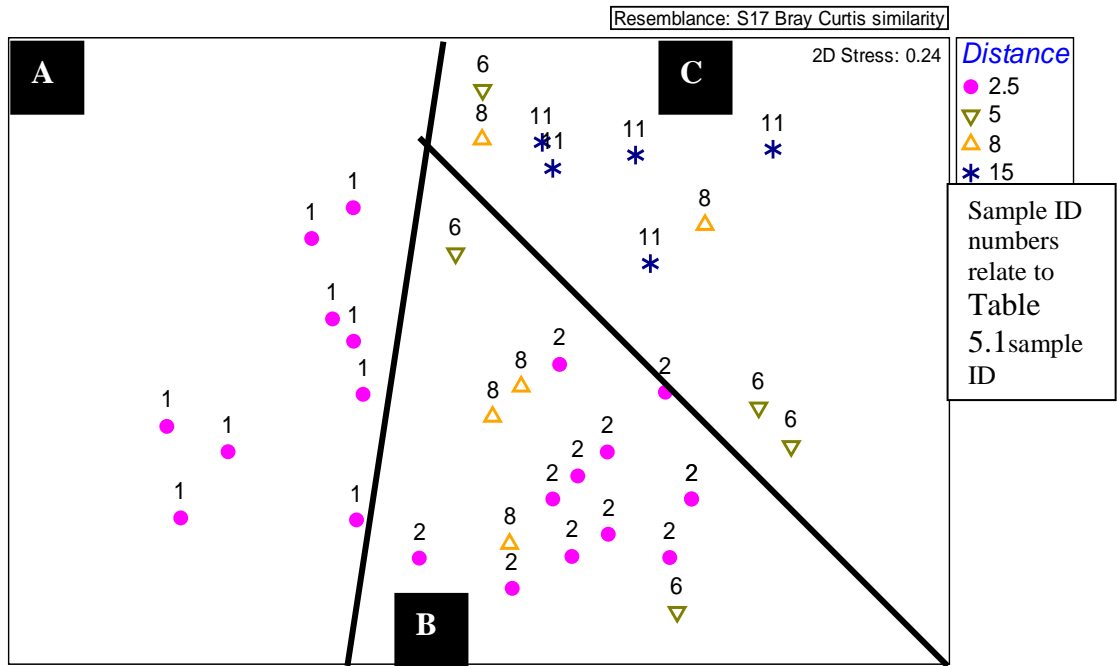
**Interpreted MDS groups – A = Background, B = Infiltration Gallery**

**Figure 5-20** 2D MDS Plot for amplified Microbial rDNA/DGGE banding patterns for Perth MAR Background (BG), extraction (17) and Infiltration Gallery (GW/GE) Fermentative Cultures over time



**Figure 5-21** 2D MDS Plot for amplified Microbial rDNA/DGGE banding patterns for all Perth MAR Fermentative cultures (excluding background, extraction and infiltration gallery) over time





**Interpreted MDS groups - A = FLB\_01 at 2.5m, B = Predominantly FLB\_02 at 2.5m, C = FLB\_11 at 15m, FLB\_06 at 5m and FLB\_08 at 8m**

## 5.5 Perth MAR Microbial RESULTS for Sulphate-Reducing Cultures

### 5.5.1 Microbial Diversity of Perth MAR Infiltration Site Sulphate-Reducing Cultures via DGGE DNA banding patterns

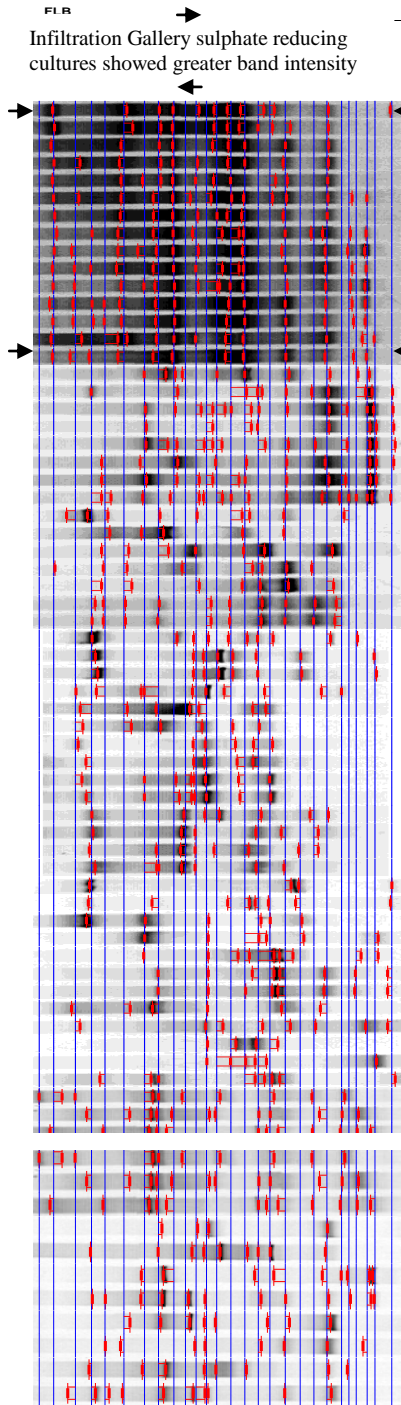
The DGGE DNA/PCR banding patterns for all sulphate-reducing cultures shown in Figure 5-22 illustrates that there were twenty seven band class types ranging from FLB\_1.5 to FLB\_97.6. The band class types visually varied in band intensity and types present within and between observation wells over time. Notably the sulphate reducing band class types from the infiltration gallery showed much greater band intensity compared with all other sampling stations. A MDS was undertaken on the binary matrix data to ascertain similarities and differences in microbial population structure between samples.

### 5.5.2 Multidimensional Scaling analysis of Microbial DGGE rDNA banding patterns from Perth MAR Sulphate-Reducing cultures over time

The 2D MDS plot shown in Figure 5-23 indicates that the sulphate reducing bacterial populations from the infiltration gallery (Group A) were very tightly grouped over time which was in contrast to the fermentative cultures (Figure 5-17) and non-culture bacterial water samples (Figure 5-6). Although more widely dispersed, the background, extraction and 2.5m sulphate reducing bacterial populations could be separated into groups as indicated. The sulphate-reducing cultures differ from the fermentative cultures (Figure 5-17) in that the two sampling stations at 2.5m (FLB\_01 and 02) were not so clearly distinct (Group D). In addition, unlike the fermentative bacterial populations, the sulphate-reducing cultures from the extraction well formed a distinct group over time (Group C).

**Figure 5-22** DGGE of bacterial rDNA/PCR banding patterns and associated binary matrix table for band matching data for all sulphate-reducing cultures from the Perth MAR Infiltration site over time.

### DGGE Gels



### Band Class Types and Associated Binary Matrix Table

Band Class	FLB15	FLB55	FLB115	FLB158	FLB195	FLB247	FLB303	FLB340	FLB382	FLB414	FLB443	FLB472	FLB489	FLB538	FLB576	FLB612	FLB644	FLB686	FLB727	FLB764	FLB799	FLB840	FLB859	FLB879	FLB909	FLB930	FLB976	
FLB15	1																											
FLB55		1																										
FLB115			1																									
FLB158				1																								
FLB195					1																							
FLB247						1																						
FLB303							1																					
FLB340								1																				
FLB382									1																			
FLB414										1																		
FLB443											1																	
FLB472												1																
FLB489													1															
FLB538														1														
FLB576															1													
FLB612																1												
FLB644																	1											
FLB686																		1										
FLB727																			1									
FLB764																				1								
FLB799																					1							
FLB840																						1						
FLB859																							1					
FLB879																								1				
FLB909																									1			
FLB930																										1		
FLB976																											1	

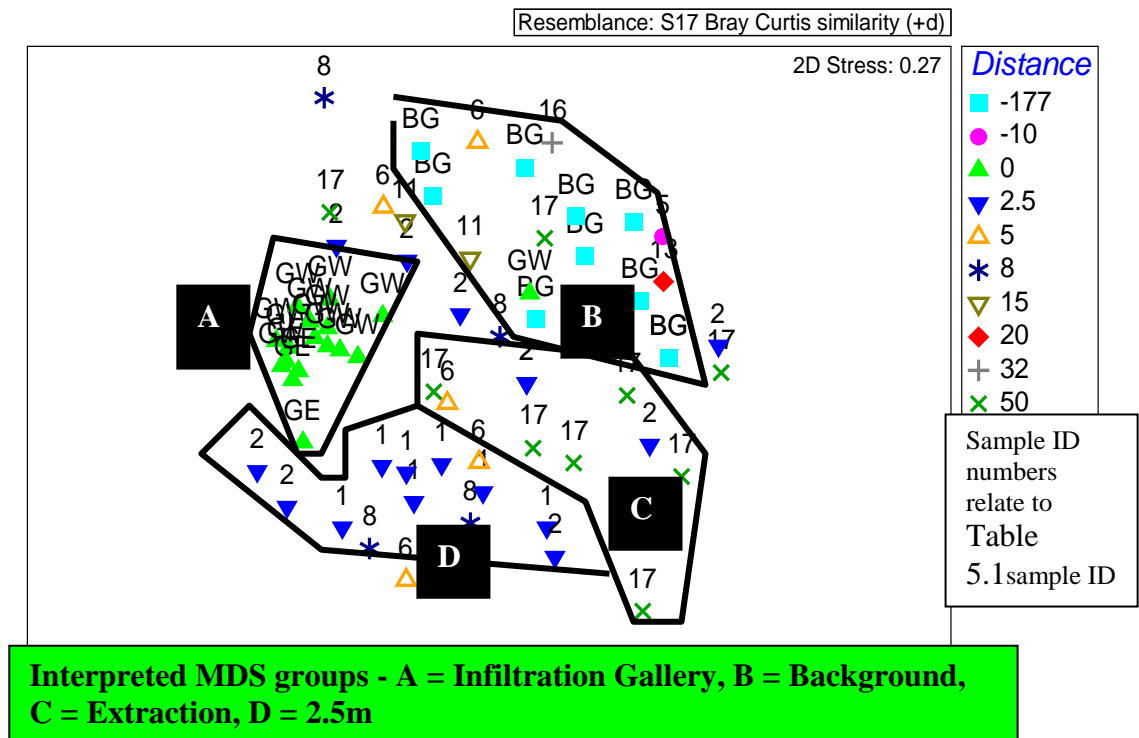
### Sample ID

1430	FLB_SRB	FLB_GW	25/01/06
1431	FLB_SRB	FLB_GW	06/02/06
1432	FLB_SRB	FLB_GW	20/02/06
1433	FLB_SRB	FLB_GW	20/02/06
1434	FLB_SRB	FLB_GW	07/03/06
1435	FLB_SRB	FLB_GW	07/03/06
1436	FLB_SRB	FLB_GW	20/03/06
1438	FLB_SRB	FLB_GW	03/04/02
1439	FLB_SRB	FLB_GW	03/04/02
1440	FLB_SRB	FLB_GW	03/04/02
1441	FLB_SRB	FLB_GW	03/04/02
1442	FLB_SRB	FLB_GW	03/04/02
1443	FLB_SRB	FLB_GE	03/04/06
1444	FLB_SRB	FLB_GE	07/03/06
1445	FLB_SRB	FLB_GE	03/04/06
1450	FLB_SRB	FLB_GE	03/04/06
1451	FLB_SRB	FLB_01	24/01/06
1452	FLB_SRB	FLB_01	06/02/06
1453	FLB_SRB	FLB_01	06/02/06
1454	FLB_SRB	FLB_01	20/02/06
1455	FLB_SRB	FLB_01	20/03/06
1456	FLB_SRB	FLB_01	20/03/06
1458	FLB_SRB	FLB_01	07/03/06
1459	FLB_SRB	FLB_02	24/01/06
1460	FLB_SRB	FLB_02	06/02/06
1461	FLB_SRB	FLB_02	06/02/06
1462	FLB_SRB	FLB_02	07/03/06
1463	FLB_SRB	FLB_02	20/03/06
1464	FLB_SRB	FLB_02	03/04/06
1465	FLB_SRB	FLB_02	03/04/06
1470	FLB_SRB	FLB_BG	24/01/06
1471	FLB_SRB	FLB_BG	06/02/06
1472	FLB_SRB	FLB_BG	06/02/06
1473	FLB_SRB	FLB_BG	20/02/06
1474	FLB_SRB	FLB_BG	20/02/06
1475	FLB_SRB	FLB_BG	07/03/06
1477	FLB_SRB	FLB_BG	20/03/06
1478	FLB_SRB	FLB_BG	20/03/06
1479	FLB_SRB	FLB_BG	03/04/06
1480	FLB_SRB	FLB_BG	03/04/06
1481	FLB_SRB	FLB_13	20/03/06
1482	FLB_SRB	FLB_05	20/02/06
1483	FLB_SRB	FLB_16	06/02/06
1484	FLB_SRB	FLB_17	03/04/06
1489	FLB_SRB	FLB_17	24/01/06
1490	FLB_SRB	FLB_17	06/02/06
1491	FLB_SRB	FLB_17	20/02/06
1492	FLB_SRB	FLB_17	07/03/06
1493	FLB_SRB	FLB_17	20/03/06
1494	FLB_SRB	FLB_17	03/04/06
1495	FLB_SRB	FLB_17	03/04/06
1497	FLB_SRB	FLB_17	03/04/02
1498	FLB_SRB	FLB_02	20/02/06
1499	FLB_SRB	FLB_02	20/03/06
1500	FLB_SRB	FLB_02	03/04/06
1501	FLB_SRB	FLB_08	24/01/06
1502	FLB_SRB	FLB_08	20/02/06
1503	FLB_SRB	FLB_08	07/03/06
1504	FLB_SRB	FLB_08	20/03/06
1502	FLB_SRB	FLB_08	20/02/06
1503	FLB_SRB	FLB_08	07/03/06
1504	FLB_SRB	FLB_08	20/03/06
1509	FLB_SRB	FLB_06	24/01/06
1510	FLB_SRB	FLB_GW	24/01/06
1511	FLB_SRB	FLB_06	20/02/06
1512	FLB_SRB	FLB_06	20/02/06
1513	FLB_SRB	FLB_06	07/03/06
1514	FLB_SRB	FLB_06	20/03/06
1515	FLB_SRB	FLB_11	06/02/06
1517	FLB_SRB	FLB_11	20/02/06

5.5.3 Plotting centroid PCO analysis of amplified Microbial rDNA/DGGE banding patterns for all Perth Sulphate-reducing cultures over time

Figure 5-24 shows the overall similarity between the sulphate reducing bacterial population structures over time between the different distances. PCO 1 and PCO 2 described 66.4% of the total variation in sulphate reducing community structures between these distances over time. The results shown in Figure 5-24 indicate a suggested nutrient gradient from the positive control (infiltration gallery) and terminates at the negative control (background bore). Sulphate reducing bacterial populations from 2.5m and 5m were placed on route between the infiltration gallery and background well. These results were therefore more indicative of the chemical gradient suggested in the PCA analysis in Figure 5-1. Exceptions include the overall sulphate reducing bacterial population structure from the 8m which were not placed next to the 5m and 8m samples in Figure 5-24. Thus the PCO centroid analysis suggests that the sulphate reducing community structure from 8m were different to other sampling stations at similar distances.

**Figure 5-23** 2D MDS Plot for amplified Microbial rDNA/DGGE banding patterns for all Perth MAR Sulphate-Reducing Cultures over time.



5.5.4 PERMANOVA – Statistical significance test for assessing microbial variation of Perth MAR Sulphate-reducing culture DGGE/rDNA banding patterns for all distances over time .

The statistical design for analysing the variation in sulphate-reducing community structures consisted of factors (i) time (ii) distance and (iii) an interaction of time and distance. This experimental design was used for all PERMANOVA analyses for non-culture water and culture samples.

The results from Table 5.14 indicate that the sulphate reducing community structures were significantly different due to an interaction of distance and time. The results also indicate that there was a significant difference in sulphate reducing population structures from distance independently. In comparisons differences in sulphate reducing bacterial populations based on sampling event was not as significant (p= 0.07).

The pair-wise tests for the independent affect of distance alone are shown in detail in Appendix 13. These results show that all pair-wise comparisons were predominantly significant. Borderline significance values were obtained for pair-wise comparisons between 2.5m & 5m and 2.5m and 8m. Pair-wise test for the interaction of distance and time based on **distance** are shown in Appendix 14. These results indicate that the infiltration gallery and background bores only contributed to a significant difference in sulphate reducing bacterial community structures based on an interaction of distance and time. Pair-wise tests for the interaction of distance and time based on **time** are shown in detail in Appendix 15. These results show that a significant difference in sulphate reducing bacterial community structures occurred between all amalgamated sampling times.

**Table 5.14** Type 3 PERMANOVA – Perth MAR Sulphate-Reducing Cultures

Source	df	SS	MS	F	P (permutation)
Distance	5	37181	7436	6.55	0.001
Sampling Event	2	3946	1973	1.73	0.07
Distance x Sampling Event	10	21554	2155	1.90	0.001
Res	44	49963	1135		
Total	61	1.1538E5			

Experimental design included time and distance as fixed factors and an interaction of these as a variable factor.

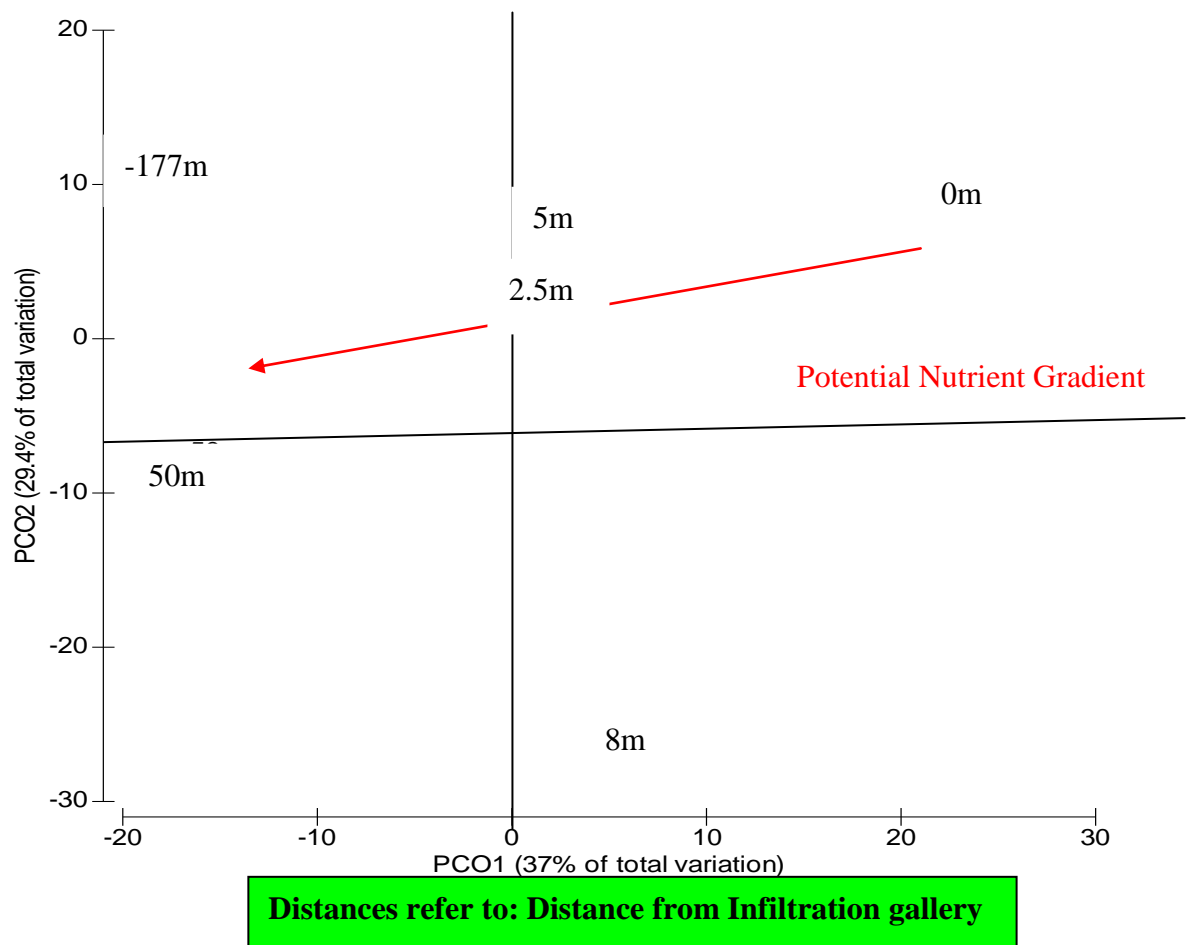
- Samples from 15m, 20m and 32m were removed due to limited replication over time.
- Individual sampling events were amalgamated as follows:

Sample 01 = 24<sup>th</sup> January to 6<sup>th</sup> February (day 68-81)

Sample 02 = 20<sup>th</sup> February to March (day 95 – 110)

Sample 03 = 20<sup>th</sup> March to 3<sup>rd</sup> April (day 123 – 137)

**Figure 5-24 – 2D PCO plot for the reduced CENTROID similarity matrix based on distance from the infiltration gallery for microbial variation (rDNA/DGGE banding patterns) of Perth MAR sulphate-reducing cultures over time**



- The similarity matrix was calculated with the removal of samples from 15m, 20m and 32m as a consequence of limited replication over time.

**Table 5.15** PERMDISP – Deviation of sample dispersion from the Centroid for Perth MAR Sulphate-Reducing Cultures

	F Statistic	P (permutation)
Sampling Event	0.15	0.89
Distance	14.17	0.001

5.5.5 PERMDISP – Statistical test for assessing microbial homogeneity of dispersion for all Perth MAR Sulphate-reducing culture DGGE/rDNA banding patterns for all distances.

PERMDISP was used to statistically test for homogeneity of multivariate dispersion of sulphate reducing bacterial DGGE rDNA banding patterns over time for each distance. Table 5.15 illustrates that the P value for the **distance**-based F statistics was very significant for homogeneity of dispersion. In contrast, homogeneity of multivariate dispersion was highly insignificant for **sampling event** (P=0.89). The difference in sulphate reducing bacteria population dispersion between distances was primarily accounted for by the infiltration galleries as detailed in the pair-wise comparison (Appendix 16). Comparison of pair-wise tests between the PERMANOVA (Appendix 13 & 14) and PERMDISP (Appendix 16) statistical tests show that they were not identical. Therefore these results indicate that the microbial populations isolated from sulphate-reducing media were not similar over distance as a consequence of location (PERMANOVA) and dispersion (PERMDISP).

5.5.6 Separate MDS analyses between (i) background and infiltration gallery (i) background, infiltration gallery and extraction bore and (iii) all other samples excluding background, infiltration gallery and extraction bore.

(i) Background vs. Infiltration Gallery -

**Figure 5-25 separates sulphate reducing community structures over time into two groups, the infiltration gallery (Group A) and background (Group B). In addition the MDS plot visually demonstrates the difference in dispersion of sulphate reducing bacterial population structures over time between the infiltration gallery and background samples. The infiltration gallery sulphate reducing community structures were tightly clustered away from the background populations. This visual interpretation supports the PERMDISP results shown in Appendix 16 which indicated that the dispersion of sulphate reducing bacterial community structures from the infiltration gallery was significantly different to all other populations over time.**

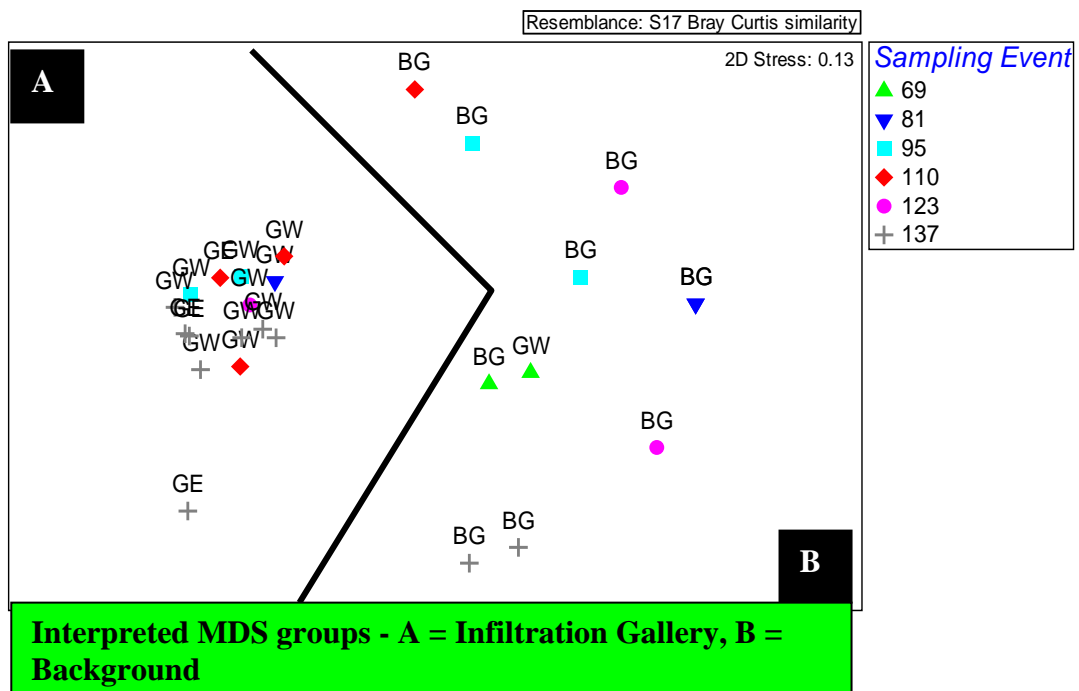
Figure 5-25 also demonstrates that one infiltration gallery sulphate reducing community structure (sampling event 69) was clustered with the background populations. This result therefore replicates the change in microbial population dynamics for fermentative cultures at sampling 69 (Figure 5-19) and the change in microbial populations for non-culture water bacterial samples at the infiltration gallery (Figure 5-15).

(ii) **Background vs. Infiltration Gallery vs. Extraction Bore** - The results from Figure 5-26 shows that the infiltration gallery sulphate reducing community structures (Group A), background (Group B) and extraction (Group C) populations form discrete groups. These results are in contrast to Figure 5-20 where fermentative bacterial populations from the extraction bore clustered with some background community structures.

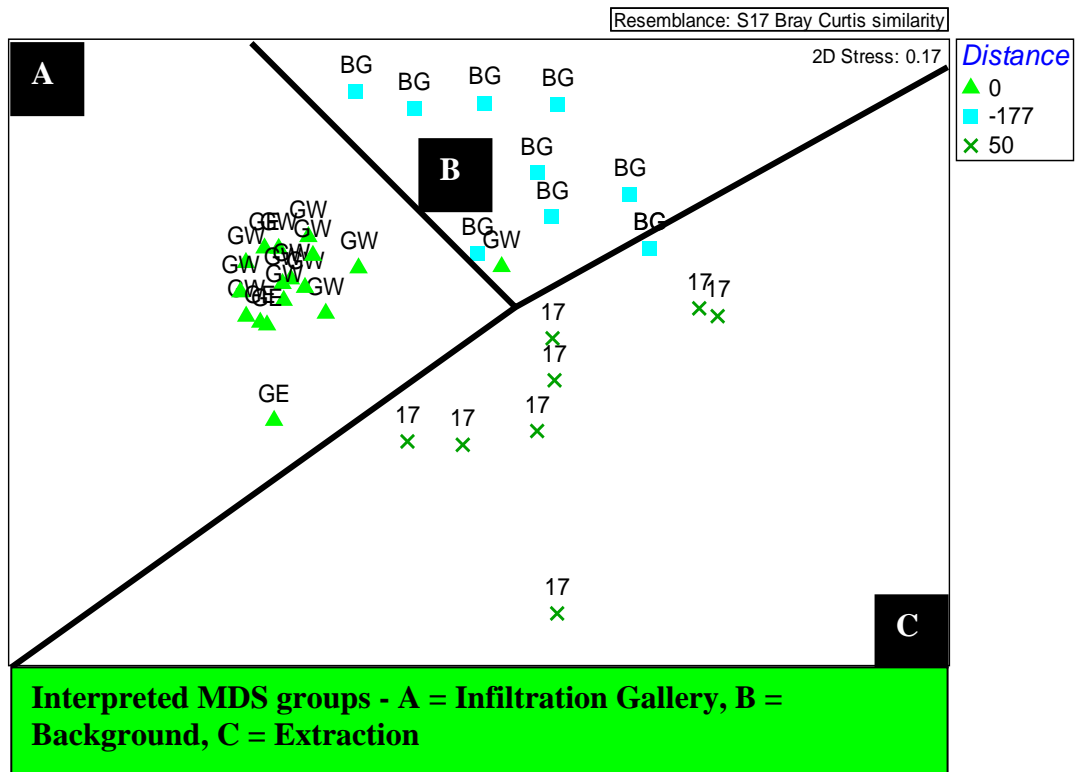


(iii) **Comparison of all sites excluding Background, Infiltration Gallery and Extraction Well** - Figure 5-27 shows that many of the sulphate reducing bacterial community structures from 2.5m to 32m do not form distinct distance-based clusters. An exception is Group A where FLB\_01 sulphate reducing bacterial populations at 2.5m were clustered together. Despite this grouping FLB\_01 sulphate reducing bacterial community structures were not distinctly positioned away from all other communities and therefore were relatively similar in population structure to all other distance-based samples.

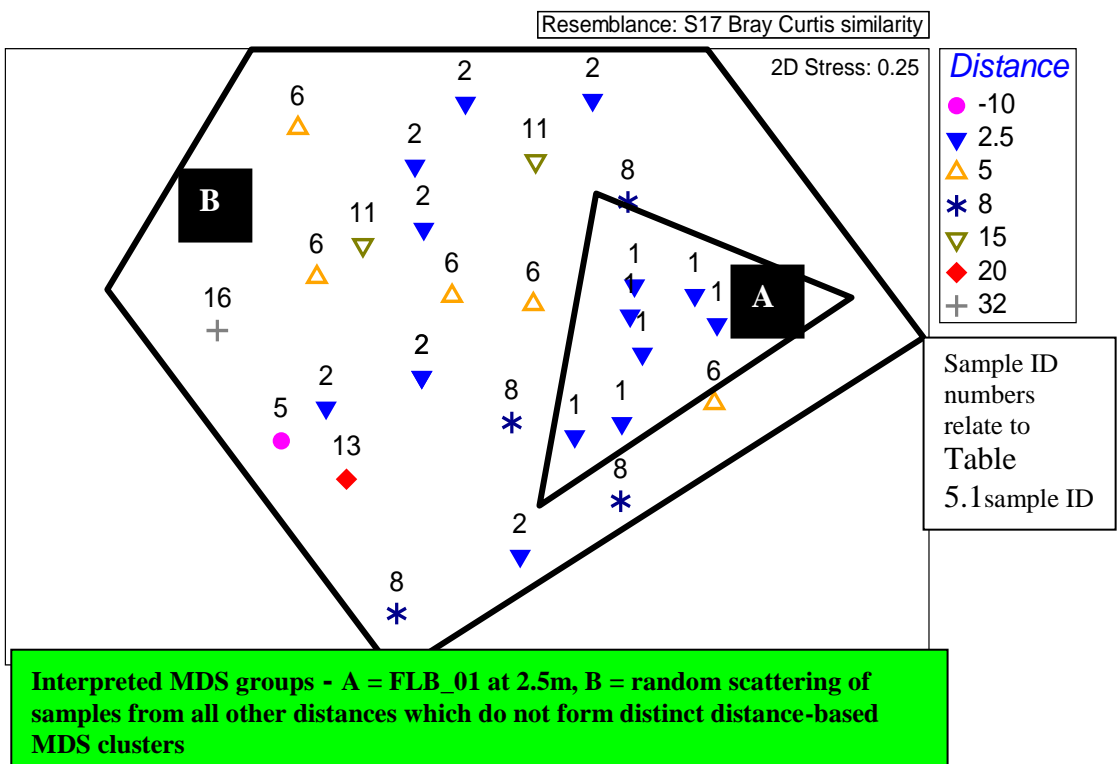
**Figure 5-25** 2D MDS Plot for amplified Microbial rDNA/DGGE banding patterns for Perth MAR Background (BG) and Infiltration Gallery (GW/GE) Sulphate-Reducing Cultures over time



**Figure 5-26** 2D MDS Plot for amplified Microbial rDNA/DGGE banding patterns for Perth MAR Background (BG), Extraction (17) and Infiltration Gallery (GW/GE) Sulphate-Reducing Cultures over time



**Figure 5-27** 2D MDS Plot for amplified Microbial rDNA/DGGE banding patterns for Perth MAR Sulphate reducing cultures (excluding background, extraction and infiltration gallery) over time



## 5.6 Perth MAR Microbial RESULTS for Nitrate-Reducing Cultures

### 5.6.1 Microbial Diversity of Perth MAR Infiltration Site for Nitrate-Reducing Cultures via DGGE rDNA banding patterns

The DGGE rDNA/PCR banding patterns for all nitrate-reducing cultures shown in Figure 5-18 illustrate that there were twenty five band class types ranging from FLB\_5.6 to FLB\_89.2. These band class types vary in intensity and diversity between sampling events and observation bores. Notably dominant band class were evident between band class types FLB: 49.5 to 59.6. A MDS was undertaken on the binary matrix data to ascertain similarities and differences in microbial population structure between samples.

### 5.6.2 Multidimensional Scaling analysis of microbial DGGE rDNA banding patterns from Perth MAR Nitrate-Reducing Cultures over time

The 2D MDS plot shown in Figure 5-29 indicates that there were some distinct distance-based nitrate-reducing bacterial population groups. The infiltration gallery nitrate-reducing bacterial community structure formed Group A which demonstrated similar clustering to that shown for fermentative bacterial population (Figure 5-17). Figure 5-29 showed that the background nitrate reducing community structures formed two distinct population groups namely Group B and C. This result is in contrast to fermentative bacterial populations (Figure 5-17) and sulphate reducing community structures (Figure 5-23).

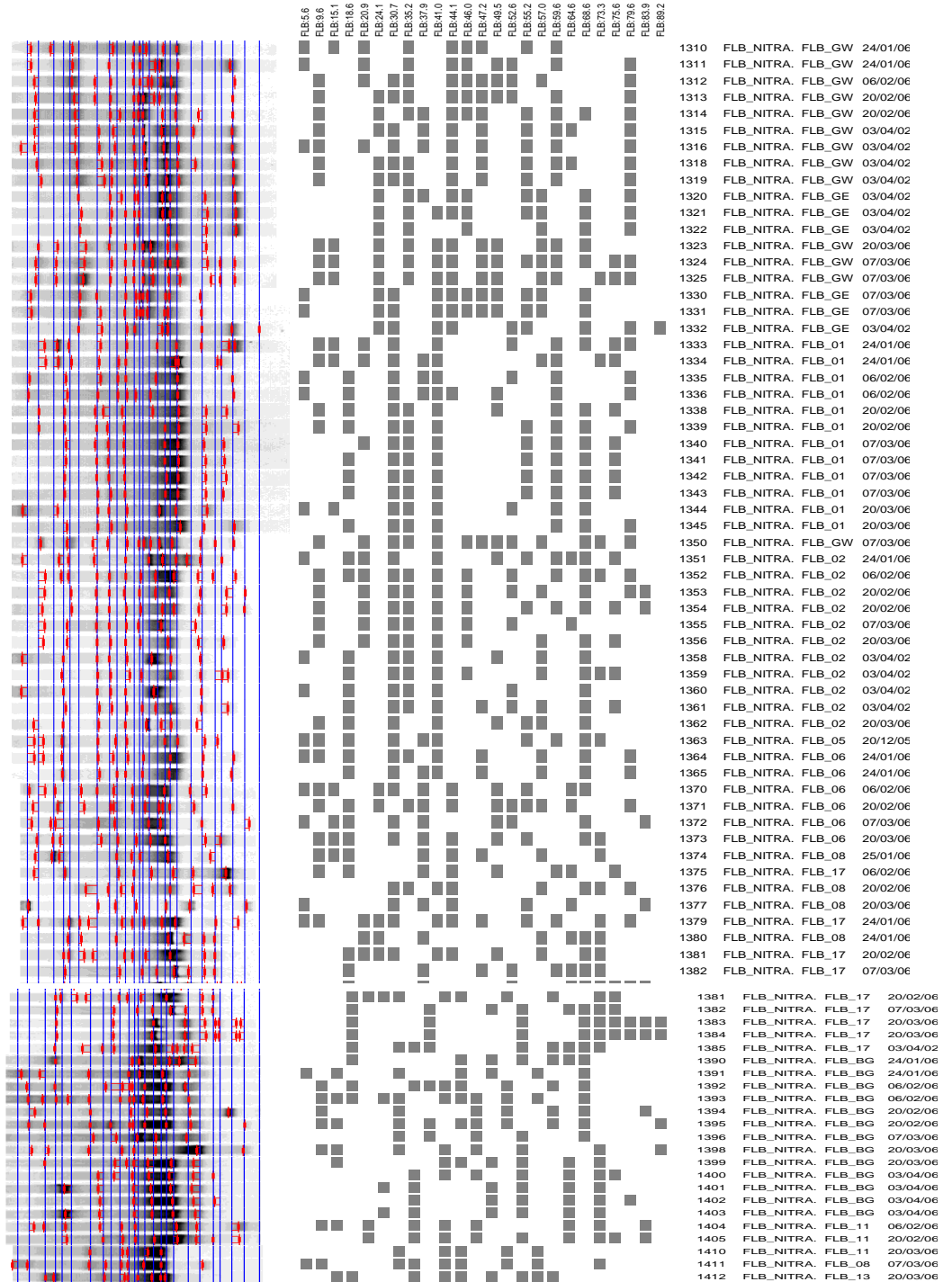
Nitrate reducing bacterial populations from 2.5m predominantly formed Group D which is in contrast to fermentative bacterial populations which split FLB\_01 and 02 into two distinct population groups. Although the nitrate reducing community structures at 2.5m were placed in group D, populations from FLB\_02 were more variable over time than indicated for nitrate-reducing bacterial populations in Figure 5-29. These results suggest nitrate-reducing community structures from 2.5m were more distinct. Nitrate reducing bacterial populations from 5m, 8m, 15m, 20m and the extraction well (50m) were randomly positioned across the MDS plot and thus did not form distinct distance-based population groups.

**Figure 5-28** DGGE of bacteria rDNA/PCR banding patterns and associated binary matrix table for band matching data for all nitrate-reducing cultures from the Perth MAR infiltration site over time.

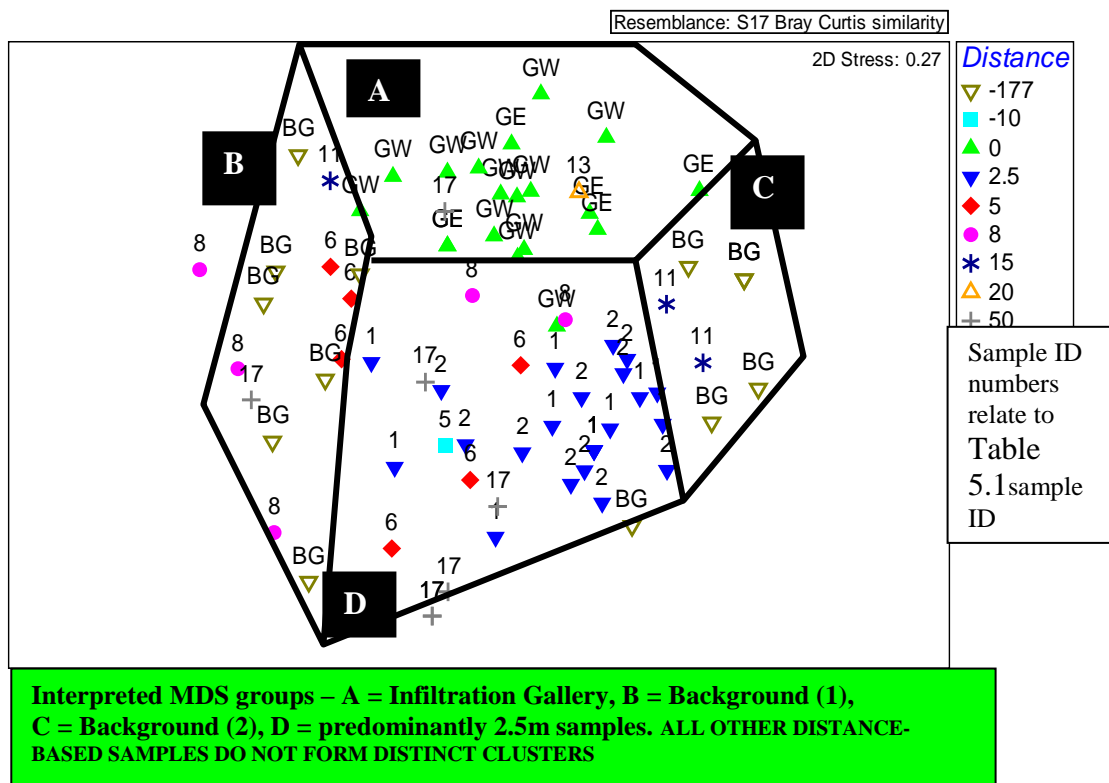
DGGE Gels

Band Class Types and Associated Binary Matrix Table

Sample ID



**Figure 5-29** 2D MDS Plot for amplified Microbial rDNA/DGGE banding patterns for all Perth MAR Nitrate-reducing Cultures over time.



### 5.6.3 *Plotting centroid PCO analysis of amplified microbial rDNA/DGGE banding patterns for all Perth MAR Nitrate-reducing Cultures over time*

Figure 5-30 shows the overall similarity between the nitrate reducing bacterial population structures over time between the different distances. In contrast with the non-culture water samples (Figure 5-9), fermentative cultures (Figure 5-18) and sulphate reducers (Figure 5-24) PCO 1 and PCO2 describe greater than 70% of the total variation in nitrate reducing community structure over distance and time. These results suggest that nitrate-reducing bacterial populations demonstrated less dynamic variation over distance and time than shown for non-culture water samples and fermentative and sulphate-reducing bacterial populations. The nitrate reducing bacterial community structure also differed as the 2.5m nitrate reducing populations were not placed in close proximity to the infiltration gallery community structure. In addition, in contrast to all other analyses, the background nitrate reducing bacterial populations displayed greater similarity in population structure to the infiltration gallery communities compared with all other observation wells. These results indicate that the nitrate-reducing bacterial populations behaved in a very different fashion over distance and time than shown for the non-culture water bacterial populations and fermentative and sulphate-reducing community structure.

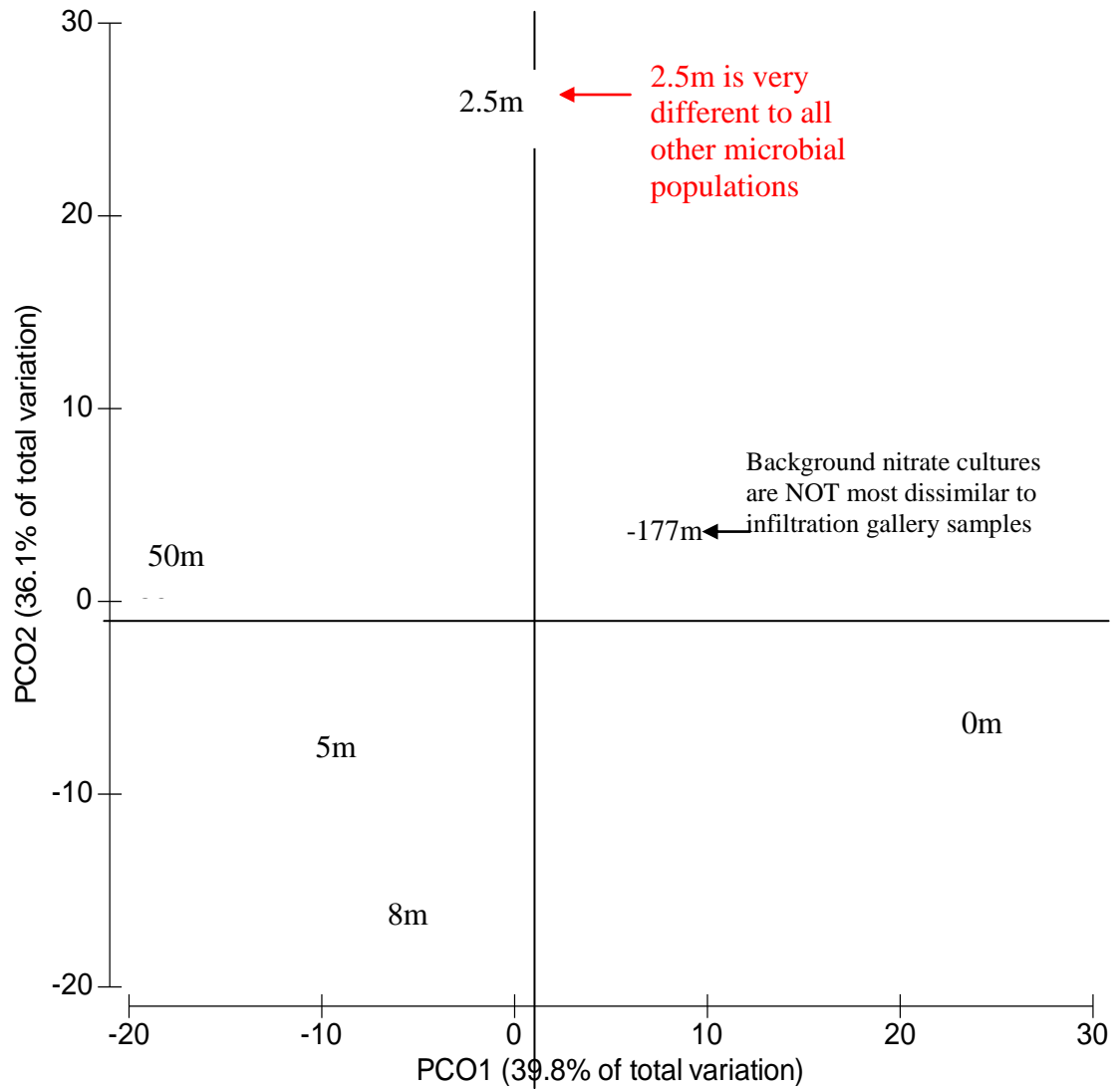
5.6.4 PERMANOVA – Statistical significance test for assessing microbial variation of Perth MAR Nitrate-reducing culture DGGE/rDNA banding patterns for all distances over time.

The statistical design for analysing differences in nitrate reducing population dynamics consisted of factors (i) time (ii) distance and (iii) an interaction of time and distance. This experimental design was used for all PERMANOVA analyses for non-culture water and culture samples.

The results from Table 5.16 indicate that there were significant differences between nitrate reducing bacterial community structures as a result of an interaction of distance and time (Distance x Sampling Event). The results also indicate that the nitrate reducing bacterial populations significantly varied from the independent affect of distance between the observation bores. In contrast the nitrate-reducing bacterial communities did not significantly change over time as the sampling event was not highly significant ( $p=0.1$ ). These results were therefore similar to the sulphate-reducing outcomes shown in Table 5.14.

The pair-wise comparisons for Table 5.16 are shown in detail in Appendix 17. These results indicate that the nitrate reducing communities structures over time from the 2.5m observation well were very significantly different to all other nitrate reducing bacterial communities. In addition all other pair-wise comparisons between the independent affects of distance were predominantly significant with the exceptions of 50 & 5m, 50 & 8m, and 5m & 8m. These results suggest that nitrate reducing bacterial populations from the extraction bore were not very distinct from all other nitrate reducing bacterial communities over time. These results support the non-specific grouping of extraction bore samples shown in Figure 5-29. The extraction bore samples from nitrate-reducing bacteria therefore contrasted with sulphate-reducing samples which formed a distinct population group (Figure 5-23). In addition the nitrate-reducing cultures contrasted with fermentative bacteria. Although the fermentative extraction well bacterial populations did not form visually discrete MDS groups (Figure 5-17), the fermentative cultures were significantly different to all other fermentative microbial communities from different observation wells (Appendix 9). The pair-wise comparisons for the independent affect of **time** for Table 5.16 are also shown in Appendix 17. A significant result was obtained between sampling events 1 & 3.

**Figure 5-30** – 2D PCO plot for the reduced CENTROID similarity matrix based on distance from the infiltration gallery for microbial variation (rDNA/DGGE banding patterns) of Perth MAR nitrate-reducing cultures over time.



**Distances refer to: Distance from Infiltration gallery**

**Table 5.16** Type 3 PERMANOVA – Perth MAR Nitrate-Reducing Cultures



Source	df	SS	MS	F	P (permutation)
Distance	5	35780	7156	7.00	0.001
Sampling Event	2	3306	1653	1.61	0.106
Distance x Sampling Event	10	31661	3166	3.08	0.001
Res	55	56385	1025		
Total	72	1.3244E5			

Experimental design included time and distance as fixed factors and an interaction of these as a variable factor

- Individual sampling events were amalgamated as follows”

Sample 01 = 24<sup>th</sup> January to 6<sup>th</sup> February (day 68 – 81)

Sample 02 = 20<sup>th</sup> February to 7<sup>th</sup> March (day 95 -110)

Sample 03 = 20<sup>th</sup> march to 3<sup>rd</sup> April (day 123 – 137)

**Table 5.17** PERMDISP – Deviation of sample dispersion from the Centroid for Perth MAR Nitrate-reducing cultures.

	F Statistic	P (permutation)
Sampling Event	1.85	0.161
Distance	10.93	0.001

5.6.5 PERMDISP – Statistical test for assessing microbial homogeneity of dispersion for all Perth MAR Nitrate-reducing culture DGGE/rDNA banding patterns for all distances over time..

PERMDISP was used to statically test for homogeneity of multivariate dispersion of nitrate reducing bacterial DGGE rDNA banding patterns over time for each distance.

Table 5.17 illustrates that the P value for the **distance**-based F statistics was significant for homogeneity of dispersion. In contrast a highly significant difference was not obtained for sampling event. These results thus suggest that the nitrate reducing bacterial populations were relatively homogenous over time at each distance but there was a significant difference overall in the homogeneity of dispersion between the various distances. The Pair-wise tests for both distance and sampling event are shown in detail in Appendix 19. Comparison of pair-wise tests between the PERMANOVA (Appendix 17 and 18) and PERMDISP (Appendix 19) statistical tests show that they were not identical. Therefore these results indicate that the microbial populations isolated from nitrate-reducing media were not similar over distance as a consequence of location (PERMANOVA) and dispersion (PERMDISP).

5.6.6 Separate MDS analyses between (i) background and infiltration gallery (ii) background, infiltration gallery and extraction bore and (iii) all other samples excluding background, infiltration gallery and extraction bore.

(i) Background vs. Infiltration Gallery - **Figure 5-31 separated the background nitrate reducing bacterial populations into two main clusters (Group A and B) which is in contrast to the fermentative (Figure 5-19) and sulphate reducing (**

**Figure 5-25) cultures. The infiltration gallery nitrate reducing bacteria formed a single population (Group A). Exceptions include the infiltration gallery samples from day 69 which were clustered away from all other samples as also detailed for the fermentative and sulphate-reducing cultures. This result therefore replicates the change in microbial population dynamics at sampling event 69 for fermentative cultures (Figure 5-19), sulphate-reducing cultures (**

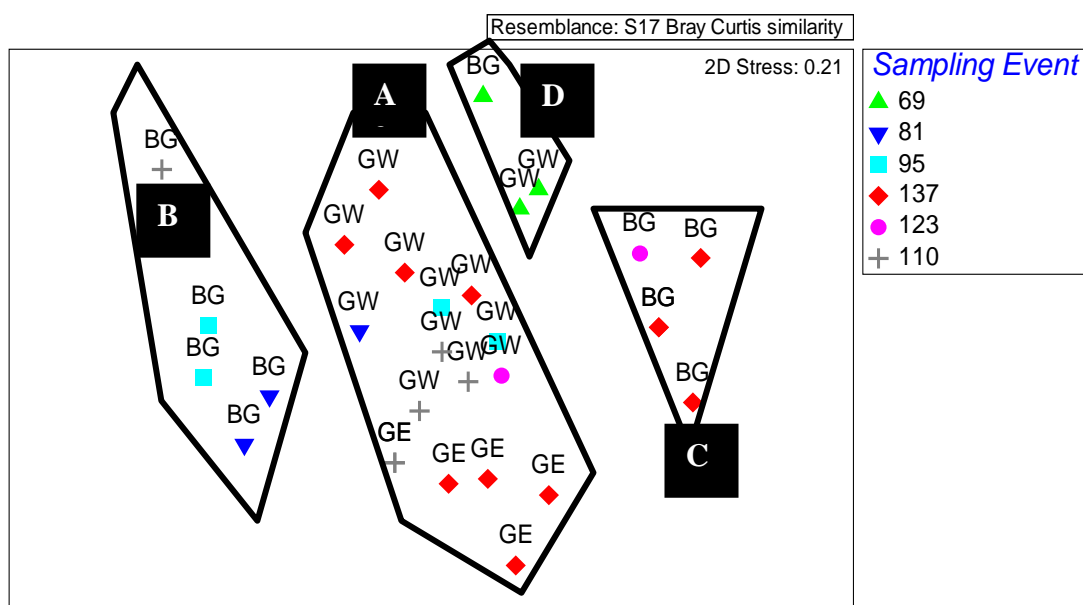
Figure 5-25) and the change in microbial populations for non-culture water samples at the infiltration gallery (Figure 5-15).

(ii) **Background vs. Infiltration Gallery vs. Extraction Bore - Figure 5-32 separated the background nitrate reducing bacterial populations into two main clusters (Groups B**

and C) and the infiltration gallery into one main population cluster (Group A). Although the extraction well nitrate reducing community structures formed Group D their separation from the background and infiltration gallery populations was not as distinct as shown for the sulphate-reducing cultures in Figure 5-26. Despite some temporal variation the nitrate reducing extraction well bacterial populations did not closely cluster with the background communities as shown for the fermentative cultures Figure 5-20.

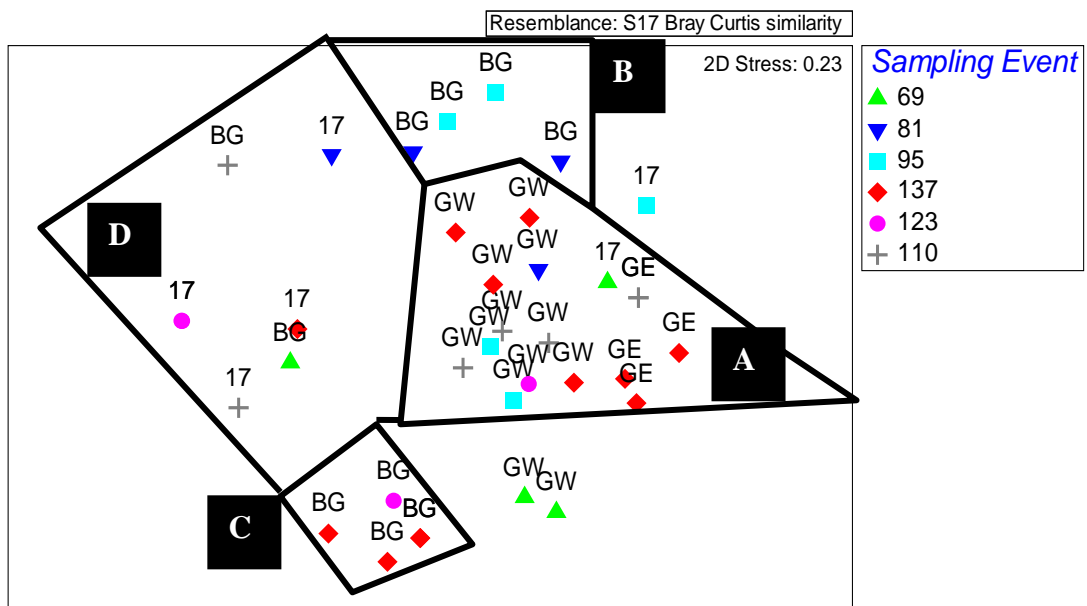
(iii) **Comparison of all sites excluding Background, Infiltration Gallery and Extraction Well** - Figure 5-33 indicates that nitrate reducing bacterial populations from 2.5m were grouped together (Group A). Nitrate reducing bacterial populations from 5m and 8m were more randomly dispersed over time but were generally clustered away from the 2.5m communities in population Group B.

**Figure 5-31** 2D MDS Plot for amplified Microbial rDNA/DGGE banding patterns for Perth MAR Background (BG) and Infiltration Gallery (GW/GE) Nitrate-Reducing Cultures



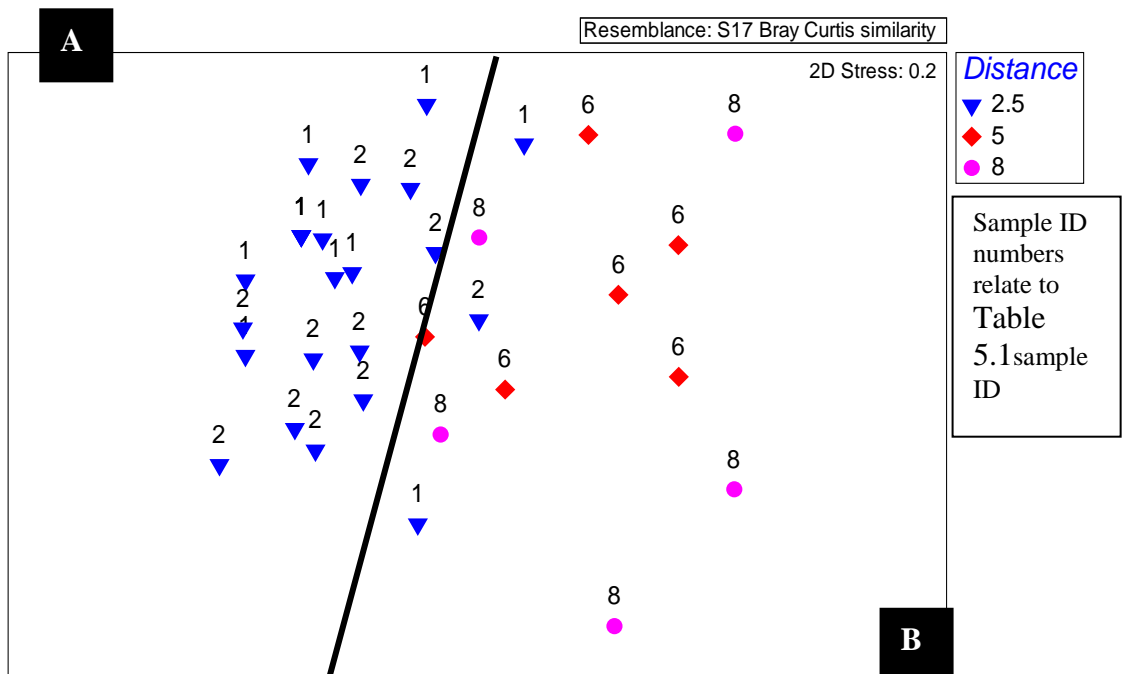
**Interpreted MDS groups – A = Infiltration Gallery, B = Background (1), C = Background (2), C = Infiltration Gallery and Background at sampling event 69**

**Figure 5-32** 2D MDS Plot for amplified Microbial rDNA/DGGE banding patterns for Perth MAR Background (BG), Extraction (17) and Infiltration Gallery (GW/GE) Nitrate-Reducing Cultures



**Interpreted MDS groups – Group A = Infiltration Gallery, Group B = Background (1), Group C = Background (2). Extraction well samples predominately form Group D.**

**Figure 5-33** 2D MDS Plot for amplified Microbial rDNA/DGGE banding patterns for all Perth MAR nitrate-reducing cultures (excluding background, extraction and infiltration gallery) over Time.



**Interpreted MDS groups – A = 2.5m samples – B = three 2.5m samples and all 5m and 8m samples**

## 5.7 Perth MAR Microbial Culture DISCUSSION

### 5.7.1 *Infiltration, Background and Extraction Bore*

#### 5.7.1.1 Infiltration Gallery verses Background

Cultures isolated from the background and infiltration gallery were distinct as shown in the overall MDS plots for fermentative bacteria (Figure 5-17), sulphate-reducing (Figure 5-23) and nitrate-reducing (Figure 5-29) bacteria. These culture results support outcomes described for non-culture water samples (Figure 5-6). These results indicated that isolated microbial populations from negative and positive control samples were dissimilar for studies using both culture and non-culture methods.

The dissimilarity between the background and infiltration gallery samples was also evaluated by PCO centroid analysis. The fermentative cultures (Figure 5-18), sulphate-reducing cultures (Figure 5-24) and non-culture water samples (Figure 5-9) placed the background samples in a different dimensional PCO space to that of the infiltration gallery. These results therefore suggested that the infiltration gallery and background well microbial populations were very different. In addition, PERMANOVA pair-wise

comparisons suggested there was a significant difference in microbial populations between these distances. These results thus suggest that non-culture water samples and culture fermentative and sulphate reducing microbial populations were significantly dissimilar between the infiltration gallery and background groundwater. The results thus indicated that treated effluent contained unique microbial populations which were associated with wastewater which were not present in native unimpacted groundwater. These results therefore support evidence described for non-culture water samples where discriminating band class types were identified between observation wells (Figure 5-13 A-M).

Pathogenic studies undertaken at the Perth MAR site have shown that *Enterococci faecalis* was detected at distance from the plume source (Toze, 2007). These results indicated that microbes that may be associated with wastewater have the potential to possibly migrate with the plume. The migrating plume may thus be an avenue for the transportation of allochthonous microbes associated with treated effluent. Unique microbes associated with wastewater that are transported by the plume (that are not present in native groundwater) therefore have the potential to be used as microbial indicators of MAR impact on groundwater. It is therefore suggested that unique band class types associated with wastewater are identified in future studies. Despite identifying unique band class types (5.3.2.1), a small percentage (3.6%) of non-culture band class types occurred across hydraulically unconnected observation wells (5.3.2.2). These results indicated that ubiquitous microbes naturally occurred in both groundwater and wastewater. Figure 5-13 (A-M) demonstrated that there were also many band class types which were isolated from the infiltration gallery that were also present in hydraulically connected observation wells at 2.5m to 32m distance. These non-culture water sample results perhaps indicated that there were similar microbes in wastewater and unimpacted groundwater. Plume migration may therefore have resulted in wastewater promoting the dominance of specific indigenous bacterial populations in response to these enhanced nutrient concentrations.

The PCO centroid analysis for nitrate-reducing cultures (Figure 5-30) was very unique compared with non-culture water samples (Figure 5-9), fermentative bacteria (Figure 5-18) and sulphate reducing (Figure 5-24) cultures. The background bacterial populations were placed in closest proximity to the centroid infiltration gallery community structure. These results suggested that they contained similar nitrate-

reducing bacteria. As the infiltration gallery and background well were hydraulically unconnected they represented very different ecological environments. Thus these results indicated that these nitrate reducing bacterial populations were naturally ubiquitous in both habitats. Nitrate reducing bacteria such as *Pseudomonas* and *Alcaligenes* are ubiquitous in water environments including groundwater and wastewater. Therefore nitrate-reducing bacterial populations microbiologically had a greater potential than the fermentative and sulphate-reducing bacterial groups to contain similar microbial species that were present in both the negative (background) and positive (infiltration gallery) control samples. The ubiquitous nature of some nitrate-reducing bacterial species may therefore explain why the centroid PCO background nitrate reducing bacterial community structure was found to be very similar to the infiltration gallery. Thus similar nitrate reducing bacterial populations may have occurred in two very contrasting water environments which were hydraulically unconnected. Despite similarity between the infiltration gallery and background groundwater for nitrate-reducing bacterial populations being suggested by the PCO analysis in Figure 5-30, the PERMANOVA pair-wise tests (Appendix 17) suggested that overall there was a significant difference between these two community structures. Therefore despite greater similarity compared with fermentative and sulphate-reducing cultures, the nitrate reducing bacterial populations were significantly different overall between these two sampling stations

#### 5.7.1.2 Background verses Extraction Well verses Infiltration Gallery

The extraction well fermentative bacterial populations did not form a unique cluster in the overall MDS (Figure 5-17). Despite demonstrating temporal variation the extraction well fermentative bacterial populations did cluster with background fermentative communities (Group C) in a specific MDS (Figure 5-20) which analysed the infiltration gallery, background and extraction well bacterial population structures. PERMANOVA pair-wise comparisons based on distance (Appendix 9) suggested that the extraction and background well fermentative bacterial populations were significantly different. In contrast the PERMANOVA pair-wises tests based on an interaction of distance and time (Appendix 10) indicated that the background and extraction well did not contain significantly different fermentative bacterial populations. These results therefore suggested that (i) the extraction bore populations which clustered with the background fermentative populations in Figure 5-20 (Group C) were not significantly different

whereas (ii) the extraction well populations placed in Group C were significantly different to the background bacterial populations placed in Group B.

Fermentative bacteria are able to inhabit a diverse range of habitats that may vary greatly in redox potential. For example unlike the nitrate-reducing and sulphate-reducing bacteria, fermentative bacteria are not limited to success within a defined redox zone. Chapelle *et al.*, (1996) showed that hydrogen concentrations were less prone to variations by groundwater flow as fermentative bacteria constantly produced hydrogen in all redox zones. The hydrogen was then utilised by a different range of respirative bacteria, the microbial group of which was dependent on the redox zone. Therefore perhaps there is less competition between fermentative bacterial populations compared to respirative communities e.g. different fermentative bacterial populations are not competing for hydrogen at different uptake affinities within preferential redox zones (Table 2.1). Therefore it is suggested that fermentative bacterial populations are not as greatly affected by plume migration which was shown to change environmental parameters such as nutrients concentrations and redox potential (Figure 5-1 and Figure 5-4).

Fermentative bacteria are also able to utilise complex organic matter which as already discussed may be the only available carbon in oligotrophic environments such as the background groundwater. The background groundwater fermentative bacterial populations perhaps displayed greater temporal variation than shown for the extraction well community structures as there were diverse populations adapted to utilising a wide range of complex organic matter. Thus, these adapted bacterial populations changed in response to small changes in nutrient types and concentrations in an oligotrophic environment as already discussed (section 4.6.12).

In contrast, the infiltration gallery fermentative bacteria (Figure 5-20) were perhaps less prone to changes in community structure as they had a constant supply of more easily assimilable organic matter compared with the background well. The fermentative bacterial populations from the extraction well also showed greater temporal stability compared with the background fermentative community structures (Figure 5-20). The extraction well fermentative bacterial populations were perhaps less changeable than the background community structures over time due to the dominant and consistent band class type (Figure 5-5) that occurred in the non-culture water samples. This



dominant microbial species may have competitively excluded many bacterial types resulting in population stability. The elevated concentrations of total iron (Figure 5-4H) at the extraction well compared with most other observation wells may not have been conducive for the growth of many fermentative bacterial populations. If the water was being drawn from deeper depths that contained greater iron concentrations, extraction may have provided the anaerobic/aerobic interface favourable to iron oxidising bacteria.

The nitrate-reducing bacterial populations were similar to the fermentative communities in that the extraction well populations did not form a clear-cut, distinct group in the overall MDS (Figure 5-29). Although a distinct population group in the overall MDS was not apparent, the extraction well nitrate reducing bacterial populations did display some grouping (Group D) in the MDS analysis, which incorporated the background, infiltration gallery and extraction well samples only (Figure 5-32). Similar to fermentative bacterial populations, the PERMANOVA pair-wise tests demonstrated that the extraction well contained significantly different nitrate-reducing communities to the background bore.

The nitrate-reducing bacterial populations from the extraction well differed from the fermentative communities in that they showed greater temporal variability (Figure 5-20). In addition, the nitrate-reducing PERMANOVA pair-wise tests in contrast with fermentative bacterial populations, suggested that the extraction well community structures were not significantly different to all distances, namely 5m and 8m. Thus, the nitrate-reducing community structures from the extraction well demonstrated greater variability over time despite being similar to nitrate reducing bacterial populations at 5m and 8m. It is unclear why the nitrate-reducing bacterial populations were similar between 5m, 8m and the extraction well. Nitrate concentrations were very different between these sampling stations (Figure 5-4E) suggesting they contained different nitrate-reducing bacteria. As previously mentioned nitrate-reducing bacteria are ubiquitous in aquatic environments and it has already been suggested that species such as *Pseudomonas* and *Alcaligenes* were potentially present in the treated effluent and in background groundwater. It is suggested that different ubiquitous nitrate-reducing bacterial populations were present between 5m, 8m which separated these communities from the infiltration gallery and background well. The similarity in nitrate reducing bacterial populations between the extraction well, 5m and 8m suggests that nitrate-

reducing cultures isolated at greater distances from the infiltration gallery were more similar to each other than shown for the infiltration gallery. Therefore in this instance, the absence of such microbial species may indicate a different redox zone within groundwater. Because these differences arise at greater distances from the infiltration gallery it is possible that such microbial species were indicative of groundwater that had received a lesser impact from the nutrient plume. Thus, it is possible that some microbial species may be used as indicators of plume migration and directly related to the observed changes in 'chemical signature' e.g. extent of impact from nutrient plume along the flow path.

The sulphate-reducing bacterial populations contrasted with the fermentative and nitrate-reducing communities in that the extraction well sulphate reducing bacteria predominantly formed a distinct population group in the overall MDS (Figure 5-23). The sulphate-reducing bacterial results therefore showed greater similarity to non-culture water samples in that the background, extraction and infiltration gallery samples all formed distinct population groups in the overall MDS (Figure 5-6). It may therefore be speculated that sulphate-reducing bacterial populations were predominantly the main bacterial type isolated from groundwater, as these observation wells demonstrated greater similarity in their overall microbial patterns to non-culture groundwater. Further studies will identify microbial groups present in non-culture water samples by DGGE membrane hybridisation using specific probes targeted at bacterial groups such as sulphate-reducing bacteria

The PCO centroid analyses for all fermentative (Figure 5-18), sulphate reducing (Figure 5-24), nitrate reducing (Figure 5-30) and non-culture water bacterial populations (Figure 5-9) demonstrated that the extraction well microbial populations were most dissimilar to the infiltration gallery. All PERMANOVA pair-wise comparisons also indicated that there was a significant difference between the microbial populations at the infiltration gallery to those present at the extraction well. Therefore both culture and non-culture microbial populations from the extraction bore were most dissimilar to those present at the infiltration gallery. Thus extracted water which may be used for irrigation purposes in the future was (i) most microbiologically dissimilar to wastewater and (ii) predominantly more closely resembled background microbial populations. These results thus suggest the microbial constituents of wastewater had markedly changed through migration and residence time within the

aquifer. With the exception of nitrate-reducing cultures these results match chemical analyses (Figure 5-2 and Figure 5-3) in that sampling stations deemed to be unaffected by plume migration (background and extraction bore) were very different to the infiltration gallery. Thus the background and extraction well samples were microbiologically and chemically different to microbial populations isolated from the nutrient source (infiltration gallery). These results therefore suggest that there was a relationship between groundwater microbiology and geochemistry.

5.7.2 *Nutrient-rich treated effluent displayed reduced temporal variability in microbial population structures compared to native groundwater – Culture vs. non-Culture*

The DGGE/DNA banding patterns shown in Figure 5-16, Figure 5-22 and

Figure 5-28 demonstrated that there was a great deal of bacterial diversity for each specific microbial group cultured. There was also variation between band intensity for band class types but greater band intensity was not visually associated to specific observation bores. An exception to this observation was that sulphate-reducing

bacterial populations isolated from the infiltration gallery visually demonstrated greater band intensity compared with all other observation wells. These results suggested that the sulphate reducing bacterial populations were very successful growth-wise in culture media from infiltration gallery samples.

**Sulphate-Reduction - Comparisons between MAR and ASR** - Visual comparisons of DGGE/DNA banding patterns for Perth MAR sulphate reducing bacterial populations between the infiltration gallery and background (Figure 5-22) indicated that (i) the background groundwater contained less diversity (fewer bands) but (ii) showed greater temporal change. In contrast the infiltration gallery sulphate-reducing cultures were unique as they were very uniform over time (Figure 5-23).

Sulphate reducing bacteria are naturally associated with wastewater treatment (Gerardi, 2003). The stability of sulphate reducing bacterial populations at the Perth infiltration gallery indicated that species within this metabolic group were well established within the wastewater treatment process. It would therefore be prudent to undertake further tests for the presence of these sulphate reducing bacteria within native groundwater populations via DGGE/DNA membrane hybridization and DNA sequencing. Sulphate reducing bacterial populations associated with treated effluent that are absent from native groundwater should be identified. Thus again it is suggested that unique bacteria identified as being associated with wastewater only, warrant their potential use as microbial indicators of plume migration. Additionally, bacterial populations that respond to the migrating plume which preferentially selects dominant species also have the potential to be used as microbial indicators of MAR.

The sulphate reducing results for MAR at Perth contrasts with Adelaide ASR results. Background sulphate reducing cultures from the Adelaide ASR site were more successful in culture media than described for the injection well during the injection phase. The enhanced levels of oxygen at the injection well during the injection cycle was suggested to be a major factor for the absence of consistent sulphate reducing cultures from these samples (section 4.6.8). In contrast the infiltration galleries in Perth represented relatively low oxygen concentrations (Figure 5-4F) which is a required condition for active sulphate reduction. In addition, the nutrient sources differed at the Perth MAR site which used secondary treated as opposed to tertiary effluent in Adelaide. The ambient sulphate concentrations were also lower at the Perth MAR site

although it was unlikely that either were microbiologically limiting. These results therefore suggest that MAR infiltration galleries using secondary treated effluent favoured sulphate-reducing bacterial populations to a much greater extent compared with the injection cycle of ASR using tertiary effluent. It is suggested that these differences were due to site specific aquifer conditions and the differences between the aquifer recharge and recovery techniques namely infiltration versus direct injection. It is suggested that this scenario is only relevant during the injection phase of ASR. During storage at the Adelaide site the enhanced reducing conditions presented more favourable conditions for sulphate reducing bacteria to proliferate during ASR.

Figure 5-4D shows that although sulphate concentrations at the Perth MAR site did vary over time, sulphate never became microbiologically limiting. The rationale for this is that there were no steep inclines and declines and thus no considerable changes in sulphate concentrations (which were never below 50 mg/L). These results may therefore suggest that there may have been a steady state utilisation of sulphate via sulphate reducing bacteria and replenishment via biogeochemical cycling. Through plume migration and microbial activity it is suggested that the redox zonation of the aquifer was in temporal flux during this 'snap-shot' analysis of MAR infiltration using treated effluent. It is proposed that over time the aquifer will attain stability in redox zonation as the chemical gradient and microbial populations may stabilise through consistent flow and input of nutrients over time. In contrast to analysing sulphate variation only, aquifer geochemistry (PCA chemical signature) showed temporal variability (Figure 5-1 and Figure 5-2) due to plume migration which intensified over time and via microbial activity. Thus it is anticipated that these initial stages of MAR would create greater variability prior to the aquifer approaching a steady state system of treated effluent recharge and extraction. Therefore it was difficult to ascertain whether active sulphate reducing bacteria was occurring *in situ* within the aquifer. What is clear is that sulphate reducing bacteria were present *in situ* as all observation wells contained sufficient numbers for successful culturing. The correct redox and nutrient conditions of the media provided advantageous conditions for their proliferation.

### 5.7.3 Comparison of all Microbial Groups Cultured using multivariate statistics and Comparisons between MAR and ASR –

The background fermentative bacterial populations displayed greater temporal variability than the infiltration gallery communities as shown in Figure 5-19 and Figure 5-21. Similar to the fermentative bacterial community structures (Figure 5-19) the background sulphate-reducing populations (

Figure 5-25) displayed much greater temporal variation than shown for the infiltration gallery communities. As already discussed, the infiltration gallery sulphate-reducing bacterial populations were very uniform over time. Therefore the background sulphate-reducing bacterial communities displayed much greater temporal variability. In contrast with all other analyses the background nitrate reducing bacterial populations showed greater distinction in that they were split into two discrete population subgroups (Figure 5-29, Figure 5-31, Figure 5-32 - Groups B & C). Despite this greater variation, the background populations from nitrate-reducing communities (Figure 5-31) were similar to the fermentative (Figure 5-19) and sulphate-reducing bacterial populations (

Figure 5-25). These bacterial populations all visually displayed greater temporal variation than described for the infiltration gallery communities. Thus nutrient-rich treated effluent displayed reduced temporal variability in microbial population structures than described for native groundwater. These results therefore support evidence from the Adelaide tertiary wastewater ASR site where the background well (300m) bacterial populations showed greater temporal variability compared with the injection well.

The Perth MAR and Adelaide ASR culture studies suggested nutrient poor environments produced greater microbial temporal diversity when analysing culture DNA from ambient groundwater. The background groundwater was nutrient poor and thus electron acceptors and donators were often scarce (Figure 5-4, A-S and

Figure 4-9, A-K). As suggested for the Adelaide study, the transfer from a nutrient poor environment to nutrient rich culture media was likely to result in strong microbial growth due to advantageous conditions in the artificial media (section 4.6.12). It was also suggested that microbial populations from nutrient poor environments may fiercely compete for available resources. It was hypothesised that microbial populations from nutrient poor environments may have a greater affinity for nutrients due to the low concentrations naturally found in their native habitat. It was also proposed that organic carbon within their native habitat would be more refractory in nature and thus it was hypothesised that indigenous microbial population were better adapted for efficient uptake of organic carbon. Thus a number of microbial species may have this adaptation in order to competitively compete in nutrient poor ambient groundwater. Diversity may therefore arise because different microbial species have different nutrient affinities for dissimilar nutrient 'species' present in groundwater. The microbial utilisation of nutrient 'species' and abiotic environmental fluxes over time can therefore cause dynamic shifts in available nutrients consequently impacting on the growth potential of different microbes and thus changes in the microbial population structure. As suggested for the Adelaide study changes in nutrient dynamics may appear to be transient within an environment that predominantly contains low nutrient concentrations (Figure 5-4 A-

S). Thus microbial populations dynamically change in response to changes in ambient nutrients. Therefore the balance of predominating bacterial species within the groundwater environment may change depending upon the available nutrients. These changes would thus explain the greater temporal changes in microbial populations described in ambient groundwater.

#### 5.7.4 Temporal Stability of Microbial Populations in Non-culture water samples compared to culture dependant analysis for ambient groundwater

The DNA bands isolated from non-culture background samples (Figure 5-5) demonstrated that the microbial populations from background groundwater were very stable over time. The similarity in microbial types was also demonstrated in the tight clustering of bacterial populations for the background samples shown in MDS (Figure 5-6) and PCO (

Figure 5-7B) analysis. Despite temporal stability, these microbial populations did vary substantially in band intensity over time as visually shown in Figure 5-5. Due to the potential for PCR bias there has been much debate as to whether DGGE/DNA band intensity can be linked to original *in situ* numbers (Muyzer and Smalla, 1998). It is hypothesised for the following rationale that band intensity may perhaps be linked to microbial numbers e.g. greater band intensity equates to a microbial species which represents a greater proportion of the total microbial population *in situ*.

Microbial species which represent a greater proportion of the original sample (greater band intensity of the original sample) will be at a competitive advantage once transferred to advantageous nutrient media. Once transferred to nutrient rich media, the greater initial numbers they represent from the total microbial population *in situ* may result in competitive exclusion of other microbial types within the media. Competitive exclusion of microbial types will therefore vary depending upon which microbial species were dominating the overall microbial population *in situ* despite containing



similar overall diversity. It is suggested that predominating microbial species *in situ* would perhaps be related to the nutrient 'species' present *in situ*. Therefore, as already suggested, competitive exclusions and thus predominating microbial species occur via differences in nutrient uptake efficiency (sections 4.6.12 and 5.7.1.1). Conversely there may be specific bacterial populations that are able to rapidly adapt to the artificial medium and therefore, despite initially being present in lower numbers perhaps they can increase in number more quickly. Madsen *et al.*, 1992 showed that indigenous microbes from a shallow contaminated aquifer acclimated to environmental conditions after an initial lag phase. Rates of biodegradation increased with acclimation thus indicating the adaptation potential of indigenous bacteria.

In summary, it is suggested that future studies should relate intensity of non-culture background water samples to changes in the microbial background populations in culture. A relationship between non-culture and culture studies should be investigated. Greater band intensity *in situ* (non-culture) may result in greater dominance within culture media. Variations in band intensity (Figure 5-5) perhaps occur due to variations in microbial responses to individual nutrients/environmental parameters (Figure 5-4 A-S). Competitive exclusion of microbial types once inoculated into media may thus occur due to greater initial numbers. Thus a change in the 'starting balance' of *in situ* microbial populations transcends into dynamic changes in microbial populations in background culture studies.

It is hypothesised that the infiltration gallery samples resulted in less temporal variation as wastewater contained microbial populations already adapted to enhanced nutrients loads which was less refractory in nature. Therefore the transfer of these microbial populations into nutrient rich media did not upset the balance of microbial populations substantially. Consequently, microbial populations already adapted to a nutrient rich environment resulted in decreased observed temporal variation.

### 5.7.5 Issues Relating to Sampling Methodology to Ensure Consistent DGGE analysis is obtained

#### 5.7.5.1 Problems associated with analysis of background microbial populations in non-culture groundwater samples

During this particular study it became apparent that a greater volume of groundwater was required to capture sufficient bacterial cells from background samples i.e. those samples not impacted by recharged water. As a result DNA was not obtained from all sampling events for non-culture studies. Therefore the hypothesis testing significant differences between culture and non-culture results was not undertaken due to insufficient data for statistical analyses.

Future studies will filter a minimum of 4L as opposed to 1L for background groundwater. This should result in consistent DGGE/DNA data from all background non-culture samples. Thus this will then provide sufficient data for statistical comparison between culture and non-culture studies. Therefore statistical tests can be undertaken between the observed temporal stability of non-culture water samples compared with culture background samples.

It is probable that the low nutrient status of the background groundwater resulted in low microbial numbers in background groundwater. Problems were encountered during this study in the collection of adequate amounts of DNA from background samples, in particular from the Adelaide site which resulted in the non-culture tests from Adelaide not being included. Thus greater volumes of sample would be required for filtering in order to capture greater bacterial mass and thus greater DNA concentrations in any future studies. In contrast DNA was obtained from all sampling event for culture studies. Thus despite apparent low bacterial numbers *in situ* (non-culture) the actual bacterial biomass was sufficient for growth within culture media allowing a population study to be undertaken.

#### 5.7.5.2 Samples Sizes across a MAR Site

MAR creates a migrating plume that ranges in nutrient concentration from its initial penetration to groundwater. Thus the environmental conditions of a MAR site markedly varies over distance through the zone of treatment compared to negative unimpacted groundwater. Through migration the plume will also vary over time as the

nutrient concentration may increase as groundwater becomes more impacted and as the plume migrates to greater distances. Thus optimising the volume of groundwater required is problematic in order to capture sufficient bacterial cells especially as greater volumes will also concentrate PCR inhibitors within groundwater and wastewater. The inhibitex tablet within the Qiagen DNA extraction kit was very successful at removing PCR inhibitors for molecular population studies. In future studies it is recommended that a minimum of 4L of groundwater be concentrated (e.g. by tangential flow filtration TFF) for groundwater with low nutrient concentrations (determined by PCA chemical signature analysis) and thus low bacterial numbers. For impacted groundwater in close proximity to the infiltration gallery 1L of groundwater was sufficient for DNA extraction. Due to the high bacterial numbers at the infiltration gallery the filter generally became clogged at approximately 300L. These concentrates consistently produced sufficient quantity and purity of DNA using the Qiagen mini stool kit for molecular studies.

## 5.8 Conclusions:

1. All cultures from the infiltration gallery notably changed in bacterial population structure at sampling event 69. Thus these results support observed notable changes in non-culture water samples and aquifer geochemistry at the infiltration gallery for this sampling event. These results strongly indicate that culture, non-culture and aquifer geochemistry results can be replicated at the same observation well thus indicating the reliability of this study for biogeochemical interpretation.
2. Excluding the nitrate-reducing cultures, bacterial populations cultured from fermentative and sulphate-reducing media matched chemical analyses in that observation bores deemed to be unaffected by plume migration (background and extraction bore) were very different to the infiltration gallery. Thus the background and extraction well samples were microbiologically and chemically different to microbial populations isolated from the nutrient source (infiltration gallery).
3. Similar to the non-culture water samples, culture analyses predominantly indicated significant differences in microbial population structures between observation wells as determined by PERMANOVA. The PERMANOVA 'F' statistic for the various culture groups was less than shown for the non-culture water samples. This was most likely due to the reduction in sampling events evaluated. Additionally the sampling period analysed for culture analysis was contained within one PCA group C in Figure 5-2 thus demonstrating less chemical variation over time than shown for the non-culture water sampling period.
4. The culture analyses were predominantly similar to the non-culture water samples in that microbial populations isolated from observation wells located between the infiltration gallery and extraction well (e.g. 2.5m to 32m) did not form distinct communities over distance and time.
5. The sulphate reducing microbial community from the infiltration gallery showed temporal stability in population structure which was unique compared

with all other observation wells. Because these sulphate reducing populations appeared to be associated with wastewater their use as a microbial indicator for plume migration and change in biodiversity may be investigated. .

## **SECTION 7 – Overall Conclusions**

The introduction of nutrients and other compounds into groundwater systems can have a number of biotic and abiotic consequences. Geochemical changes during Aquifer Storage and Recovery (ASR) have been well documented but changes in microbial biodiversity have not been previously studied. The aim of this study was to evaluate changes in microbial population dynamics in response to changes in aquifer geochemistry created by the recharge of reclaimed water into distinct and geographically separated aquifers. In an attempt to dissect the dynamics of biogeochemical interactions in response to Managed Aquifer Recharge (MAR) a multitude of multivariate statistical tools were employed.

The first study site was undertaken in Adelaide where research into Aquifer Storage and Recovery (ASR) had occurred prior to this study. ASR cycles using tertiary treated wastewater had thus previously penetrated the confined aquifer at this water reclamation site. Therefore background groundwater at each observation bore was not necessarily representative of ambient groundwater. Consequently in order to monitor the response of indigenous groundwater micro-organisms to ASR, background groundwater was inoculated into chambers. These chambers were then put into the observation wells for the duration of the injection, storage and recovery phases. Samples were taken periodically during these ASR cycles. Because chamber devices were used, bacterial populations associated with sediment could be compared to free-living communities by the addition of aquifer material into some chambers. Bacterial cultures targeted at biogeochemical cycles associated with groundwater systems were cultured from these samples.

There were a number of technical difficulties that were predominantly overcome during the first study site relating to the bacterial population dynamics associated with ASR. It was determined that a greater volume of groundwater was required for non-culture population studies which was subsequently optimised at the Perth study site. Therefore the Adelaide ASR site was reliant on culture analysis only for microbial population studies.

The volume of groundwater required for sufficient concentration of bacterial cells from groundwater was later optimised during the second study site in Perth. Despite an attempt to optimise bacterial recovery from groundwater at the Perth site, the technique was not always totally infallible at obtaining DNA from all samples. The DNA extraction technique was optimised to obtain maximum DNA recovery with sufficient purity for molecular studies by removing PCR inhibitors within groundwater and within treated effluent. This work encountered the difficulty in optimising experimental conditions at a site where environmental conditions markedly changed over distance and time. Both the concentration and types of both chemical and microbial species displayed notable spatial and temporal differences.

Despite initial difficulties in molecular optimisation, sufficient non-culture bacterial DNA was obtained across numerous observation wells over time at the Perth infiltration site. Adequate DNA was collected for both culture and non-culture samples which permitted PERMANOVA statistical analysis for testing significant differences between bacterial populations over distance and time. In contrast to the Perth site, the Adelaide ASR study site did not incorporate sufficient observation wells that could be compared between sampling events over time for PERMANOVA analysis. In addition, unlike the Perth culture analysis, the ASR bacterial growth within culture media did not consistently occur for all observation wells over time. The very contrasting geochemical changes that occurred during the ASR cycles created conditions where not all bacterial types targeted could be detected for all sampling events. As a consequence there was insufficient data for PERMANOVA significance testing between bacterial community structures observed by multidimensional scaling plots.

Regardless of these technical difficulties encountered, which were predominantly overcome, there were a number of consistent observations made between non-culture and culture analyses. There were also consistent observations that suggested a direct link between microbial population dynamics and aquifer geochemistry. The results indicated biogeochemical interactions for each site and between the two different wastewater reclamation sites. Replicate microbial DNA extractions and subsequent PCR, DGGE and statistical analyses were also shown to be reproducible. Additionally molecular analysis of microbial samples from different observation wells which were at the same distance (but at slightly different depth) were also shown to change similarly

over time. These results indicated that the methodologies optimised during this study were reliable and consistent for biogeochemical interpretations.

At the Adelaide ASR site it was shown that there were very contrasting differences in aquifer geochemistry between the different cycles of ASR. After the storage phase aquifer biogeochemistry demonstrated that reclaimed wastewater had returned to ambient conditions with a concomitant change in microbial populations. During recovery, the bacterial culture community structure more closely resembled the background and 50m well microbial populations. These results suggested that both aquifer geochemistry and microbial community structure had returned to an 'ambient status' during the recovery of reclaimed water. Thus significant improvements in the quality of tertiary treated effluent had occurred during ASR at the Adelaide site during the course of this experiment.

At the Perth MAR site, a forced hydraulic gradient extracted 5x the volume of aquifer recharge via infiltration with secondary treated effluent. Microbial and chemical analyses indicated that the background and extraction wells were unaffected by infiltration of treated effluent. The hydraulic gradient ensured that extracted water did not contain chemicals (analysed for this study) and microbial populations that were shown to be associated with wastewater. In contrast, the microbial populations in closest vicinity to the infiltration gallery became progressively more similar to communities at the infiltration gallery as the plume became more concentrated with time. The geochemical analysis demonstrated a migrating chemical plume over distance and time. Between the infiltration gallery and extraction bore it was shown that there was a distance dependent successional change in microbial community structure concomitant with chemical plume migration. The results suggested that the quality of reclaimed wastewater notably improved via filtration and lateral movement through the aquifer.

Important comparisons between bacterial and geochemical data included notable changes in bacterial populations and aquifer geochemistry at the Perth infiltration gallery for sampling event 68/69. These results showed that a considerable MDS/PCA shift occurred for this sampling event which was replicated in all culture, non-culture and geochemical analyses at the Perth infiltration site. Additionally, the notable change in aquifer geochemistry which occurred during the recovery phase during ASR at the



Adelaide site was also replicated by a considerable change in microbial population dynamics. These results strongly indicated that there was a direct relationship between microbial population dynamics and aquifer geochemistry in response to MAR at both sites. Despite complex biogeochemical interactions this study demonstrated the value of using multivariate statistics to dissect dynamic variation between many biotic and abiotic variables. These analyses indicated that changes in microbial populations were more dynamic than changes in aquifer geochemistry from recharge of treated wastewater into aquifers.

This study also highlighted another noteworthy observation between culture and non-culture analyses. Similarities and replicated changes observed between bacterial community structures for culture and non-culture analyses emphasised the value of using culture media for combined geochemical and molecular population studies. Population analysis using cultures targeted at biogeochemical cycles could be directly related to geochemical information to dissect the interactions occurring in groundwater without the specific identification of individual bacterial strains. For example nitrate reducing cultures could be directly compared with the nitrate, nitrite and ammonia chemical information whereas non-culture analysis requires hybridization or sequence analysis to determine any microbial links to aquifer nitrate reducing redox zones e.g. *pseudomonas* probe.

This study was undertaken to evaluate the overall biogeochemical interactions occurring in response to recharge of treated wastewater during MAR. Further work is now required to specifically identify some of the non-culture and culture microbial populations. Techniques such as DGGE membrane hybridizations using specific bacterial probes and DNA sequencing methods can be utilised which were optimised during this study, but time prevented further work. Additionally, further information may be extrapolated from this study and future similar studies by utilising still further sophisticated multivariate statistical analyses that can link geochemical data to changes in microbial populations such as canonical correspondence analysis (CAP) and distance-based multivariate multiple regression e.g. dbRDA and DISTLM (Anderson and Robertson, 2003; Anderson and Willis, 2003; McArdle and Anderson, 2001) .

The hypotheses outlined for this work which were described in 2.11 are specifically addressed as follows—

- I. *A spatial and temporal chemical plume develops from the infiltration gallery (Perth) and Injection well (Adelaide).* A spatial and temporal chemical plume was determined from the infiltration gallery (Perth) and injection well (Adelaide) via principal component analyses which determined changes in the overall chemical signature.
- II. *The nutrient plume from MAR changes bacterial diversity.* The results indicated that the nutrient plume from MAR changed groundwater bacterial diversity compared with microbial populations isolated from ambient groundwater. The PERMANOVA tests demonstrated that there were significant differences for culture and non-culture bacterial population structures over distance and time at the Perth infiltration site. MDS plots also demonstrated that cultured bacterial populations were notably different over distance and time at the Adelaide ASR site.
- III. *Microbial and chemical changes correspond over distance and time thus demonstrating aquifer biogeochemical changes/trends in response to MAR.* Microbial populations between the infiltration gallery and extraction bore demonstrated a distance dependent successional change in community structure concomitant with chemical plume migration. The microbial populations in closest vicinity to the infiltration gallery became progressively more similar to communities at the infiltration gallery over time. Changes in bacterial community structures could also be linked to changes in aquifer geochemistry at the Adelaide ASR site during the recovery phase. Microbial and chemical changes therefore often corresponded over distance and time.
- IV. *Changes in groundwater nutrients from MAR will cause a shift in microbial community structure and species dominance.* Most notably the Perth MAR and Adelaide ASR culture studies both suggested oligotrophic ambient groundwater produced greater microbial temporal variation compared with the nutrient source. Additionally sulphate reducing bacteria from both sites and nitrate-

reducing bacteria associated with aquifer material at the Adelaide ASR site were noticeably unique. These bacterial populations showed greater band intensity thus indicating greater dominance within the 'sample gene pool' and furthermore demonstrated temporal stability in community structure. Overall however, there was no obvious consistent increase or decrease in biodiversity (more or less band class types), only that there were shifts in bacterial community structures associated with the chemical plume. The results indicated that MAR did result in some dominant microbial populations with increased concentrations of nutrients. Dominance was determined by a decrease in temporal variation in community structure in unaffected or least impacted observation wells. Thus the results suggested that stability in bacterial population structure associated with enhanced nutrients indicated that they were able to competitively exclude other bacterial types that were less adapted to the nutrient rich conditions. Additionally, the results suggested that there was a change in the 'balance' of microbial community structure associated with nutrients as indicated by differential MDS clustering of bacterial populations and significant PERMANOVA results between observation wells.

V. *Groundwater microbial populations unaffected by plume migration are more similar in community structure than groundwater microbial populations subjected to MAR nutrients.* At either site studied groundwater microbial populations unaffected by plume migration were more similar in community structure than groundwater microbial populations subjected to MAR nutrients. The microbial results showed that observation wells which were less impacted by treated wastewater had microbial populations more similar to those described for ambient groundwater. For example the 50m and 300m wells were more similar than the injection and 4m wells at the Adelaide ASR site. The centroid PCO analysis also demonstrated the successional change in microbial community structure at the Perth infiltration site. These results indicated that groundwater which was less impacted by the nutrient plume contained more similar microbial populations.

VII. *The development of microbial communities in response to MAR is different between attached compared with free-living bacterial populations.* The development of microbial communities in response to MAR was different

between populations attached to solid surfaces compared with free-living bacterial populations. The addition of aquifer material into chambers resulted in increased stability in microbial population structure over time. Thus the potential for biofilm development and nutrient concentration perhaps favoured some well adapted bacterial populations. These results thus support bacterial temporal stability in community structure associated with nutrients at the source of the plume compared with ambient groundwater. Overall therefore, the results suggest an increase in nutrient concentration results in more stable microbial community structures over time.

This study has shown that microbial populations and the geochemical processes associated with MAR can be studied and compared. It is hoped that the outcomes of this study will enhance other studies in the biogeochemical processes involved in water quality changes (e.g. nutrient removal, pathogen decay and biodegradation of trace organics) as well as controlling biological clogging of MAR schemes.

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## SECTION 9 APPENDICES

### Appendix 1

#### PERMANOVA Permutational MANOVA

*Resemblance worksheet*

Name: Resem37

Data type: Similarity

Selection: All

Sums of squares type: Type III

Permutation method: Permutation of residuals under the reduced model

Number of permutations: 999

#### PAIRWISE TESTS

Term: Distancecex\$sam for pairs of levels of factor: Distance

Groups	df	SS	MS	t	P(perm)
2.5, 15	1	73.525	73.525	0.30488	0.975
<b>2.5, 50</b>	<b>2</b>	<b>4116.5</b>	<b>2058.3</b>	<b>1.4841</b>	<b>0.027</b>
2.5, 5	2	3356	1678	1.229	0.148
2.5, 8	2	3430.3	1715.1	1.2995	0.095
<b>2.5, -50</b>	<b>2</b>	<b>6610.4</b>	<b>3305.2</b>	<b>2.0843</b>	<b>0.001</b>
<b>2.5, 0</b>	<b>2</b>	<b>3835.5</b>	<b>1917.8</b>	<b>1.3119</b>	<b>0.07</b>
15, 50	1	276.12	276.12	0.55076	0.862
15, 5	1	2221.4	2221.4	1.3192	0.129
15, 8	1	2066.3	2066.3	1.4036	0.089
<b>15, -50</b>	<b>1</b>	<b>3837.3</b>	<b>3837.3</b>	<b>2.6233</b>	<b>0.001</b>
15, 0	1	341.68	341.68	0.53596	0.896
50, 5	2	4243.4	2121.7	1.2289	0.155
<b>50, 8</b>	<b>2</b>	<b>5294.6</b>	<b>2647.3</b>	<b>1.5028</b>	<b>0.015</b>
<b>50, -50</b>	<b>2</b>	<b>5429</b>	<b>2714.5</b>	<b>1.7817</b>	<b>0.005</b>
<b>50, 0</b>	<b>2</b>	<b>4816.6</b>	<b>2408.3</b>	<b>1.3775</b>	<b>0.052</b>
5, 8	2	3625.2	1812.6	1.1229	0.279
5, -50	2	4256.6	2128.3	1.3533	0.104
<b>5, 0</b>	<b>2</b>	<b>5566.1</b>	<b>2783.1</b>	<b>1.3659</b>	<b>0.042</b>
<b>8, -50</b>	<b>2</b>	<b>5774</b>	<b>2887</b>	<b>1.703</b>	<b>0.006</b>
<b>8, 0</b>	<b>2</b>	<b>5005.2</b>	<b>2502.6</b>	<b>1.3847</b>	<b>0.037</b>
<b>-50, 0</b>	<b>2</b>	<b>10342</b>	<b>5171.2</b>	<b>2.1299</b>	<b>0.001</b>

## Appendix 2

### PERMANOVA Permutational MANOVA

*Resemblance worksheet*

Name: Resem37

Data type: Similarity

Selection: All

Sums of squares type: Type III

Permutation method: Permutation of residuals under the reduced model

Number of permutations: 999

#### PAIRWISE TESTS

Term: Distance	Groups	df	SS	MS	t	P (perm)
	2.5, 15	0	0		No test	
	2.5, 50	1	14962	14962	4.0013	0.001
	2.5, 5	1	3880.8	3880.8	1.869	0.008
	2.5, 8	1	2271.4	2271.4	1.4955	0.056
	2.5, -50	1	9726.7	9726.7	3.5755	0.001
	2.5, 0	1	11116	11116	3.1584	0.001
	15, 50	0	0		No test	
	15, 5	0	0		No test	
	15, 8	0	0		No test	
	15, -50	0	0		No test	
	15, 0	0	0		No test	
	50, 5	1	6738.4	6738.4	2.1901	0.002
	50, 8	1	7171.4	7171.4	2.4735	0.001
	50, -50	1	10272	10272	3.4659	0.001
	50, 0	1	14983	14983	3.4359	0.001
	5, 8	1	4715.1	4715.1	1.8111	0.004
	5, -50	1	6564.8	6564.8	2.3767	0.002
	5, 0	1	6842.5	6842.5	2.1417	0.001
	8, -50	1	6843.4	6843.4	2.622	0.001
	8, 0	1	5601.6	5601.6	2.0717	0.002
	-50, 0	1	9946.5	9946.5	2.9539	0.001

## Appendix 3

### PERMANOVA Permutational MANOVA

*Resemblance worksheet*

Name: Resem48

Data type: Similarity

Selection: All

Sums of squares type: Type III

Permutation method: Permutation of residuals under the reduced model

Number of permutations: 999

#### PAIRWISE TESTS

Term: Distance

Groups	df	SS	MS	t	P (perm)
2.5, 15	0	0		No test	
2.5, 5	1	3880.8	3880.8	1.869	0.01
2.5, 8	1	2271.4	2271.4	1.4955	0.054
15, 5	0	0		No test	
15, 8	0	0		No test	
5, 8	1	4715.1	4715.1	1.8111	0.003

### PERMANOVA Permutational MANOVA

*Resemblance worksheet*

Name: Resem37

Data type: Similarity

Selection: All

Sums of squares type: Type III

Permutation method: Permutation of residuals under the reduced model

Number of permutations: 999

#### PAIRWISE TESTS

Term: \$sam

Groups	df	SS	MS	t	P (perm)
1, 2	0	0		No test	
1, 3	0	0		No test	
2, 3	1	3072.3	3072.3	1.6889	0.008

## Appendix 4

### Pairwise Tests

Groups	R Statistic	Significance Level %	Possible Permutations	Actual Permutations	Number >= Observed
<b>0, 2.5</b>	<b>0.684</b>	<b>0.4</b>	<b>2430</b>	<b>999</b>	3
<b>0, 5</b>	<b>0.814</b>	<b>0.2</b>	<b>540</b>	<b>540</b>	1
<b>0, 8</b>	<b>0.508</b>	<b>1.6</b>	<b>450</b>	<b>450</b>	7
0, 15	1	11.1	9	9	1
<b>0, 50</b>	<b>1</b>	<b>8.3</b>	<b>12</b>	<b>12</b>	1
0, -50	1	33.3	3	3	1
2.5, 5	0.294	8.9	540	540	48
2.5, 8	0.075	25.1	450	450	113
2.5, 15	0.304	33.3	9	9	3
2.5, 50	0.319	19.4	36	36	7
2.5, -50	1	33.3	3	3	1
<b>5, 8</b>	<b>0.542</b>	<b>0.3</b>	<b>1485</b>	<b>999</b>	2
5, 15	1	33.3	3	3	1
5, 50	1	33.3	3	3	1
5, -50	0.473	22.2	9	9	2
8, 15	0.313	22.2	9	9	2
8, 50	0.837	11.1	9	9	1
<b>8, -50</b>	<b>0.641</b>	<b>6.7</b>	<b>15</b>	<b>15</b>	1
15, 50	1	11.1	9	9	1
15, -50	1	33.3	3	3	1
50, -50	1	33.3	3	3	1

## Appendix 5

### TESTS FOR DIFFERENCES BETWEEN Sampling Day GROUPS (across all Distance groups)

#### Global Test

Sample statistic (Global R): 0.317

Significance level of sample statistic: 0.5%

Number of permutations: 999 (Random sample from a large number)

Number of permuted statistics greater than or equal to Global R: 4

#### Pairwise Tests

Groups	R Statistic	Significance Level %	Possible Permutations	Actual Permutations	Number >= Observed
2, 13	0.238	14	315	315	44
2, 32	1	11.1	9	9	1
2, 60	1	33.3	3	3	1
2, 69	1	11.1	9	9	1
2, 81	1	11.1	9	9	1
2, 95	1	33.3	3	3	1
2, 110	0.407	12.2	90	90	11
2, 123	0.75	11.1	9	9	1
2, 137	0.333	14	100	100	14
2, 68	1	33.3	3	3	1
2, 33	0.25	30	10	10	3
13, 32	0.473	3	405	405	12
13, 60	1	33.3	3	3	1
13, 69	1	11.1	9	9	1
13, 81	1	11.1	9	9	1
13, 95	1	33.3	3	3	1
13, 110	0.033	31.1	135	135	42
13, 123	0.5	33.3	9	9	3
13, 137	0.277	15	900	900	135
13, 68	0.316	14.8	27	27	4
13, 33	0.238	23.7	135	135	32
13, 53	1	33.3	3	3	1
32, 60	1	33.3	3	3	1
32, 69	1	11.1	9	9	1
32, 81	1	11.1	9	9	1
32, 95	1	33.3	3	3	1
32, 110	1	11.1	9	9	1
32, 123	0.75	11.1	9	9	1
32, 137	0.316	10.3	300	300	31
32, 68	1	11.1	9	9	1
32, 33	1	33.3	3	3	1
60, 123	1	33.3	3	3	1
60, 137	0.333	25	4	4	1
69, 110	-1	100	3	3	3
69, 123	0	66.7	9	9	6
69, 137	0.333	31.3	16	16	5
81, 110	0.5	22.2	9	9	2
81, 123	0	66.7	9	9	6
81, 137	0.111	43.8	16	16	7
81, 33	-0.5	88.9	9	9	8
81, 26	1	25	4	4	1
95, 110	1	33.3	3	3	1
95, 123	0	66.7	3	3	2

95, 137	0	50	4	4	2
95, 33	0	66.7	9	9	6
95, 26	0.889	25	4	4	1
110, 123	0.016	48.1	27	27	13
110, 137	-0.382	92.5	40	40	37
110, 68	0	66.7	3	3	2
110, 33	-0.013	55.6	27	27	15
110, 26	0.778	25	4	4	1
123, 137	0.021	44	100	100	44
123, 68	-1	100	3	3	3
123, 33	1	11.1	9	9	1
123, 26	0.556	25	4	4	1
137, 68	-0.333	75	4	4	3
137, 33	-1	100	3	3	3
137, 26	1	25	4	4	1
33, 26	0.833	10	10	10	1
33, 53	1	33.3	3	3	1

*Failed Pairwise Tests*

Groups	Error
2, 26	Groups too small
2, 53	Groups too small
13, 26	Groups too small
32, 26	Groups too small
32, 53	Groups too small
60, 69	Groups too small
60, 81	Groups too small
60, 95	Groups too small
60, 110	Groups too small
60, 68	Groups too small
60, 33	Groups too small
60, 26	Groups too small
60, 53	Groups too small
69, 81	Groups too small
69, 95	Groups too small
69, 68	Groups too small
69, 33	Groups too small
69, 26	Groups too small
69, 53	Groups too small
81, 95	Groups too small
81, 68	Groups too small
81, 53	Groups too small
95, 68	Groups too small
95, 53	Groups too small
110, 53	Groups too small
123, 53	Groups too small
137, 53	Groups too small
68, 33	Groups too small
68, 26	Groups too small
68, 53	Groups too small
26, 53	Groups too small

## Appendix 6

### PAIRWISE COMPARISONS (PERMDISP – SAMPLING EVENT)

Groups	t	P(perm)
(95,33)	0.1295	0.925
(95,69)	2.2459	0.221
(95,137)	0.78176	0.652
(95,110)	4.6713E-2	0.981
(95,81)	0.82533	0.506
(95,13)	0.56474	0.837
(95,32)	0.42283	0.783
(95,2)	0.11859	0.941
(95,123)	0.76292	0.559
*(95,53)	6.5593	1E-3
*(95,60)	4.8256	1E-3
(95,68)	0.3384	0.564
(95,26)	2.6674	0.135
(33,69)	2.2763	0.27
(33,137)	0.67697	0.555
(33,110)	0.23545	0.838
(33,81)	0.78808	0.506
(33,13)	0.41219	0.745
(33,32)	0.65853	0.551
(33,2)	0.32301	0.776
(33,123)	0.98274	0.389
*(33,53)	6.4449	5E-3
*(33,60)	4.9209	1.5E-2
(33,68)	0.51657	0.703
*(33,26)	3.1634	5.1E-2
*(69,137)	4.016	2.3E-2
(69,110)	2.2952	0.251
*(69,81)	3.1298	2.6E-2
(69,13)	4.1687	0.138
(69,32)	2.3183	0.387
(69,2)	2.3436	0.264
(69,123)	3.0307	0.213
(69,53)	4.7535E8	0.331
*(69,60)	Infinity	1E-3
(69,68)	1.5461	0.65
(69,26)	0.62879	0.845
(137,110)	1.0291	0.361
(137,81)	0.37908	0.737
(137,13)	0.60116	0.636
(137,32)	1.6123	0.15
(137,2)	1.1563	0.307



*(137,123)	2.2247	6E-2
*(137,53)	10.337	6E-3
*(137,60)	7.8778	3.4E-2
(137,68)	1.2196	0.38
*(137,26)	4.7122	1.1E-2
(110,81)	1.0846	0.412
(110,13)	0.8451	0.452
(110,32)	0.48063	0.699
(110,2)	9.7857E-2	0.934
(110,123)	0.80558	0.542
*(110,53)	6.7745	2E-3
*(110,60)	5.2222	2E-2
(110,68)	0.39959	0.808
*(110,26)	3.3696	4.7E-2
(81,13)	0.92949	0.628
(81,32)	1.5245	0.196
(81,2)	1.1859	0.374
*(81,123)	2.0321	8.4E-2
*(81,53)	7.6866	2.3E-2
(81,60)	5.7087	0.109
(81,68)	1.1166	0.393
*(81,26)	3.6065	4.8E-2
(13,32)	1.5338	0.247
(13,2)	0.99244	0.385
(13,123)	2.1046	0.16
*(13,53)	11.272	3E-3
*(13,60)	8.6884	2.8E-2
(13,68)	1.1887	0.559
*(13,26)	5.621	9E-3
(32,2)	0.39455	0.74
(32,123)	0.33286	0.774
*(32,53)	7.4818	5E-3
*(32,60)	5.7459	2.3E-2
(32,68)	5.9026E-2	0.961
*(32,26)	3.1556	7E-2
(2,123)	0.73266	0.555
*(2,53)	7.0288	2E-3
*(2,60)	5.4158	7E-3
(2,68)	0.33965	0.81
*(2,26)	3.3841	4.4E-2
*(123,53)	10.43	1.8E-2
*(123,60)	7.9976	3.5E-2
(123,68)	0.17212	0.918
*(123,26)	3.4056	5.1E-2
(53,60)	4.036E7	1
*(53,68)	5.0754	4.5E-2
(53,26)	2.7523	0.476
(60,68)	3.7528	0.116
(60,26)	2.0976	0.434
(68,26)	2.1195	0.24

## Appendix 7

### *PAIRWISE COMPARISONS (PERMDISP – DISTANCE)*

Groups	t	P(perm)
(8,50)	1.0381	0.363
(8,0)	1.9295	9E-2
(8,5)	0.78715	0.51
(8,-50)	2.8837E-2	0.979
(8,15)	2.8987	1.8E-2
(8,2.5)	2.2257	4.4E-2
(8,7)	3.79	3.4E-2
(8,32)	5.6111	2.4E-2
(8,-10)	6.1311	1E-3
(50,0)	3.0683	1.2E-2
(50,5)	1.7919	0.114
(50,-50)	1.1231	0.285
(50,15)	1.9928	0.109
(50,2.5)	1.2277	0.284
(50,7)	3.4018	7.9E-2
(50,32)	5.4962	5.5E-2
(50,-10)	5.2294	2E-3
(0,5)	1.0302	0.367
(0,-50)	2.0354	0.101
(0,15)	4.7723	1E-3
(0,2.5)	4.1406	1E-3
(0,7)	5.5685	9E-3
(0,32)	7.4366	2.7E-2
(0,-10)	8.9813	1E-3
(5,-50)	0.81961	0.546
(5,15)	3.5103	1E-2
(5,2.5)	2.9279	1E-2
(5,7)	4.144	2.4E-2
(5,32)	5.8186	2.4E-2
(5,-10)	6.7988	1E-3
(-50,15)	2.7956	2.9E-2
(-50,2.5)	1.9536	7.7E-2
(-50,7)	7.4098	3E-3
(-50,32)	10.899	6E-3
(-50,-10)	8.2102	1E-3
(15,2.5)	0.78908	0.523
(15,7)	1.0762	0.525
(15,32)	2.6738	9E-2
(15,-10)	1.7124	0.19
(2.5,7)	1.5883	0.43
(2.5,32)	3.133	3.9E-2

(2.5,-10)	2.6649	2.3E-2
(7,32)	Infinity	1E-3
(7,-10)	0.22614	0.877
(32,-10)	4.0618	1E-3

*MEANS AND STANDARD ERRORS*

Group	Average	SE
8	44.817	2.1347
50	41.546	2.2791
0	49.824	1.5792
5	47.172	2.0932
-50	44.732	1.368
15	32.708	4.0776
2.5	36.603	2.8335
7	23.529	0
32	0	NaN
-10	24.476	2.1304

## Appendix 8 –

Correlation values for Environmental Chemicals including gallery information.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1															
2	0.02														
3	0.08	0.23													
4	0.46	0.36	0.02												
5	0.19	0.13	0.12	0.35											
6	0.44	0.20	0.12	0.55	0.40										
7	0.21	0.12	0.09	0.50	0.67	0.45									
8	0.40	0.37	0.21	0.34	0.25	0.93	0.31								
9	0.08	0.41	0.19	0.10	0.39	0.34	0.15	0.36							
10	0.04	0.06	0.21	0.26	0.42	0.24	0.47	0.19	0.02						
11	0.03	0.18	0.19	0.07	0.21	0.39	0.11	0.40	0.51	0.21					
12	0.14	0.22	0.12	0.31	0.58	0.45	0.69	0.40	0.31	0.70	0.01				
13	0.14	0.09	0.15	0.48	0.82	0.34	0.71	0.15	0.18	0.43	0.08	0.50			
14	0.30	0.02	0.24	0.27	0.04	0.37	0.05	0.31	0.18	0.13	0.11	0.11	0.08		
15	0.45	0.16	0.18	0.45	0.12	0.93	0.21	0.93	0.22	0.11	0.33	0.25	0.08	0.47	
16	0.06	0.14	0.02	0.36	0.72	0.34	0.86	0.22	0.27	0.36	0.17	0.57	0.68	0.18	0.08

- Red indicates negative relationships
- Bold represents > 0.67 correlation in negative and positive directions.

Legend for appendix 8

1 =	ORP	7 =	DOC	13 =	P_SR
2 =	pH	8 =	Econd	14 =	SO <sub>4</sub>
3 =	Dissolved Oxygen	9 =	Fe	15 =	TDS
4 =	Temperature	10 =	N-NH <sub>3</sub>	16 =	TOC
5 =	Alkalinity	11 =	N-NO <sub>3</sub>		
6 =	Chloride	12 =	N-TK		

## Appendix 9

### Fermenting Cultures

#### PERMANOVA

#### Permutational MANOVA

*Resemblance worksheet*

Name: Resem31

Data type: Similarity

Selection: All

Sums of squares type: Type III

Permutation method: Permutation of residuals under the reduced model

Number of permutations: 999

#### PAIRWISE TESTS

Term: Distance

Groups	df	SS	MS	t	P(perm)
15, 50	0	0		No test	
15, 2.5	0	0		No test	
15, 5	0	0		No test	
15, 8	0	0		No test	
15, -177	0	0		No test	
15, 0	0	0		No test	
<b>50, 2.5</b>	<b>1</b>	<b>6839.7</b>	<b>6839.7</b>	<b>2.0874</b>	<b>0.002</b>
<b>50, 5</b>	<b>1</b>	<b>7040.2</b>	<b>7040.2</b>	<b>2.478</b>	<b>0.004</b>
<b>50, 8</b>	<b>1</b>	<b>3761.8</b>	<b>3761.8</b>	<b>2.094</b>	<b>0.012</b>
<b>50, -177</b>	<b>1</b>	<b>5210.9</b>	<b>5210.9</b>	<b>1.7863</b>	<b>0.015</b>
<b>50, 0</b>	<b>1</b>	<b>6672</b>	<b>6672</b>	<b>2.417</b>	<b>0.002</b>
<b>2.5, 5</b>	<b>1</b>	<b>6849.4</b>	<b>6849.4</b>	<b>2.0892</b>	<b>0.001</b>
2.5, 8	1	2033	2033	1.1653	0.252
<b>2.5, -177</b>	<b>1</b>	<b>7954.8</b>	<b>7954.8</b>	<b>2.1786</b>	<b>0.001</b>
<b>2.5, 0</b>	<b>1</b>	<b>5464.3</b>	<b>5464.3</b>	<b>1.9436</b>	<b>0.004</b>
5, 8	1	1910.4	1910.4	1.6791	0.092
<b>5, -177</b>	<b>1</b>	<b>7149.9</b>	<b>7149.9</b>	<b>2.0893</b>	<b>0.001</b>
<b>5, 0</b>	<b>1</b>	<b>7492.3</b>	<b>7492.3</b>	<b>2.6031</b>	<b>0.001</b>
<b>8, -177</b>	<b>1</b>	<b>4771.8</b>	<b>4771.8</b>	<b>1.7795</b>	<b>0.001</b>
<b>8, 0</b>	<b>1</b>	<b>5064.9</b>	<b>5064.9</b>	<b>2.2671</b>	<b>0.001</b>
<b>-177, 0</b>	<b>1</b>	<b>7886</b>	<b>7886</b>	<b>2.3499</b>	<b>0.001</b>

## PERMANOVA

### Permutational MANOVA

*Resemblance worksheet*

Name: Resem31

Data type: Similarity

Selection: All

Sums of squares type: Type III

Permutation method: Permutation of residuals under the reduced model

Number of permutations: 999

#### PAIRWISE TESTS

Term: New Time

Groups	df	SS	MS	t	P(perm)
1, 2	1	4051.2	4051.2	1.6015	0.019
1, 3	0	0		No test	
2, 3	0	0		No test	

## Appendix 10 –

### Fermenting Cultures

#### PERMANOVA Permutational MANOVA

*Resemblance worksheet*

Name: Resem31

Data type: Similarity

Selection: All

Sums of squares type: Type III

Permutation method: Permutation of residuals under the reduced model

Number of permutations: 999

#### PAIRWISE TESTS

Term: DistancexNew Time for pairs of levels of factor: Distance

Groups	df	SS	MS	t	P(perm)
15, 50	1	952.12	952.12	0.90066	0.57
15, 2.5	1	1087.6	1087.6	0.83581	0.619
<b>15, 5</b>	<b>1</b>	<b>4305.3</b>	<b>4305.3</b>	<b>1.985</b>	<b>0.003</b>
<b>15, 8</b>	<b>1</b>	<b>3921</b>	<b>3921</b>	<b>2.2083</b>	<b>0.022</b>
15, -177	1	1803.1	1803.1	1.058	0.397
15, 0	1	1467.6	1467.6	1.144	0.275
50, 2.5	2	2648.8	1324.4	0.91853	0.571
50, 5	2	3873.2	1936.6	1.2997	0.173
<b>50, 8</b>	<b>2</b>	<b>4497</b>	<b>2248.5</b>	<b>1.6189</b>	<b>0.052</b>
50, -177	2	3896.8	1948.4	1.0923	0.327
50, 0	2	2471.6	1235.8	1.0402	0.377
2.5, 5	2	3450.4	1725.2	1.0485	0.35
<b>2.5, 8</b>	<b>2</b>	<b>6267.5</b>	<b>3133.7</b>	<b>1.4468</b>	<b>0.034</b>
<b>2.5, -177</b>	<b>2</b>	<b>6080.6</b>	<b>3040.3</b>	<b>1.3469</b>	<b>0.068</b>
2.5, 0	2	4729.6	2364.8	1.2786	0.109
5, 8	2	3576.6	1788.3	1.6246	0.101
5, -177	2	1916.1	958.06	0.76478	0.82
5, 0	2	566.6	283.3	0.50619	0.924
8, -177	2	4836.6	2418.3	1.2668	0.128
8, 0	2	2317.3	1158.7	1.0843	0.332
-177, 0	2	3624.2	1812.1	1.1265	0.243

#### PERMANOVA Permutational MANOVA

*Resemblance worksheet*

Name: Resem31

Data type: Similarity

Selection: All

Sums of squares type: Type III

Permutation method: Permutation of residuals under the reduced model

Number of permutations: 999

#### PAIRWISE TESTS

Term: DistancexNew Time for pairs of levels of factor: New Time

Groups	df	SS	MS	t	P(perm)
1, 2	6	9143.2	1523.9	0.9822	0.54
1, 3	5	8613.9	1722.8	1.1473	0.148
<b>2, 3</b>	<b>5</b>	<b>12991</b>	<b>2598.3</b>	<b>1.3824</b>	<b>0.009</b>

## Appendix 11 –

### Fermenting Cultures

#### PERMDISP

#### Distance-based test for homogeneity of multivariate dispersions

*Resemblance worksheet*

Name: Resem25

Data type: Similarity

Selection: All

Number of groups: 6

Number of samples: 67

#### *DEVIATIONS FROM CENTROID*

F: 4.8344

P(perm): 0.007

#### *MEANS AND STANDARD ERRORS*

Group	Average	SE
-177	41.657	1.6482
0	31.498	2.1925
2.5	41.43	1.5333
7	39.1	2.3836
11	32.818	5.4255
-50	30.549	3.6504

#### Variability in dispersion for distance.

#### *PAIRWISE COMPARISONS*

Groups	t	P(perm)
<b>(-177,0)</b>	<b>3.6556</b>	<b>1E-3</b>
(-177,2.5)	9.5189E-2	0.934
(-177,7)	0.90564	0.384
<b>(-177,11)</b>	<b>2.0897</b>	<b>7.6E-2</b>
<b>(-177,-50)</b>	<b>3.2272</b>	<b>1E-2</b>
<b>(0,2.5)</b>	<b>3.8197</b>	<b>4E-3</b>
<b>(0,7)</b>	<b>2.3321</b>	<b>4.9E-2</b>
(0,11)	0.2742	0.837
(0,-50)	0.23396	0.862
(2.5,7)	0.84371	0.436
(2.5,11)	2.1357	0.148
<b>(2.5,-50)</b>	<b>3.1558</b>	<b>1.9E-2</b>
(7,11)	1.2468	0.324
(7,-50)	2.0528	9.1E-2
(11,-50)	0.35761	0.711

## Appendix 12 –

### Fermenting Cultures

#### PERMDISP

Distance-based test for homogeneity of multivariate dispersions

*Resemblance worksheet*

Name: Resem25

Data type: Similarity

Selection: All

Number of groups: 3

Number of samples: 67

*DEVIATIONS FROM CENTROID*

F: 2.58

P(perm): 0.081

*MEANS AND STANDARD ERRORS*

Group	Average	SE
1	41.77	1.7909
2	45.813	1.2568
3	41.095	1.7489



## Appendix 13 -

Sulphate-reducing cultures

### PERMANOVA Permutational MANOVA

*Resemblance worksheet*

Name: Resem7

Data type: Similarity

Selection: All

Sums of squares type: Type III

Permutation method: Permutation of residuals under the reduced model

Number of permutations: 999

#### PAIRWISE TESTS

Term: Distance

Groups	df	SS	MS	t	P(perm)
-177, 0	1	12388	12388	4.7474	0.001
-177, 2.5	1	9239.1	9239.1	2.4462	0.001
-177, 5	1	4650.3	4650.3	2.0819	0.002
-177, 8	1	7541.7	7541.7	2.7478	0.001
-177, 50	1	6156	6156	2.3604	0.001
0, 2.5	1	8464.8	8464.8	2.7669	0.001
0, 5	1	5452	5452	3.2632	0.001
0, 8	1	5727.4	5727.4	3.6281	0.001
0, 50	1	14592	14592	4.7564	0.001
2.5, 5	1	3682.9	3682.9	1.4237	0.065
2.5, 8	1	3712.2	3712.2	1.4254	0.059
2.5, 50	1	6711.6	6711.6	1.9934	0.001
5, 8	1	5664.4	5664.4	1.9136	0.031
5, 50	1	6506.8	6506.8	2.174	0.001
8, 50	1	4218.2	4218.2	1.7772	0.013

## Appendix 14 -

### Sulphate reducing cultures

#### PERMANOVA

#### Permutational MANOVA

*Resemblance worksheet*

Name: Resem7

Data type: Similarity

Selection: All

Sums of squares type: Type III

Permutation method: Permutation of residuals under the reduced model

Number of permutations: 999

#### PAIRWISE TESTS

Term: Distance x New Time for pairs of levels of factor: Distance

Groups	df	SS	MS	t	P (perm)
-177, 0	2	3296.7	1648.3	1.7317	0.007
-177, 2.5	2	6259.1	3129.5	1.4237	0.052
-177, 5	2	4933.1	2466.6	1.5162	0.033
-177, 8	2	4257.1	2128.5	1.4598	0.032
-177, 50	2	6705	3352.5	1.7419	0.008
0, 2.5	2	4885.5	2442.8	1.4864	0.032
0, 5	2	2699	1349.5	1.6235	0.022
0, 8	2	2166.9	1083.5	1.578	0.02
0, 50	2	6627	3313.5	2.2666	0.001
2.5, 5	2	1971.1	985.57	0.73648	0.88
2.5, 8	2	1586.7	793.37	0.65895	0.942
2.5, 50	2	6030.9	3015.4	1.3362	0.059
5, 8	2	2983.9	1492	0.9821	0.491
5, 50	2	4572.3	2286.2	1.2887	0.122
8, 50	2	2770.2	1385.1	1.0184	0.438

## Appendix 15 –

### Sulphate-reducing cultures

#### PERMANOVA Permutational MANOVA

*Resemblance worksheet*

Name: Resem7

Data type: Similarity

Selection: All

Sums of squares type: Type III

Permutation method: Permutation of residuals under the reduced model

Number of permutations: 999

#### PAIRWISE TESTS

Term: New Time

Groups	df	SS	MS	t	P(perm)
1, 2	1	3002.7	3002.7	1.6161	0.021
1, 3	1	2313.4	2313.4	1.4114	0.092
2, 3	1	758.68	758.68	0.8296	0.64

#### PERMANOVA Permutational MANOVA

*Resemblance worksheet*

Name: Resem7

Data type: Similarity

Selection: All

Sums of squares type: Type III

Permutation method: Permutation of residuals under the reduced model

Number of permutations: 999

#### PAIRWISE TESTS

Term: DistancexNew Time for pairs of levels of factor: New Time

Groups	df	SS	MS	t	P(perm)
1, 2	5	11124	2224.7	1.391	0.01
1, 3	5	10153	2030.6	1.3223	0.034
2, 3	5	10449	2089.7	1.3768	0.007

## Appendix 16 –

### Sulphate-reducing cultures

#### PERMDISP

Distance-based test for homogeneity of multivariate dispersions

*Resemblance worksheet*

Name: Resem7

Data type: Similarity

Selection: All

Number of groups: 5

Number of samples: 62

#### *DEVIATIONS FROM CENTROID*

F: 14.167

P(perm): 0.001

#### *PAIRWISE COMPARISONS*

Groups	t	P(perm)
<b>(-177,0)</b>	<b>3.7511</b>	<b>3E-3</b>
<b>(-177,2.5)</b>	<b>1.8945</b>	<b>8.7E-2</b>
(-177,7)	1.6222	0.124
(-177,50)	1.7463	0.102
<b>(0,2.5)</b>	<b>6.3656</b>	<b>1E-3</b>
<b>(0,7)</b>	<b>5.1091</b>	<b>1E-3</b>
<b>(0,50)</b>	<b>5.3208</b>	<b>2E-3</b>
(2.5,7)	5.2219E-2	0.967
(2.5,50)	8.1286E-2	0.928
(7,50)	2.2346E-2	0.978

#### *MEANS AND STANDARD ERRORS*

Group	Average	SE
-177	33.695	2.5606
0	20.303	2.2817
2.5	40.118	2.1174
7	40.313	3.2283
50	40.41	2.8886

## Appendix 17 –

Nitrate-reducing cultures

### PERMANOVA Permutational MANOVA

*Resemblance worksheet*

Name: Resem18

Data type: Similarity

Selection: All

Sums of squares type: Type III

Permutation method: Permutation of residuals under the reduced model

Number of permutations: 999

#### PAIRWISE TESTS

Term: Distance

Groups	df	SS	MS	t	P(perm)
<b>2.5, 50</b>	<b>1</b>	<b>6443.5</b>	<b>6443.5</b>	<b>2.6716</b>	<b>0.001</b>
<b>2.5, 5</b>	<b>1</b>	<b>6081.1</b>	<b>6081.1</b>	<b>2.606</b>	<b>0.001</b>
<b>2.5, 8</b>	<b>1</b>	<b>7526.2</b>	<b>7526.2</b>	<b>3.006</b>	<b>0.001</b>
<b>2.5, -50</b>	<b>1</b>	<b>10636</b>	<b>10636</b>	<b>3.4044</b>	<b>0.001</b>
<b>2.5, 0</b>	<b>1</b>	<b>16523</b>	<b>16523</b>	<b>4.3822</b>	<b>0.001</b>
50, 5	1	1786.9	1786.9	1.0708	0.357
50, 8	1	2093.7	2093.7	1.206	0.233
<b>50, -50</b>	<b>1</b>	<b>3244.5</b>	<b>3244.5</b>	<b>1.5955</b>	<b>0.061</b>
<b>50, 0</b>	<b>1</b>	<b>9471.9</b>	<b>9471.9</b>	<b>2.9832</b>	<b>0.001</b>
5, 8	1	994.59	994.59	0.81071	0.694
<b>5, -50</b>	<b>1</b>	<b>3336.6</b>	<b>3336.6</b>	<b>1.6081</b>	<b>0.048</b>
<b>5, 0</b>	<b>1</b>	<b>6434.4</b>	<b>6434.4</b>	<b>2.4591</b>	<b>0.001</b>
<b>8, -50</b>	<b>1</b>	<b>3068.5</b>	<b>3068.5</b>	<b>1.5934</b>	<b>0.046</b>
<b>8, 0</b>	<b>1</b>	<b>5274</b>	<b>5274</b>	<b>2.3</b>	<b>0.002</b>
<b>-50, 0</b>	<b>1</b>	<b>5377.3</b>	<b>5377.3</b>	<b>2.2692</b>	<b>0.001</b>

### PERMANOVA Permutational MANOVA

*Resemblance worksheet*

Name: Resem18

Data type: Similarity

Selection: All

Sums of squares type: Type III

Permutation method: Permutation of residuals under the reduced model

Number of permutations: 999

#### PAIRWISE TESTS

Term: new\_time

Groups	df	SS	MS	t	P(perm)
1, 2	1	1888.2	1888.2	1.326	0.141
<b>1, 3</b>	<b>1</b>	<b>2783.2</b>	<b>2783.2</b>	<b>1.5887</b>	<b>0.031</b>
2, 3	1	255.26	255.26	0.52733	0.845

## Appendix 18 -

Nitrate-reducing cultures

### PERMANOVA Permutational MANOVA

*Resemblance worksheet*

Name: Resem18

Data type: Similarity

Selection: All

Sums of squares type: Type III

Permutation method: Permutation of residuals under the reduced model

Number of permutations: 999

#### PAIRWISE TESTS

Term: Distancexnew\_time for pairs of levels of factor: Distance

Groups	df	SS	MS	t	P(perm)
<b>2.5, 50</b>	<b>2</b>	<b>3412.1</b>	<b>1706.1</b>	<b>1.3747</b>	<b>0.071</b>
<b>2.5, 5</b>	<b>2</b>	<b>3469.7</b>	<b>1734.9</b>	<b>1.3919</b>	<b>0.046</b>
<b>2.5, 8</b>	<b>2</b>	<b>5178.1</b>	<b>2589.1</b>	<b>1.7631</b>	<b>0.002</b>
<b>2.5, -50</b>	<b>2</b>	<b>12512</b>	<b>6256.2</b>	<b>2.611</b>	<b>0.001</b>
<b>2.5, 0</b>	<b>2</b>	<b>4645.3</b>	<b>2322.6</b>	<b>1.643</b>	<b>0.009</b>
50, 5	2	3228.1	1614.1	1.0178	0.463
50, 8	2	5300	2650	1.3568	0.111
<b>50, -50</b>	<b>2</b>	<b>7307.5</b>	<b>3653.8</b>	<b>1.6931</b>	<b>0.011</b>
50, 0	2	2356.7	1178.4	1.0522	0.381
5, 8	2	4845.9	2423	1.2654	0.144
<b>5, -50</b>	<b>2</b>	<b>5538.3</b>	<b>2769.1</b>	<b>1.465</b>	<b>0.049</b>
5, 0	2	3701.9	1851	1.3189	0.077
<b>8, -50</b>	<b>2</b>	<b>11493</b>	<b>5746.5</b>	<b>2.1805</b>	<b>0.001</b>
<b>8, 0</b>	<b>2</b>	<b>5605.9</b>	<b>2802.9</b>	<b>1.6768</b>	<b>0.011</b>
<b>-50, 0</b>	<b>2</b>	<b>12743</b>	<b>6371.7</b>	<b>2.4701</b>	<b>0.001</b>

### PERMANOVA Permutational MANOVA

*Resemblance worksheet*

Name: Resem18

Data type: Similarity

Selection: All

Sums of squares type: Type III

Permutation method: Permutation of residuals under the reduced model

Number of permutations: 999

#### PAIRWISE TESTS

Term: Distancexnew\_time for pairs of levels of factor: new\_time

Groups	df	SS	MS	t	P(perm)
<b>1, 2</b>	<b>5</b>	<b>11333</b>	<b>2266.6</b>	<b>1.4528</b>	<b>0.002</b>
<b>1, 3</b>	<b>5</b>	<b>17576</b>	<b>3515.2</b>	<b>1.7854</b>	<b>0.001</b>
<b>2, 3</b>	<b>5</b>	<b>17787</b>	<b>3557.4</b>	<b>1.9686</b>	<b>0.001</b>

## Appendix 19 –

### Nitrate-reducing cultures

#### PERMDISP FOR DISTANCE

F: 10.93

P(perm): 0.001

##### PAIRWISE COMPARISONS

Groups	t	P(perm)
(2.5,50)	2.0216	0.102
(2.5,5)	1.0524	0.418
<b>(2.5,8)</b>	<b>3.3278</b>	<b>2E-2</b>
<b>(2.5,-50)</b>	<b>6.7309</b>	<b>1E-3</b>
(2.5,0)	0.81144	0.484
(50,5)	0.87807	0.482
(50,8)	1.0563	0.351
<b>(50,-50)</b>	<b>2.7824</b>	<b>2E-2</b>
(50,0)	1.4939	0.219
<b>(5,8)</b>	<b>3.3709</b>	<b>9E-3</b>
<b>(5,-50)</b>	<b>5.3194</b>	<b>1E-3</b>
(5,0)	0.49965	0.682
(8,-50)	1.546	0.295
<b>(8,0)</b>	<b>2.9398</b>	<b>2.6E-2</b>
<b>(-50,0)</b>	<b>6.0943</b>	<b>1E-3</b>

#### PERMDISP FOR SAMPLING EVENT

F: 1.8534

P(perm): 0.161

##### PAIRWISE COMPARISONS

Groups	t	P(perm)
<b>(69,81)</b>	<b>2.3393</b>	<b>2.3E-2</b>
<b>(69,95)</b>	<b>2.6806</b>	<b>9E-3</b>
(69,110)	0.92231	0.441
(69,123)	0.10785	0.9
(69,137)	1.2041	0.211
(81,95)	0.10782	0.898
(81,110)	0.99907	0.418
<b>(81,123)</b>	<b>2.0819</b>	<b>5.6E-2</b>
(81,137)	1.649	0.143
(95,110)	1.1017	0.337
<b>(95,123)</b>	<b>2.3819</b>	<b>3.1E-2</b>
<b>(95,137)</b>	<b>1.8329</b>	<b>7.2E-2</b>
(110,123)	0.78286	0.519
(110,137)	0.1002	0.925
(123,137)	1.0038	0.327

## Appendix 20

### Bolivar Chemical Data

	DOC	S04	NH4	N03	N02	P_SR	Tot. P	Sampling
	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	Event
INJ	20.2	195	0.32	0.54	0.005	0.293	0.566	1
INJ	20.2	195	0.32	0.54	0.005	0.293	0.566	29
INJ	24	194	0.44	0.582	0.023	1.41	1.65	43
INJ	21.2	187	0.62	1.66	0.01	2.4	3.01	57
INJ	20.3	174	0.75	2.05	0.041	2.94	3.69	71
INJ	19.8	175	0.66	3.39	0.048	3.1	3.53	116
INJ	18.1	175	0.34	5.4	0.005	2.04	2.27	159
INJ	17.7	168	0.76	3.6	0.153	1.72	1.9	188
INJ	16.2	174	4.55	0.041	0.02	1.21	1.42	218
INJ	12.3	184	1.27	0	0.005	0.582	0.755	232
INJ	7.8	233	2.03	0.004	0.005	0.192	0.44	282
INJ	5.5	273	1.96	0	0.005	0.216	0.222	324
19450	8.3	250	13.2	0	0.005	0.083	0.151	1
19450	16.9	204	0.465	0.079	0.005	0.057	0.12	29
19450	18.3	189	0.793	0.21	0.02	0.127	0.148	43
19450	16.9	199	1.458	0.48	0.048	0.372	0.525	57
19450	17.6	181	3.41	0	0.005	0.038	1.67	71
19450	17.7	176	3.11	1.96	0.19	1.36	1.54	116
19450	16	173	1.01	3.33	0.124	1.08	1.16	159
19450	15.2	165	3.4	3.37	0.222	0.88	0.98	188
19450	16.2	174	4.55	0.041	0.02	1.21	1.42	218
19450	12.3	184	1.27	0	0.005	0.582	0.755	232
19450	7.8	233	2.03	0.004	0.005	0.192	0.44	282
19450	5.5	273	1.96	0	0.005	0.216	0.222	324
19181	6.4	260	7.38	0	0.005	0.028	0.041	1
19181	8.3	220	8.84	0	0.005	0.005	0.036	29
19181	9.4	222	13.7	0	0.005	0.019	0.045	43
19181	9.9	217	15.4	0.003	0.005	0.007	0.046	57
19181	10.5	213	19.2	0	0.005	0.039	0.056	71
19181	10.4	221	20.2	0	0.005	0.012	0.049	116
19181	12	218	20.1	0	0.005	0.011	0.038	159
19181	12	215	16.2	0	0.005	0.013	0.039	188
19181	11.2	202	14	0	0.005	0.005	0.042	218
19181	10.1	227	8.82	0	0.005	0.005	0.057	232
19181	10.4	211	14.7	0.004	0.005	0.012	0.028	282
19181	9.5	218	14.5	0	0.005	0.024	0.038	324
19449	3	221	0.128	0	0.005	0.005	0.03	1
19035	0.9	302	0.048	0.048	0.005	0.005	0.019	1
19035	0.9	297	0.048	0.048	0.005	0.005	0.019	29
19035	0.9	302	0.048	0.048	0.005	0.005	0.019	43



19035	0.9	302	0.048	0.048	0.005	0.005	0.019		57
19035	0.9	302	0.048	0.048	0.005	0.005	0.019		71
19035	0.9	302	0.048	0.048	0.005	0.005	0.019		116
19035	0.9	302	0.048	0.048	0.005	0.005	0.019		159
19035	0.9	302	0.048	0.048	0.005	0.005	0.019		188
19035	0.9	302	0.048	0.048	0.005	0.005	0.019		218
19035	0.9	302	0.048	0.048	0.005	0.005	0.019		232
19035	0.9	302	0.048	0.048	0.005	0.005	0.019		282
19035	0.9	302	0.048	0.048	0.005	0.005	0.019		324

Bolivar Chemical Data

	TKN	Fe	Eh	DO	Econd	pH	Temp	TOC	Cl	Sampling
	mg/L	mg/L	mV	mg/L	ms/m	pH	°C	mg/L	mg/L	Event
INJ	2.34	0.03	971	5.05	2000	7.29	21.4	21.2	482	1
INJ	2.34	0.03	971	5.05	2000	7.29	21.4	21.2	482	29
INJ	3.19	0.056	865	7	1943	6.7	20.5	25.5	430	43
INJ	3.45	0.067	740	5.24	1917	6.84	17.7	22	425	57
INJ	6.04	0.16	765	8.58	1980	6.67	15.8	20.8	411	71
INJ	5.32	0.04	816	4.5	1800	6.85	15.5	20.3	339	116
INJ	2.1	0.067	973	6.42	1853	6.94	13.5	18.8	386	159
INJ	7.62	0.196	844	5.34	1866	7.4	11.3	18.7	431	188
INJ	6.89	0.574	46	0.05	1815	7.54	13.9	16.5	394	218
INJ	2.72	0.734	56	0	2130	7.54	17.9	12.3	464	232
INJ	2.58	0.572	134	0.03	2340	7.26	19.9	7.9	668	282
INJ	2.97	0.634	56	0	3200	7.15	21.5	5.4	849	324
19450	14.4	0.741	33	0.08	2580	6.58	22.7	8.6	600	1
19450	2.18	0.329	377	0.15	2220	7.39	22.3	17.2	463	29
19450	2.32	0.281	136	0.67	2120	7.46	20.6	18.9	426	43
19450	2.02	0.315	135	2.51	1965	7.88		17	421	57
19450	5.1	0.102	408	2	2029	6.94	18	17.8	389	71
19450	4.61	0.141	565	1.2	2000	7.05	17.8	17.6	398	116
19450	2.49	0.114	225	1.46	1959	7.19	15.6	16.2	391	159
19450	5	0.083	475	1.95	1893	7.19	13.8	15.4	426	188
19450	6.89	0.574	46	0.05	1815	7.54	13.9	16.5	394	218
19450	2.72	0.734	56	0	2130	7.54	17.9	12.3	464	232
19450	2.58	0.572	134	0.03	2340	7.26	19.9	7.9	668	282
19450	2.97	0.634	56	0	3200	7.15	21.5	5.4	849	324
19181	8.59	0.684	20	0.22	2590	6.56	22.6	6.5	637	1
19181	14.1	0.422	94	5.55	2420	7.32	20.6	8.4	527	29
19181	16.6	0.711	26	0.29	2360	7.46	22.5	9.5	474	43
19181	21.1	0.712	50	0.76	2240	7.72		9.7	482	57
19181	20.3	0.698	55	2	2450	7.03	22.7	10.5	467	71
19181	21.5	0.801	40	1.27	2390	7.07	21.6	10.7	472	116
19181	20.6	0.717	53	0.65	2390	7.11	20.9	12.2	465	159
19181	16.6	0.79	32	1.08	2300	7.15	20.3	12.4	514	188
19181	14.9	0.757	45	0.2	2250	7.61	19.6	11.1	405	218
19181	14.4	1.19	58	1	2280	7.65	20.9	10.2	411	232

19181	15.1	1.05	57	0.03	2390	7.5	21.7	10.5	509	282
19181	16.8	0.789	47	0	2520	7.44	20.9	9.5	538	324
19449	0.35	1.2	97	0.38	2280	6.67	22.5	3.2	648	1
19035	0.08	1.37	98	0.24	3460	7.19	24.6	0.9	1040	1
19035	0.08	1.37	52	0.42	3490	6.81	25.4	0.9	974	29
19035	0.08	1.37	98	0.24	3460	7.19	24.6	0.9	1040	43
19035	0.08	1.37	98	0.24	3460	7.19	24.6	0.9	1040	57
19035	0.08	1.37	98	0.24	3460	7.19	24.6	0.9	1040	71
19035	0.08	1.37	98	0.24	3460	7.19	24.6	0.9	1040	116
19035	0.08	1.37	98	0.24	3460	7.19	24.6	0.9	1040	159
19035	0.08	1.37	98	0.24	3460	7.19	24.6	0.9	1040	188
19035	0.08	1.37	98	0.24	3460	7.19	24.6	0.9	1040	218
19035	0.08	1.37	98	0.24	3460	7.19	24.6	0.9	1040	232
19035	0.08	1.37	98	0.24	3460	7.19	24.6	0.9	1040	282
19035	0.08	1.37	98	0.24	3460	7.19	24.6	0.9	1040	324

## Appendix 21

### Perth Chemical Data

Site (m from Infiltration Gallery)	Fe mg/L	N-NH3 mg/L	N_N03 mg/L	N-TK mg/L	P_SR mg/L	S04 mg/L	TOC mg/L	Sampling Event
0.00	0.06	0.17	2.50	1.10	11.00	56.00	9.00	1
0.00	0.06	0.86	4.10	1.70	12.00	57.40	9.00	1
0.00	0.14	0.18	2.40	1.40	9.70	54.70	8.00	1
0.00	0.16	0.79	4.10	1.40	13.00	56.00	9.00	1
0.00	0.06	0.39	3.80	0.96	11.00	53.10	10.00	2
0.00	0.08	0.65	4.10	1.40	13.00	66.90	12.00	2
0.00	0.06	0.39	1.70	2.30	9.90	52.90	12.00	13
0.00	0.06	0.14	1.00	1.30	5.10	52.10	13.00	32
0.00	0.10	3.60	0.48	5.40	4.60	61.30	13.00	69
0.00	0.07	1.80	1.60	2.90	8.80	76.20	15.00	81
0.00	0.08	0.41	1.70	1.90	11.00	57.70	12.00	95
0.00	0.04	0.40	5.80	1.90	9.90	80.30	9.00	110
0.00	0.04	0.32	5.70	1.60	6.90	79.60	9.00	110
0.00	0.08	0.04	1.70	1.50	7.00	65.70	15.00	124
0.00	0.08	0.03	1.80	1.50	7.20	68.50	14.00	124
0.00	0.70	1.60	0.26	2.80	4.34	73.10	9.00	137
0.00	1.10	0.60	0.30	1.70	3.33	70.60	9.00	137
1.00		1.20	3.90	3.80	0.01	49.00		-1
1.00		0.01	2.20	0.02	0.01	60.00		-2
1.00		0.01	0.46	0.12	0.01	69.90	4.00	-3
1.00	0.05	0.00	3.70	0.54	0.01	57.50	4.00	2
1.00	0.04	0.00	4.20	0.36	0.01	52.60	10.00	13
1.00	0.03	0.00	2.80	0.81	0.01	57.30	5.00	32
1.00	0.04	0.00	4.60	0.71	0.02	55.70	11.00	68
1.00	0.07	0.00	6.30	0.67	0.01	72.10	11.00	81
1.00	0.10	0.00	5.80	0.78	0.85	67.30	15.00	95
1.00	0.01	0.00	4.30	0.71	1.40	74.80	5.00	110
1.00	0.04	0.00	2.60	1.50	2.00	68.00	7.00	123
1.00	0.02	0.00	2.50	0.79	2.60	70.00	6.00	137
2.00		0.01	5.00	1.40	0.01	67.00		-1
2.00		0.05	1.90	0.26	0.01	72.50		-3
2.00	0.04	0.00	4.20	0.47	0.00	69.00	4.00	2
2.00	0.02	0.00	5.10	0.00	0.01	52.70	4.00	13
2.00	0.02	0.00	3.50	1.10	0.01	56.50	2.00	32
2.00	0.02	0.00	1.80	2.40	0.01	54.10	7.00	68
2.00	0.02	0.00	4.80	0.33	0.01	65.20	6.00	81
2.00	0.02	0.00	4.50	0.46	0.00	66.70	8.00	95
2.00	0.00	0.00	2.00	1.40	0.00	69.10	4.00	110
2.00	0.01	0.00	1.70	1.50	0.00	84.20	5.00	123
2.00	0.01	0.00	2.20	0.70	0.00	70.60	4.00	137
5.00	0.10	0.06	0.79	1.50	0.01	57.40	3.00	0

5.00	0.00	0.00	0.39	0.04	0.00	65.60	2.00	2
5.00	0.00	0.00	1.50	0.76	0.01	62.30	7.00	13
5.00	0.00	0.00	1.30	0.35	0.02	58.40	0.00	32
5.00	0.04	0.00	1.30	2.50	0.01	58.90	7.00	68
5.00	0.00	0.00	3.60	0.44	0.00	64.10	9.00	95
6.00		0.01	1.30	0.06	0.01	84.00	6.00	-2
6.00		0.04	1.50	0.17	0.01	99.70	5.00	-3
6.00	0.05	0.08	3.60	0.30	0.00	63.10	3.00	2
6.00	0.03	0.00	2.00	1.50	0.01	52.70	6.00	13
6.00	0.07	0.00	2.40	0.73	0.01	56.10	12.00	32
6.00	0.01	0.00	1.30	2.10	0.01	51.40	7.00	68
6.00	0.02	0.00	3.70	0.54	0.01	62.60	7.00	81
6.00	0.00	0.00	3.70	0.32	0.00	66.60	9.00	95
6.00	0.00	0.00	3.40	0.41	0.00	66.40	3.00	110
6.00	0.04	0.00	2.70	2.10	0.01	82.10	6.00	123
6.00	0.02	0.00	1.80	1.20	0.01	69.90	5.00	137
7.00	0.10	0.01	0.35	0.52	0.01	69.60	4.00	0
7.00	0.02	0.00	4.40	0.54	0.01	71.50	5.00	2
7.00	0.01	0.00	3.30	0.00	0.01	57.40	2.00	13
7.00	0.01	0.00	3.40	1.20	0.00	54.20	3.00	32
8.00		0.01	0.01	0.09	0.01	65.00	6.00	-2.00
8.00		0.01	0.01	0.09	0.01	76.90	4.00	-3
8.00	0.20	0.03	3.00	0.41	0.02	75.60	7.00	2
8.00	0.04	0.01	2.80	1.10	0.01	60.10	4.00	13
8.00	0.00	0.00	1.50	0.43	0.00	62.00	2.00	32
8.00	0.04	0.00	1.20	1.40	0.01	56.20	8.00	68
8.00	0.05	0.00	3.90	0.14	0.01	63.80	9.00	95
8.00	0.00	0.00	4.90	0.14	0.01	64.70	3.00	110
8.00	0.02	0.00	2.40	1.40	0.01	83.50	9.00	123
9.00		0.01	0.19	0.13	0.01	65.60	6.00	-3
9.00	0.02	0.03	4.95	0.04	0.01	69.60	5.00	2
9.00	0.01	0.01	3.60	0.85	0.01	62.00	6.00	13
9.00	0.03	0.02	3.50	1.10	0.02	56.40	5.00	33
10.00	0.30	0.01	0.49	0.48	0.01	72.70	4.00	-3
10.00	0.02	0.00	4.80	0.02	0.01	80.00	5.00	1
10.00	0.02	0.02	4.20	1.30	0.01	60.40	6.00	33
11.00		0.02	0.01	0.10	0.01	75.00	6.00	-2.00
11.00	0.30	0.01	0.01	0.11	0.01	71.20	4.00	0
11.00	1.50	0.00	0.00	0.14	0.00	80.00	0.00	1
11.00	0.01	0.04	0.01	0.06	0.02	67.80	1.00	33
11.00	4.30	0.00	0.00	0.16	0.01	72.50	5.00	81
11.00	4.60	0.00	0.00	0.06	0.01	68.30	7.00	95
11.00	3.90	0.00	0.01	0.07	0.00	65.70	2.00	110
11.00	2.90	0.00	0.00	0.09	0.00	78.10	0.00	123
15.00	2.70	0.00	0.00	0.10	0.00	78.50	1.00	1
15.00	3.20	0.00	0.00	0.24	0.00	74.90	0.00	34
16.00	0.10	0.01	0.01	0.10	0.01	70.30	11.00	0

16.00	0.94	0.00	0.03	0.07	0.00	80.00	2.00	1
16.00	3.60	0.00	0.01	0.20	0.00	79.70	3.00	137
17.00	1.70	0.32	0.75	0.33	0.01	65.60	2.00	1
17.00	0.01	0.28	0.95	0.48	0.01	59.10	2.00	33
17.00	6.00	0.38	0.63	0.60	0.01	51.40	8.00	68
17.00	3.30	0.54	0.31	0.55	0.01	56.90	7.00	81
17.00	3.50	0.33	0.96	0.41	0.01	58.00	7.00	95
17.00	3.60	0.32	0.76	0.49	0.00	55.90	4.00	110
17.00	3.60	0.27	0.55	0.68	0.00	66.30	8.00	124
17.00	4.00	0.24	0.67	0.69	0.00	64.00	2.00	137
-177.00	0.26	0.00	0.01	0.04	0.01	72.80	3.00	13
-177.00	0.29	0.00	0.00	0.01	0.02	73.50	2.00	33
-177.00	0.30	0.00	0.02	0.10	0.03	68.90	8.00	68
-177.00	0.03	0.00	0.05	0.10	0.01	78.30	3.00	81
-177.00	0.05	0.00	0.06	0.06	0.00	70.90	7.00	95
-177.00	0.11	0.00	0.05	0.08	0.00	69.80	1.00	110
-177.00	0.40	0.00	0.08	0.09	0.01	78.10	5.00	124
-177.00	0.27	0.00	0.10	0.04	0.00	72.70	0.00	137

#### Perth Chemical Data

Site (m from Infiltration Gallery)	pH	DO	Temp	Alkalinity	Cl	DOC	Econd	Sampling
	pH	mg/L	°C	mg/L	mg/L	mg/L	ms/m	Event
0.00	6.50	2.00	25.45	132.54	189.00	10.00	98.30	1
0.00	6.53	3.74	25.97	111.91	188.00	9.00	98.40	1
0.00	6.50	3.50	25.90	171.60	187.00	13.00	97.50	1
0.00	6.54	0.35	25.88	129.47	188.00	15.00	98.40	1
0.00	6.61	3.25	26.42	129.05	174.00	11.00	89.70	2
0.00	6.63	5.64	26.36	97.31	205.00	9.00	116.00	2
0.00	6.80	5.80	24.34	135.00	234.00	10.00	123.00	13
0.00	6.89	4.38	24.99	170.00	231.00	9.00	117.00	32
0.00	7.64	6.40	24.46	145.00	304.00	13.00	144.00	69
0.00	7.04	3.30	29.90	140.00	387.00	15.00	170.00	81
0.00	6.47	2.17	27.97	110.00	303.00	11.00	122.00	95
0.00	6.23	1.45	29.73	125.00	512.00	9.00	199.00	110
0.00	6.11	2.63	29.69	125.00	507.00	9.00	194.00	110
0.00	7.20	1.20	27.10	135.00	272.00	15.00	129.00	124
0.00	7.15	1.43	27.04	135.00	286.00	14.00	129.00	124
0.00	8.19	2.82	23.82	160.00	291.00	9.00	148.00	137
0.00	8.08	2.61	23.70	155.00	289.00	9.00	147.00	137
1.00							81.10	-1
1.00	7.42	4.43	21.21				114.00	-2
1.00	7.01	3.57	20.30	245.00	187.00	3.00	117.00	-3
1.00	7.33	3.66	24.08	216.86	203.00	3.77	112.00	2
1.00	7.33	3.07	23.04	185.00	232.00	4.00	136.00	13
1.00	7.32	8.38	23.53	230.00	240.00	4.00	131.00	32
1.00	7.33	9.36	26.20	225.00	277.00	10.00	150.00	68

1.00	7.35	6.92	28.80	225.00	324.00	10.00	173.00	81
1.00	6.72	6.23	29.53	230.00	339.00	12.00	154.00	95
1.00	7.01	4.67	27.99	210.00	449.00	5.00	191.00	110
1.00	7.74	9.12	26.23	220.00	254.00	7.00	125.00	123
1.00	8.85	4.51	22.73	220.00	338.00	5.00	171.00	137
2.00	7.31	4.30	20.85				123.00	-1
2.00	6.94	3.66	20.90	230.00	210.00	3.00	126.00	-3
2.00	7.39	4.80	21.23	214.44	198.00	2.48	115.00	2
2.00	7.28	4.88	22.62	175.00	202.00	2.00	123.00	13
2.00	7.26	4.36	22.74	220.00	234.00	0.00	129.00	32
2.00	7.29	8.78	22.33	223.00	281.00	7.00	148.00	68
2.00	7.48	4.99	25.90	223.00	301.00	6.00	157.00	81
2.00	7.48	7.44	28.13	225.00	319.00	8.00	142.00	95
2.00	5.73	5.98	28.83	215.00	417.00	4.00	176.00	110
2.00	6.00	7.36	28.66	230.00	337.00	5.00	154.00	123
2.00	8.18	6.27	24.22	215.00	335.00	4.00	169.00	137
5.00	7.07	2.28	19.20	225.00	172.00	3.00	104.00	0
5.00	7.13	4.30	21.53	236.39	183.00	1.01	108.00	2
5.00	7.14	7.23	21.96	170.00	206.00	0.00	127.00	13
5.00	7.00	7.43	22.39	240.00	198.00	0.00	120.00	32
5.00	7.21	8.45	23.41	220.00	279.00	7.00	152.00	68
5.00	7.27	7.35	32.12	230.00	305.00	9.00	140.00	95
6.00	7.15	5.66	21.09		272.00		153.00	-2
6.00	6.92	4.25	20.80	235.00	275.00	4.00	145.00	-3
6.00	7.24	4.65	23.29	216.39	207.00	1.66	116.00	2
6.00	7.37	5.10	22.76	210.00	207.00	5.00	123.00	13
6.00	7.11	4.53	23.09	225.00	241.00	6.00	130.00	32
6.00	7.29	6.41	23.32	235.00	233.00	7.00	143.00	68
6.00	7.38	5.80	26.50	223.00	291.00	6.00	156.00	81
6.00	6.83	6.90	27.94	225.00	312.00	9.00	142.00	95
6.00	6.74	6.71	28.02	210.00	381.00	3.00	165.00	110
6.00	8.06	9.13	25.79	220.00	326.00	6.00	149.00	123
6.00	8.62	5.77	24.21	220.00	312.00	5.00	163.00	137
7.00	7.06	2.66	18.70	263.00	210.00	3.00	119.00	0
7.00	7.42	4.36	22.45	205.07	232.00	3.07	125.00	2
7.00	7.15	5.64	20.34	225.00	209.00	2.00	126.00	13
7.00	7.15	7.33	22.11	220.00	224.00	2.00	125.00	32
8.00	7.20	1.65	20.87		169.00		117.00	-2.00
8.00	6.90	0.33	20.70	225.00	172.00	3.00	107.00	-3
8.00	7.33	5.04	22.37	212.03	256.00	4.45	129.00	2
8.00	7.41	2.52	20.24	225.00	213.00	3.00	128.00	13
8.00	7.21	7.44	21.92	235.00	206.00	0.00	121.00	32
8.00	7.40	0.54	22.53	220.00	201.00	8.00	125.00	68
8.00	6.96	2.81	26.69	230.00	298.00	8.00	138.00	95
8.00	6.41	1.91	28.64	225.00	312.00	3.00	148.00	110
8.00	7.96	8.79	27.47	220.00	343.00	9.00	158.00	123

9.00	6.97	0.31	20.40	215.00	182.00	2.00	107.00	-3
9.00	7.26	3.74	22.07	211.42	246.00	3.00	131.00	2
9.00	7.36	3.18	20.77	210.00	208.00	5.00	125.00	13
9.00	7.08	3.01	23.53	215.00	217.00	5.00	123.00	33
10.00	6.89	3.12	22.00	250.00	210.00	3.00	125.00	-3
10.00	7.04	3.44	22.97	215.64	246.00	3.17	128.00	1
10.00	6.92	4.40	22.86	215.00	208.00	4.00	121.00	33
11.00	7.21	1.47	21.06		190.00		120.00	-2.00
11.00	7.06	2.29	18.40	263.00	190.00	3.00	113.00	0
11.00	6.88	0.55	23.18	252.41	161.00	1.42	106.00	1
11.00	6.61	0.98	24.38	245.00	169.00	0.00	110.00	33
11.00	7.14	3.09	24.70	240.00	175.00	5.00	117.00	81
11.00	6.95	4.42	24.74	250.00	171.00	5.00	105.00	95
11.00	5.28	2.82	26.44	250.00	170.00	2.00	104.00	110
11.00	6.39	5.11	32.21	255.00	183.00	0.00	101.00	123
15.00	7.01	0.53	22.27	243.10	183.00	1.84	112.00	1
15.00	7.75	2.71	21.07	220.00	232.00	0.00	119.00	34
16.00	7.11	2.01	20.10	263.00	196.00	7.00	116.00	0
16.00	7.00	1.13	23.47	249.44	180.00	2.62	111.00	1
16.00	7.41	2.70	22.99	220.00	249.00	3.00	141.00	137
17.00	6.41	1.56	23.04	234.91	192.00	2.05	109.00	1
17.00	6.97	5.33	21.38	250.00	189.00	0.00	116.00	33
17.00	7.12	2.83	21.87	240.00	186.00	8.00	118.00	68
17.00	7.40	4.38	25.30	240.00	186.00	7.00	117.00	81
17.00	5.97	4.05	25.76	235.00	196.00	7.00	106.00	95
17.00	6.44	4.42	21.54	240.00	168.00	4.00	109.00	110
17.00	7.00	4.13	20.93	235.00	188.00	8.00	112.00	124
17.00	6.97	3.87	22.24	235.00	201.00	2.00	126.00	137
-177.00	6.97	0.12	21.08	230.00	163.00	1.00	116.00	13
-177.00	6.88	1.37	21.09	250.00	163.00	2.00	108.00	33
-177.00	7.07	0.33	21.26	233.00	161.00	5.00	109.00	68
-177.00	7.35	3.55	25.70	240.00	157.00	3.00	109.00	81
-177.00	7.37	3.44	23.90	240.00	164.00	6.00	100.00	95
-177.00	7.03	2.91	23.07	245.00	166.00	1.00	103.00	110
-177.00	7.57	2.13	23.36	240.00	158.00	5.00	106.00	124
-177.00	7.23	2.59	21.35	235.00	156.00	0.00	113.00	137